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APPLICATION OF MICROBIAL ENDOPHYTES IN THE BIOCONTROL OF FUNGAL DISEASES AND FOR THE PRODUCTION OF FUNCTIONAL FOOD

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ABSTRACT

Non-pathogenic endophytic bacteria can positively affect host plants growth and health by inducing changes in their physiological responses and metabolic activities and by improving their response to biotic and abiotic stresses. At the same time, supporting the interaction between plants and plant growth-promoting bacteria (PGPB) can improve the qualitative and nutraceutical properties of agri-food products, making agriculture more environmentally and economically sustainable at the same time.
The aim of the present research work was to identify microbial endophytes with a possible application as biocontrol and biofortification agents. The project was developed in three parts.

In the first part, the volatile organic compounds (VOCs) produced by the bacterium *Streptomyces* sp. SA51 were tested against *Fusarium oxysporum* f.sp. *lactucae* (FOL) race 1, both *in vitro* and against the development of the Fusarium wilt symptoms in lettuce plants.
In vitro, fungal mycelial growth was inhibited only by 8%. In lettuce plants infected with FOL, on the other hand, the state of plant health was estimated through the McKinney index (MKI). This index showed a decrease of 25% when plants infected with FOL grew in the presence of bacterial VOCs. Even plants not infected with FOL benefited from the presence of bacterial VOCs, in fact the MKI decreased by 48 % in this case.
To identify the VOCs produced by *Streptomyces* sp. SA51, HS-SPME technique and a GC–MS analysis were performed. The analysis revealed the presence of several compounds with already known antifungal properties, such as Germacrene D and Phenylethyl Alcohol.

In the second part, the interactions between three species of baby leaf vegetables destined for the fresh-cut market (*Brassica juncea, Beta vulgaris,* and *Lactuca sativa*) and three endophytic bacteria (*Streptomyces* sp. SA51, P*seudomonas* sp. DLS65, and *Pantoea* sp. S1) were investigated. The purpose was to evaluate whether these interactions could be exploited to increase the polyphenol content of the food.
For each plant species, the seeds were treated separately with the three bacterial cell suspensions. The total polyphenolic content of the plants was extracted and estimated with the Folin-Ciocâlteu assay. Then, LC-ESI-IT-MS/MS analysis was performed to assess the qualitative variations of the polyphenolic content. Significant differences were observed in lettuce plants treated with *Streptomyces* sp. SA51 and in all the vegetable species treated with *Pantoea* sp. S1.

Finally, the last part of the research was conducted at the Austrian Institute of Technology (AIT). The focus was the vitamin B_{12}, an essential nutrient for humans that can only be produced by certain bacteria and archaea. The aim of the study was to identify endophytic bacterial strains able of synthetising vitamin B_{12} *de novo* and to verify whether they could be exploited to enrich edible plants with this vitamin.
First, an *in-silico* analysis of 70 bacterial genomes was carried out to verify the presence of the vitamin B_{12} metabolic pathway. Based on the presence and the completeness of this metabolic pathways in their genomes, 30 strains were selected and their actual ability to produce vitamin B_{12} was tested with an HPLC-DAD analysis on pure cultures extracts. Thus, 10 strains that were shown to be able to

produce detectable amounts of vitamin B_{12} were selected. The best candidates were further tested to evaluate their efficacy in producing vitamin B_{12} in lettuce plants. Vitamin B_{12} was extracted from the edible parts of plants and purified with immunoaffinity columns. Detection and quantification of vitamin B_{12} were performed by HPLC-DAD analysis. One bacterial strain was proved to be able of producing vitamin B_{12} in lettuce plants.

In conclusion, it is possible to assess that some endophytic bacteria have a great potential to be exploited both as biological control against fungal pathogens and to implement the content of molecules of nutraceutical interest in plants.

Chapter 1

Introduction

In the context of the current environmental change and global population growth, the need to develop production and distribution systems that make food available and accessible to everyone is evident. However, such systems may also participate to exhaustion of natural resources (e.g., land; water; energy), compromising biodiversity and violating the environmental balances that support life on Earth (e.g., nitrogen and carbon cycles) (Aiking, 2014; Campbell et al., 2017; Ritchie et al., 2018). These influences are ongoing and are likely to increase in the coming decades due to the relevant socio-economic changes expected to occur worldwide (Alexandratos et al., 2012; Clark & Tilman, 2017). As a possible solution to this problem, there is broad consensus that food sustainability can benefit from a transition towards greater dependence on plant-based foods, and therefore from reduced consumption of meat and animal-based products (Aiking & de Boer, 2020; Clark & Tilman, 2017; Godfray et al., 2018; Willett et al., 2019).

In light of this awareness on the part of the population, new market trends have emerged in recent years to respond to the emerging need of the audience, looking for a more ethical, healthy, and sustainable lifestyle (Willett et al., 2019).

Thus, in high income countries, the interest in food products with eco-friendly characteristics and certifications has significantly grown in recent decades (Falguera et al., 2012; Nuttavuthisit & Thøgersen, 2017). The so-called 'green' food, such as organic products (i.e., vegetables produced following biological management practices of diseases and pests), are perceived by most consumers as healthier than conventional food products, because they are poorly processed and grown naturally (Goetzke et al., 2014; Hemmerling et al., 2016; Lee & Yun, 2015).

On the other hand, especially in urban centres, consumers are increasingly demanding ready-to-eat food that allow saving time, adapting to consumers' modern lifestyles (Botonaki & Mattas, 2010; Brunner et al., 2010).

1.1 FRESH-CUT MARKET AND BABY LEAF

The fresh-cut market offers fresh and natural products, which have been packaged minimally processed and free from additives (Raffo & Paoletti, 2022). This market may offer a compromise between the need to have healthy food that requires minimal preparation time before consumption and, at the same time, the need to eat natural and ecological products.

Therefore, fresh-cut products are an excellent solution to increase the daily consumption of fruit and vegetables, thus obtaining a healthy diet rich in vitamins, minerals, fibres, and polyphenols. A big portion of the fresh-cut market is represented by baby leaf vegetables i.e., any leafy vegetable harvested at the early stage of development and in an active metabolic phase: after true leaf formation but before it is fully grown (di Gioia et al., 2017). The vegetable crops denoted as baby leaf belong to different families, such as Brassicaceae, Asteraceae, Amaranthaceae: the most common are lettuce (*Lactuca sativa* mainly Batavia type), lamb's lettuce (*Valerianella locusta* L.), wild and cultivated rocket (*Diplotaxis tenuifolia* L. and *Eruca sativa* Mill., respectively) and spinach (*Spinacia oleracea* L.). Therefore, they present a wide variety of leaf shapes, colours, textures, and flavours. In this category, the vegetable leaves are packaged whole, and the packets may include a singular species or a mix of two or more leafy vegetable.

1.2 LETTUCE

One of the main species cultivated for the fresh-cut and for the baby leaf market is lettuce (*Lactuca sativa* L.), of which several botanical varieties are known, such as *capitata*, *crispa, longifolia*, *acephala*, *angustana,* etc*.* Each variety is characterised by different plant morphology, different shape of the leaves and different colour (de Vires, 1997).

Lettuce is cultivated both in open air and in protected production facilities, including arrangement for soilless cultivation. In Italy in 2021, 338 933 tons of lettuce were produced in open air, on an area of 15 333 hectares, and 163 085 tons of lettuce were produced in protected environment, on an area of 4 592 hectares (*ISTAT*, n.d.). Crop cover serves as protection from external natural hazards such as insects or adverse weather, but also it allows for artificial manipulation of the environment in which plants grow. This helps to improve crop performance, for example by extending the time of year in which a certain crop can be grown, allowing production "out-of-season" and by increasing the number of production cycles. The result is a greater production and better quality (Gruda & Tanny, 2014). Covering crops may also protect lettuce from the wide range of fungal and bacterial pathogens to which it is prone. One of the most important diseases is downy mildew caused by the fungus *Bremia lactucae* (Regel), which can attack the plant throughout its vegetative cycle and typically forms a felt-like layer in the underside of lettuce leaf (Parra et al., 2016). *Sclerotinia sclerotiorum* (de Bary), *Sclerotinia minor* (Jagger), *Botrytis cinerea* (Pers.), *Rhizoctonia solani* (Kühn), and *Pythium tracheiphilum* (Matta) are important soil-borne fungal pathogens, which cause similar symptom in lettuce plants: the basal rot. Of these, *S. minor* and *S. sclerotiorum* are of greatest concern because they can affect a wide range of plant species and their sclerotia can remain dormant in soil for more than eight years (Mou, 2008). Finally, *Fusarium oxysporum* f. sp. *lactucae,* the causal agent of Fusarium wilt, is a host-specific pathogen and its symptoms vary in severity, from leaf chlorosis to plant death. It is promoted by high temperatures and in the last years new races of the pathogen are expanding in new areas(Gilardi et al., 2017, 2019), therefore, under climate change conditions, it poses a threat to lettuce cultivation.
Lettuce crops are also prone to bacterial pathogen such as *Pseudomonas cichorii* (Swingle), the causal agent of varnish spot, and *Xanthomonas campestris* pv. *vitians* (Brown), the causal agent of bacterial leaf spot of lettuce (Barrière et al., 2014).

Therefore, it appears clear that crops grown for the fresh-cut market, especially baby leaf crops such as lettuce, have to face several problems, like pathogens attack and intensive production. At the same time these plants, intended to feed increasingly demanding consumers, must be grown in a natural and ecological way. An answer to these requests could be found in plant growth promoting bacteria (PGPB), which help to improve crop performance in an environmentally friendly way.

1.3 PLANT GROWTH PROMOTING BACTERIA (PGPB)

Plan growth-promoting bacteria (PGPB) are well-known beneficial partners of plants, able to deliver numerous ecosystem services. To mention some of them, PGPB can increase soil fertility and agricultural productivity, improve food nutritional quality, improve health of plant and soil by

controlling pests, maintain nutrients cycles, promote crop pollination (Abhilash et al., 2016; Dubey et al., 2016; Sarma et al., 2015, They can also intervene in the remediation and management of contaminated and degraded lands (Pellegrino & Bedini, 2014), and contribute to biomass improvement both above and below ground, which is essential for water retention and erosion control (Weyens et al., 2009).
The PGPB deliver these improvements in a sustainable and environmentally friendly manner, triggering even more benefits like the reduction of pesticide pollution, nitrogen and phosphorous runoff, the reduction of watershed eutrophication and gas emissions from soil (Abhilash et al., 2012; Dubey et al., 2016).

Plants can benefit from forming associations with microorganisms present in their ecosystem (Santoyo et al., 2016). Bacteria can live both externally and internally to their host plant. When they colonise the leaf surface, they are called epiphytes, while those living within the soil, on the outside of the roots are called rhizospheric (Compant et al., 2010). Finally, bacteria living inside their host plant are called endophytes (Hardoim et al., 2008). Endophytic bacteria are considered a subclass of rhizospheric bacteria, in fact they are a specialized group of rhizobacteria that have acquired the ability to colonize their host plants (Reinhold-Hurek & Hurek, 1998). Endophytes share host plant growth promoting traits found in rhizobacteria, but their effects are generally greater than those provided by many rhizospheric bacteria (Hardoim et al., 2008).
PGPB can benefit the host plants through several different mechanisms. These can be direct, for example PGPB may favour the uptake of nutrients, or improve plant growth by modulating phytohormones both under normal and stress conditions (Ma et al., 2016). They may adopt indirect mechanisms, for example: antagonise phytopathogens through the production of antibiotic and lytic enzyme, or by creating unavailability of nutrients for pathogens (Miliute et al., 2015) (Figure 1.1).
The most important of these processes are briefly discussed below.

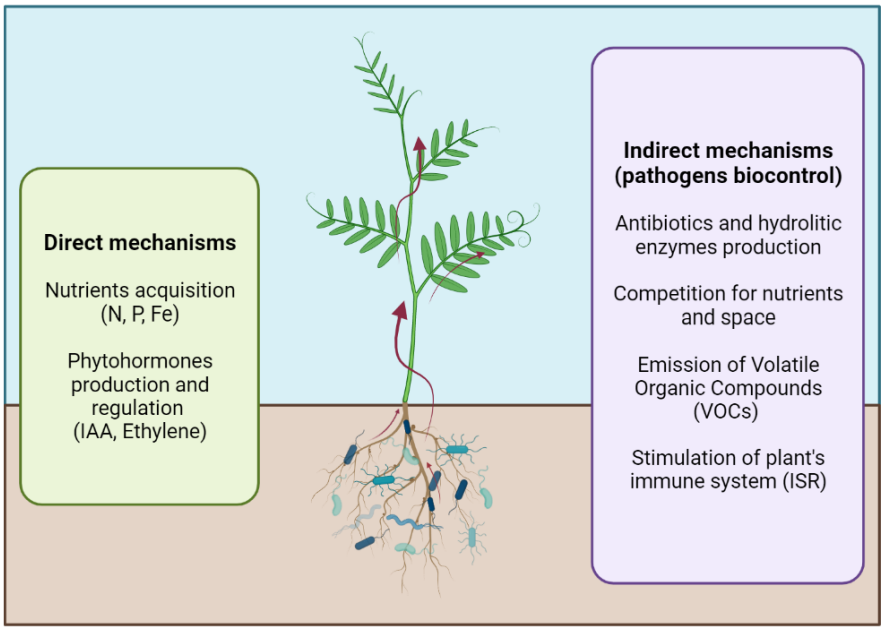

Figure 1.1: Beneficial mechanisms exerted by plant growth-promoting bacteria (PGPB) to stimulate healthy plant growth. Designed with app.biorender.com.

1.3.1 Direct mechanisms

1.3.1.1 Nutrients uptake

As already mentioned, PGPB can make it easier for plants to obtain an adequate amount of essential nutrients when they are scarce in the soils. The most important nutrients are nitrogen, phosphorus, and iron (Glick, 2012).

Plant growth depends on nitrogen (N), which is a limiting factor and its demand in agriculture is constantly growing (FAO, 2022). On the other hand, the massive use of inorganic nitrogen fertilizers has led many ecosystems to imbalance (Bishnoi, 2018). For this reason, in recent years efforts have been made to limit it by promoting sustainable agricultural practices (Araujo et al., 2011; Shah & Wu, 2019). Some bacteria are able to fix molecular nitrogen (N_2) present in the atmosphere in ammonia (NH^{3+}), which is soluble and available for plants that use it for the synthesis of various biomolecules (Soumare et al., 2020).
Biological nitrogen fixation (BNF) constitutes indeed one of the major sources of nitrogen for plants and it is a crucial step for the distribution of this element in the ecosystems (Santi et al., 2013; Sur et al., 2010). BNF is performed exclusively by prokaryotes, bacteria, and archaea, which possess a protein called nitrogenase that is highly conserved across all the different groups of bacteria involved in this process (Soumare et al., 2020). They can be classified into three categories: free-living N, associative N fixers, and symbiotic N fixers. The last two groups can be found in the rhizosphere of leguminous and non-leguminous plants (Mus et al., 2016; Santi et al., 2013). Root nodule symbiosis, in particular legume-rhizobium association, is the most effective method in N-fixing and is also the most important because it involves almost all food and fodder legumes (Soumare et al., 2020).

Phosphorous is another macronutrient crucial for the action of enzymes responsible for many physiological processes of plants (Ahemad, 2015). Although it is present in large amounts, like nitrogen, most phosphorus present in soil is insoluble. Even phosphorus applied as fertilizer, for the most part, forms complexes with soil and becomes unavailable for plants, consequently it cannot support their growth (Ezawa et al., 2002). Some PGPB are capable of solubilizing the P present in the soil and converting it into forms accessible to plants, such as orthophosphate. This is possible thanks to mechanisms like acidification, chelation, ion exchange and production of organic acids (Richardson & Simpson, 2011). The ability to solubilize phosphates is a trait common to bacteria associated with different crops. A big proportion of microbial populations associated to wheat, rice, maize, and legumes have been found to be able to solubilize mineral phosphates in plate assays, and among them a large number of PGP microbes have been reported, including members belonging to *Burkholderia*, *Enterobacter*, *Halolamina*, *Pantoea*, *Pseudomonas*, *Citrobacter,* and *Azotobacter* (Forchetti et al., 2007; Kumar et al., 2016; Nath Yadav et al., 2017; R. N. Singh et al., 2016).

Iron is essential for human life as it is involved in physiological processes such as respiration (Ma et al., 2016). Similarly, in plants Fe is required for multiple life processes, such as photosynthesis, chlorophyll synthesis, and nitrogen fixation (Kim & Rees, 1992). Iron is the fourth most abundant element in soil, where it is mainly present in the form of Fe^{3+} included in iron-carbonates, hydroxides, oxides and phosphates. Unfortunately, Fe^{3+} is hardly absorbed by plants, especially when it is present

in calcareous soils with pH between 7.5 to 8.5 (del Campillo & Torrent, 2008; Miethke & Marahiel, 2007). Fe deficiency is a worldwide limitation for crop production on alkaline soils (Falkowski et al., 2017). Some PGPB are able to produce specific low-molecular-weight (1–2 kDa) compounds called microbial siderophores, that are iron chelating agents and can bind Fe^{3+} with high specificity, plants can then acquire siderophores (Niehus et al., 2017). Thus, bacterial siderophores play a key role in supplying iron to plants under iron-deficient conditions (Ma et al., 2016). As observed, for example, by Marques et al., (2010), the production of siderophores by PGPB associated to maize plants, is strongly correlated with the increase of plant growth parameters, including biomass of shoots and roots. Although the mechanisms are not yet fully understood, Fe uptake may occur either by exchange of ligands between microbial and plant siderophores, or by direct uptake of microbial siderophores (Loper & Buyer, 1991). It has been observed that plants that lack Fe may modulate the composition of root exudates to favour the development of siderophore-producing microorganisms (Jin et al., 2010).

Moreover, some endophytic biota may also produce secondary metabolites like vitamin B_{12} (Nakos et al., 2017). Chandrasekaran et al. (2019) reported that *Bacillus subtilis* CBR05 induces vitamin B6 biosynthesis in tomato. Vitamin B6 possesses antioxidant activity, and it can modulate plant defence by regulating the antioxidant status in plants. While bacterial strain *Phyllobacterium* PEPV15 was reported to improve the yield, quality, and functionality of strawberry fruit by increasing their content in vitamin C (Flores-Félix et al., 2015).

1.3.1.2 Phytohormones regulation

Phytohormones are signal molecules that coordinate cellular activities and plant metabolism, acting on plant growth and development. Endophytic bacteria can increase the nutrient accumulation and the growth of the host plants by producing and regulating phytohormones (M. Singh et al., 2017). Overall, there are five types of plant hormones (abscisic acid, cytokinins, ethylene, gibberellins and IAA (indole-3-acetic acid)). IAA and ethylene are the most involved in the interactions between plant and bacteria.

The best known phytohormone among those produced by PGPB is IAA, which is involved in numerous physiological processes of plants such as cell-cell signalling, developmental regulation, and induction of plant defence systems (Etesami & Maheshwari, 2018; U. Singh et al., 2016; Yu et al., 2016). IAA may also promote lateral and adventitious root formation, may influence photosynthesis and metabolites biosynthesis, and may mediate resistance to stressful conditions (Glick, 2012). Furthermore, IAA can also control the synthesis of other plant hormones such as ethylene (Tatsuki et al., 2013; Woodward & Bartel, 2005). Thus, beneficial bacteria capable of modulating IAA concentration within plants can affect all these processes and in particular increase root biomass and adventitious roots production (Dias et al., 2009; Dias et al., 2017). Numerous bacterial isolates have been identified as efficient producers of IAA, including strains belonging to the genera *Psychrobacillus, Microbacterium, Lysinibacillus, Pseudomonas,* and *Bacillus* (Yu et al., 2016). An interesting example is represented by *Streptomyces* spp.. Thanks to their ability to produce IAA, these bacteria have been shown to be able to improve seeds germination, and elongation and dry weight

of roots in pea (Tokala et al., 2002), bean (Nassar et al., 2003), tomato (El-Tarabily, 2008; Passari et al., 2016), wheat (Sadeghi et al., 2012), chili (Passari et al., 2015), and rice (Gopalakrishnan et al., 2013, 2014).

Ethylene is another important phytohormone that controls several physiological and developmental processes of plants. For example, ethylene induces root initiation and nodulation, leaf senescence and abscission, cell elongation, fruit maturation, and auxin transport (Sun et al., 2016). Following biotic and abiotic stresses in the plant there is an increase in the production of ethylene which causes, among other things, the inhibition of root elongation, the development of lateral roots and formation of root hair (Dias et al., 2009). Some PGPB can produce an enzyme known as ACC (1-aminocyclopropane-1- carboxylate) deaminase. This enzyme hydrolyses ACC, which is a precursor of ethylene, into α-ketobutyrate and ammonia (NH^{3+}), that is used from the bacteria as source of nitrogen (Sun et al., 2009). Thus, the hydrolysis of ACC improves plant growth (Santoyo et al., 2016). Numerous studies on PGPB show how ACC deaminase activity is correlated with plant growth benefits, also in stress conditions (Glick, 2014; Orozco-Mosqueda et al., 2020). An interesting example of ACC activity is reported by Sun et al. (2009), who silenced the ACC deaminase gene of *B. phytofirmans* strain PsJN, growth promoter of canola (*Brassica napus*). The authors observed that the mutant was no longer able to stimulate the host's root growth. However, the reintroduction of the wild-type ACC deaminase gene restored the root growth promoting ability, confirming the crucial role of this enzyme.

It is worth mentioning here that there is evidence that some PGPB can produce gibberellic acid (GA) and cytokinin (Maheswari et al., 2013). GA is an endogenous growth regulator in plants, that stimulate stem and root elongation, flowering, and operate in breaking of dormancy in seeds (Chaudhry et al., 2017; Maheswari et al., 2013). Cytokinins, on the other hand, are a class of phytohormones that play a crucial role during the cell cycle, inducing cell division and, consequently, plant growth (Chaudhry et al., 2017; Maheswari et al., 2013). Cohen et al. (2009) showed that the GA produced by *Azospirillum lipoferum* contribute to relieve drought stress in maize plants treated with inhibitors of gibberellins synthesis. Bhore et al. (2010) instead, identified cytokinin-like compounds in the broth-extracts of two endophytes, *Psuedomonas resinovorans* and *Paenibacillus polymaxa*, isolated from *Gynura procumbens*. More work is needed to clarify a possible role of bacterial gibberellins and cytokinins in enhancing plant growth.

1.3.2 Indirect mechanisms

PGPB can also promote the growth of host plant through indirect mechanisms, the most relevant being the contrast to phytopathogens and pests (Sheoran et al., 2015). Phytopathogenic microbes pose a serious threat to sustainable agriculture and ecosystem stability worldwide, and consequently to human health. The use of microorganisms to control diseases seems to be a very promising strategy, furthermore biocontrol is an approach that respect the environment and the natural microflora of the soil (Köhl et al., 2019), as it reduces the use of agrochemicals (such as fertilizers and pesticides) (Compant et al., 2005). The biocontrol agents can act through multiple mechanisms such as competition for nutrients and space, parasitism production of antibiotics, siderophores, hydrolytic

enzymes (such as β-1, 3-glucanase, chitinases), volatile organic compounds (VOCs) or HCN or. However, a limitation of this approach is that the microorganisms, to be effective, must be able to adapt to rather variable conditions such as pH, temperature, and salinity (Nath Yadav et al., 2017).

One of the main mechanisms used by PGPB to counteract biotic stresses caused by phytopathogens is the synthesis of antibiotics (Kenawy et al., 2019), which can serve as antifungal, antibacterial, antihelminthic, antiviral, antimicrobial, phytotoxic, antioxidant, cytotoxic, and antitumor agents (Goswami et al., 2016).
Bacteria belonging to the genus *Pseudomonas* are known to produce compounds such as amphisin, 2,4-diacetylphloroglucinol (DAPG), hydrogen cyanide, oomycin A, phenazine, pyoluteorin, pyrrolnitrin, tensin, tropolone, and cyclic lipopeptides; while oligomycin A, kanosamine, zwittermicin A, and xanthobaccin are produced by *Bacillus*, *Streptomyces*, and *Stenotrophomonas* spp. (Compant et al., 2010). The importance of these compounds for the biocontrol can be understood by observing how mutant bacteria, deprived of the ability to synthesize antibiotics, lose all or most of the ability to prevent damage caused by the target phytopathogen (Heimpel & Mills, 2017). On the other hand, when it has been possible to isolate and purify specific antibiotics synthetised by PGPB, they were shown to be inhibitory of the same phytopathogens as the biocontrol PGPB strain itself (Glick, 2020). Interestingly, some antibiotics produced by PGPB find new uses as experimental pharmaceuticals. This group of bacteria may constitute a not yet exploited source of antibiotics that may result useful to control the alarming rise ascent of multidrug-resistant pathogenic bacteria (Compant et al., 2010; Narayanan & Glick, 2022).

In addition to the biocontrol mechanisms, in which PGPB produce substances that are themselves inhibitors of phytopathogens, it is possible for some PGPB to exert biocontrol by competing over nutrients (Barahona et al., 2011; Compant et al., 2005).
One element on which PGPB and pathogens compete most for is iron, which is essential for the growth of all living organisms. As already mentioned, under conditions of deficiency of bioavailable iron in soil, PGPB can produce siderophores that have a high affinity for iron chelating (Niehus et al., 2017). For this reason, siderophores are implicated in both direct and indirect promotion of plant growth by microorganisms.

More than 500 siderophores produced by microorganisms have been identified (Ali & Vidhale, 2013; Comensoli et al., 2017; Neilands, 1974) and have been grouped into three main categories based on the Fe-chelating group: hydroxamates, catecholates, and carboxylates (Schalk & Mislin, 2017). In Gram- negative bacteria Fe^{3+}-siderophore complexes bind to specific outer membrane transporters (OMTs), which transfer the complexes into the cytoplasm using the protein complex TonB-ExbBD, the periplasmic siderophore-binding proteins (SBPs), and a siderophore-permease-adenosine triphosphatase (ATPase) system (Faraldo-Gómez & Sansom, 2003; Klebba, 2016). In Gram-positive bacteria, lacking the outer membrane, the extracellular Fe-siderophore complexes bind directly to the SBPs anchored to the cell membrane, that translocate the complexes to the intracellular compartment via a siderophore-permease-ATPase system (Fukushima et al., 2014).

Microorganisms can also trigger systemic resistance in plants, allowing them a stronger and faster defence response against a possible subsequent invasion of phytopathogens (Conrath et al., 2006).

Plant systemic resistance can be divided into induced systemic resistance (ISR) and systemic acquired resistance (SAR), stimulated by non-pathogenic and pathogenic microbes, respectively (Kamle et al., 2020). SAR was first discovered in 1961 and it was recognised as a mechanism dependent on salicylic acid (SA), which accumulates and activates the expression of pathogen-related (PR) genes (Ross, 1961). In 1991, however, it was discovered that even non-pathogenic microbes were able to stimulate a resistance (Alstrom S., 1991; van Peer et al., 1991; Wei et al., 1991). The ISR was originally thought to be mechanistically similar to the pathogen-induced SAR. However, evidence has been provided that ISR is independent of both PR proteins and SA (de Vleesschauwer et al., 2008; Pieterse et al., 1996). It was therefore concluded that ISR and SAR are regulated by different signalling pathways. Therefore, although the terms SAR and ISR are commonly used interchangeable, it is more appropriate to speak of SAR when the induced resistance is triggered by a pathogen or demonstrated to be SA and PR protein dependent, while we speak of ISR when the induced resistance is triggered by a beneficial microbe or is shown to be SA independent (Pieterse et al., 2014). Using *Arabidopsis* mutants impaired in JA or ET signalling, JA and ET were shown to play a key role in PGPB-mediated regulation of ISR (Pieterse et al., 1998). However, Conn et al. (2008) observed that the same bacterium activates both systemic resistance pathways, providing resistance against two different pathogens. *A. thaliana* plants inoculated with endophytic *Actinobacteria* showed upregulation of both systemic resistance pathways, the JA/ET pathway (ISR) regulate the resistance to *Erwinia carotovora*, while resistance towards *F. oxysporum* was regulated by SA/PR proteins pathway.

Finally, an interesting feature of many bacterial species is the ability to emit VOCs. These low-molecular weight compounds can travel long distances, and some of them can play an important role in communication between microorganisms and plants, modulating pathways involved in plant defence (Wu et al., 2018), growth and development (Tahir et al., 2017). Basically, VOCs can interfere in all the field of actions of PGPB.

Exposure to VOCs can suppress the growth of plant pathogens without direct contact or just create a fungistatic condition. Some recent studies, for example, have shown that bacterial VOCs are effective against fungal pathogens such as *Aspergillus flavus, Botrytis cinerea, Aspergillus fumigatus, Penicillium citrinum, F. oxysporum,* and *Rhizopus stolonifera* (Erjaee et al., 2019; Rojas-Solís et al., 2018; Vaca et al., 2020).

Bacterial VOCs can also improve plant health and development by providing mineral nutrients, or by stimulating seed germination or plant immunity (Weisskopf et al., 2021). For example, dimethyl trisulfide and ketones, produced by a *Microbacterium* sp. associated with *A.thaliana* roots, significantly increased shoot and root growth (Cordovez et al., 2017). While VOCs derived from PGPR *Pseudomonas fluorescens* promoted the growth of *Nicotiana tabacum* and its induced systemic resistance (ISR) (Park et al., 2015). Similar effects have been shown by VOCs emitted by bacterial species from different genera, including *Pseudomonas, Bacillus, Arthrobacter,* and *Serratia* (Rani et al., 2023). Hence, bacterial VOCs intervene in plant-microbe communication and their action influences plant growth, ISR and metabolic pathways that regulate plant cellular function (Fincheira et al., 2021; Gámez-Arcas et al., 2022). Weisskopf et al. (2021) discussed the role of bacterial VOCs in the interactions with other microbes, plants, and insects, highlighting their potential biotechnological application.

Considering all the potential of PGPB, many scientists have been developing the use of beneficial bacteria as agents of biocontrol, in an effort to decrease the widespread use of chemicals as prevention to damages and losses caused by phytopathogen. Many of these beneficial microorganisms are already commercially available on market (Glick, 2012; Lahlali et al., 2022; Rana et al., 2020; Rani et al., 2023).

1.4 AIM OF THE THESIS

Considering the numerous beneficial effects that PGPB can exert on growth, nutraceutical qualities and health condition of their host plants, the aim of this work was to investigate new possible applications of endophytic bacteria to improve the quality of crops intended for the baby-leaf market, especially lettuce, in a sustainable and environmentally friendly way.

To achieve this purpose, in the first part of this thesis, we focused on the possible application of the VOCs emitted by *Streptomyces* sp. SA51, as biocontrol agent against *F. oxysporum* f.sp. *lactucae*. This bacterial strain is already known for its plant growth promoting and biocontrol activity (Vurukonda et al., 2018, 2020), however, the antagonistic effect of VOCs produced by this strain had never been tested. Therefore, given the many benefits deriving from VOCs application, and considering that VOCs emitted by some *Streptomyces* spp. are already known from literature for having antagonistic effect (Ayed et al., 2021), it was chosen to test the efficacy of VOCs produced by *Streptomyces* sp. SA51 to contrast *Fusarium oxysporum* f.sp. *lactucae* both *in vitro* and *in planta*.

Then the study proceeded with the exploration of new possible applications of different beneficial endophytes to improve the nutraceutical qualities of baby leaf crops. On one hand, the effects of three bacterial strains, from the strain collection of the Plant pathology laboratory of the University of Modena and Reggio, belonging to three different species (*Streptomyces* sp*., Pseudomonas* sp. and *Pantoea* sp.) on the polyphenol content of three plant species destined for the baby leaf market were investigated. On the other hand, a considerable number of endophytes (70) were examined to identify new bacterial strains capable of synthesizing vitamin B_{12} *de novo*. Firstly, a check was carried out on the actual ability of the bacterial strains to produce vitamin B_{12}. The best candidates were then tested for the possibility of using these bacterial endophytes for the biofortification of lettuce with vitamin B_{12}.

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Chapter 2

Mitigation of the infection induced by *Fusarium oxysporum* f.sp. *lactucae* by Volatile Organic Compounds (VOCs) produced by *Streptomyces* sp. SA51

ABSTRACT

Microorganisms can produce secondary metabolites known as VOCs, i.e., organic chemicals belonging to several chemical classes and characterised by a low molecular weight. Bacterial VOCs may be involved in a wide range of processes, such as the promotion of plant growth, the control of plant pathogens and pests, and the induction of resistance in plants. For these reasons, they are currently attracting more and more interest as a sustainable alternative to the use of synthetic plant protection products in agriculture.

Fusarium oxysporum f. sp. *lactucae* (FOL) is a soil-borne pathogenic fungus that infects all types of lettuce, therefore causing remarkable crop losses. Nowadays, four races of FOL are known. Resistant cultivars are extensively used as a strategy against this pathogen; however, they could promote the development of new and more aggressive FOL races.

In this study the biocontrol activity of VOCs produced by a strain of *Streptomyces* sp. was investigated against FOL race 1, both *in vitro* and *in planta*. VOCs produced by a culture of *Streptomyces* sp. showed an inhibitory effect against mycelial growth *in vitro* (- 8%), and they were also effective on lettuce seedlings (variety 'Chiara'). The McKinney index (MKI), used for assessing the plant health, decreased by 25%, when infected lettuce seedlings grew in the presence of bacterial VOCs. While uninfected plants grown in the presence of bacterial VOCs, showed a 48% decrease of the McKinney index. To identify the VOCs produced by the beneficial streptomyces, a solid phase micro extraction (HS-SPME), coupled to gas chromatography with mass-spectrometry detection (GC–MS) was employed.

Our research revealed a complex VOCs profile that included some interesting compounds with known antagonistic properties, such as Germacrene D and Phenylethyl alcohol. In conclusion, the VOCs-producing *Streptomyces* sp. strain demonstrated a good potential as biocontrol agent against FOL race 1 and as plant bio-stimulant for the growth of lettuce seedlings.

2.1 INTRODUCTION

Lettuce (*Lactuca sativa* L.) is an important crop for both fresh consumption and ready-to-eat market. In 2020, the Italian annual lettuce yield exceeded 0.5 million tonnes over a cultivated area of almost 20 thousand hectares (ISTAT, 2022). Commonly, lettuce plants are very susceptible to infection by pathogens, which limit their growth and development. Currently, *Fusarium oxysporum* f. sp. *lactucae* (FOL), the causal agent of the Fusarium wilt of lettuce is considered one of the most harmful pathogens (Gilardi et al., 2021). Fusarium wilt is a vascular disease-causing leaf yellowing and necrosis and, finally, plant death (Matheron et al., 2005). Resistant cultivars are extensively used to manage the disease: however, the effectiveness is compromised by the presence of four different FOL races (Garibaldi et al., 2004; Gilardi et al., 2017, 2019). Chemical fungicides are effective in the management of many pathogens: however, due to their clinical and environmental toxicity, the number of active substances allowed is very limited (Zubrod et al., 2019). An innovative alternative to chemical fungicides could be the use of biological control agents (BCAs). In particular, bacteria of the genus *Streptomyces* are successfully applied in the management of many phytopathogenic fungi such as *Pythium aphanidermatum* causing cucumber root rot (Postma et al., 2005), *Colletotrichum* sp. and *Curvularia lunata* causing *Brassica rapa* leaf spots (Wonglom et al., 2019), or *Botrytis cinerea* causing tomato gray mold (Shi et al., 2018). The use of *Streptomyces* strains could be compatible with the organic agriculture by notably reducing the environmental impact (Vurukonda et al., 2018). Nowadays only one commercial product is currently registered as a generic fungicide against soil-borne pathogenic fungi for lettuce hydroponics. The product is the LALSTOP ® K61 WP, a wettable powder made from dried spores and mycelium of *Streptomyces* strain K61.

The direct antifungal activity of streptomycetes is based on different principles: 1) inhibition or suppression of mycelial growth, as observed in several fungi, through the production of secondary bioactive metabolites, lytic enzymes or antibiotics (Mander et al., 2016; Wonglom et al., 2019; Zacky & Ting, 2013); 2) hyper parasitism *i.e.* living at the expenses of another parasite, either on or inside it (Chen et al., 2016; Jixia et al., 2005); 3) induction of plant resistance response (Abbasi et al., 2022; Tran et al., 2021); 4) competition for nutrients and space. Bacteria belonging to genus *Streptomyces* may also have a role in plant growth promotion (PGP). The PGP potential of *Streptomyces* was reported on many plants of economic interest such as tomato (Dias et al., 2017), wheat (Singh et al., 2019) and rice (Suárez-Moreno et al., 2019). In addition, *Streptomyces* are able to maintain their PGP characteristics even under adverse environmental conditions, such as salt or drought stress. This is an important feature, since, in recent years, the events of salt and drought stress are increasing due to climate changes and to inadequate agronomical practices (Nozari et al., 2021). The PGP effects may be mediated by different mechanisms that improve the host plant health, contributing to reinforce the response of the plant against pathogens. One of these strategies is correlated to nitrogen fixation and metal mobilization, for example through the production of siderophores (Gopalakrishnan et al., 2014). *Streptomyces* bacteria are also known to produce enzymes, such as amylase, chitinase, cellulase and protease, capable of decomposing complex nutrients (Jog et al., 2016), plant hormones, such as indole-3-acetic acid (IAA) that induces cell elongation and division (Manulis et al., 1994; Shi et al., 2018). In addition, *Streptomyces* species can produce a wide range of secondary metabolites. Among them it is possible to find many volatile organic compounds (VOCs):

low-molecular-weight compounds with high vapour pressures that can diffuse rapidly through air (Cordovez et al., 2015). These characteristics suggest that they might also function as remote signalling molecules acting with an ecological role in communication and competition within or between species (Fajardo & Martínez, 2008; Romero et al., 2011). Although the most characteristic VOC produced by *Streptomyces* is the geosmin, which provides the typical earthy aroma. One of the most representative studies on such subject, carried out by Cordovez et al. (2015), identified 536 different VOCs produced by 12 *Streptomyces* species.

VOCs may have many different functions, such as modulate the behaviour of the streptomycetes itself, affect the growth of other microorganisms or operate in cooperation whit them (Jones & Elliot, 2017). The greatest advantage of using VOCs is that these, to be effective, do not require a direct contact between the producing microorganism and the agricultural products (Freimoser et al., 2019). Volatiles from *Streptomyces* spp. have been proved to promote root and shoot growth of tomato plants (Dias et al., 2017) and *Arabidopsis thaliana* (Cordovez et al., 2015). (Wang et al., 2013) demonstrated that *Streptomyces alboflavus* TD-1 is effective against *Fusarium moniliforme,* probably for the action of the antifungal volatile compound dimethyl disulphide. Wonglom et al., (2019) identified 8 volatile compounds, belonging to different groups (alcohol, carboxylic acid and fatty acid), released by *Streptomyces angustmyceticus* NR8-2 and with antifungal/antimicrobial properties against *Colletotrichum* sp. and *Curvularia lunata* on Tokyo Bekana cabbage. Furthermore, VOCs produced by the PGP *Streptomyces lavendulae* SPS-33 significantly inhibited the development of *Ceratocystis fimbriata*, causal agent of black spot disease in postharvest sweet potatoes (Li et al., 2020).

The numerous studies conducted over the last decades underline the importance of testing new strains of *Streptomyces* spp. that could have a significant antimicrobial activity, in order to develop innovative bio-formulations effective against various phytopathogens and to reduce the input of chemical pesticides into the agricultural eco-systems. Consequently, in this paper we reported our study on the nature and effects of VOCs produced by a new *Streptomyces* sp. strain against FOL *in vitro* and *in planta*.

2.2 MATERIALS AND METHODS

2.2.1 Microorganisms and plant materials

A potential biocontrol agent*, Streptomyces* sp. SA51, belonging to the collection of the Plant Pathology laboratory of the University of Modena and Reggio, was used in the present experiments. The strain was isolated from the rhizosphere of an olive tree and stored at -80°C on liquid ISP2 (International Streptomyces Project-2 Medium) supplemented by glycerol 30%.
Fusarium oxysporum f. sp. *lactucae* (FOL) strain F1537, belonging to the collection of the Department of Agricultural and Food Sciences, University of Bologna, was isolated from the crown of a lettuce plant showing symptoms of Fusarium wilt. The FOL strain was stored in 30% glycerol at -80°C.
The lettuce *cv*. Chiara (ISI sementi S.p.a., Italy), susceptible to FOL, was chosen as model plant.

2.2.2 *Streptomyces* sp. SA51 cell suspension

Five days before inoculation, a fresh culture of SA51 was prepared on ISP2 agar medium (for 1 L of medium: 4 g of yeast extract, 10 g of malt extract, 4 g of dextrose and 20 g of agar) at 27°C for 48 h. A single colony of the *Streptomyces* strain was picked up and inoculated in 250 mL of ISP2 liquid medium and incubated at 27°C and 120 rpm in the dark. After 48 h, the liquid culture was centrifuged at 4,000 g for 15 minutes and the pellet was resuspended in autoclaved NaCl 0.9% to obtain an OD_{600} of 1.

2.2.3 *In vitro* assay

The effectiveness of VOCs produced by *Streptomyces* sp. SA51 on mycelial growth of FOL was tested by the double Petri dish assay (Di Francesco et al., 2020). For this purpose, ISP plates were inoculated by spreading 100 µL of *Streptomyces* cell suspension (10^6 colony-forming unit (CFU) mL^{-1}) and incubated at 27°C. The control consisted of ISP plates spread with 100 µL NaCl 0.9%. One day after bacteria inoculation, the lid of the plate was replaced by a plate of PDA with a mycelium plug (5 mm of diameter) of the FOL strain in the centre. The two plates without the lid were sealed together with a double layer of Parafilm and incubated at 27°C for seven days. In this way, the two microorganisms share the air without coming into contact with each other. For the control, FOL mycelium was coupled with plates spread with NaCl 0.9%. For the treatment and the control, ten plates (replicates) each were prepared. The mycelial growth was measured along two perpendicular diameters five and seven days after inoculation. The experiment was conducted twice. The percentage of inhibition of the mycelial growth (GI) was calculated using the following equation (Chen and Dai, 2012):

$$GI = [(d1-d2)/d1] \times 100$$

where d1 represents the average of the two control colony diameters for each plate (mm); d2 represents the average of the two treated colony diameters for each plate (mm). Percentage data were analysed statistically with RStudio using the t-test.

2.2.4 *Fusarium oxysporum* f.sp. *lactucae* (FOL) conidial suspension

Mycelium of FOL, grown on PDA for 5 days, was inoculated in 500 mL of V8 media and shaken at 120 rpm at 27°C in the dark for 5 days. The V8 liquid medium was prepared by adding 14.28 g of calcium carbonate (Sigma-aldrich) to 1L of Clarified V-8 Juice Broth, brick 1L (Campbell's). The mixture was centrifuged at 4,000 rpm for 20 minutes at 4°C. The supernatant was diluted at a ratio of 1:4 with distilled water. The liquid culture was filtered through a layer of sterile cheesecloth to separate the mycelium from the conidia and centrifuged at 4,000 g for 15 minutes. The pellet was suspended in NaCl 0.9%, the conidia were counted by a Thoma cell counting chamber and the suspension was diluted to obtain a concentration of 1×10^7 conidia mL^{-1}.

2.2.5 *In vivo* experiment

The effectiveness of VOCs produced by *Streptomyces* sp. SA51 against FOL was tested on lettuce plants. For this purpose, four treatments were prepared. The experiment was carried out two times and each treatment consist of 10 lettuce plants. The treatments were:

- FOL+*Str*: lettuce plants inoculated with FOL and grown in presence of VOCs produced by *Streptomyces* sp..
- FOL+ISP2: lettuce plants inoculated with FOL and grown in absence of VOCs produced by *Streptomyces* sp..
- W+*Str*: lettuce plants not inoculated with FOL and grown in presence of VOCs produced by *Streptomyces* sp..
- W+ISP2: lettuce plants not inoculated with FOL and grown in absence of VOCs produced by *Streptomyces* sp..

Seeds of lettuce *cv*. Chiara (ISI sementi S.p.a., Italy) were surface sterilised by soaking in ethanol 70% for 2 minutes and in 5% hypochlorite for 5 minutes and rinsed in sterilized water for 1 minute for 5 times. The seeds were then gently dried on filter paper and sowed in plug trays (60 wells/tray) filled with sterile peat (Potgrond H, Klasmann-Deilmann GmbH, Geeste, Germany) (features of peat: pH (in H_2O): 6; electric conductibility: 0.45 dS/m; dry bulk density: 160 kg/m^3; porosity: 85% v/v.) and placed in growth chamber with 16/8 hours day/night at 24 °C and 20 °C, respectively. Twenty days old lettuce seedlings were removed from the substrate and the roots were immediately dipped for 30 minutes in 100 mL of the FOL conidial suspension (1×10^7 conidia mL^{-1}), prepared as previously described, or in water for the negative control. The seedlings were placed in single pots filled with sterile peat (Potgrond H, Klasmann-Deilmann GmbH, Geeste, Germany) mixed with expanded perlite (Agrilit 3, Agraria di vita, Pescia, Italy, granulometry: 2 – 6 mm, density: 70 – 90 kg/m^3 +/- 20%, pH: 6,5 – 7,5) in a 5:1 ratio. For each treatment, the pots were arranged around the perimeter of four transparent boxes. In two boxes (FOL+Str and W+Str), three Petri dishes, containing ISP2 media inoculated 2 days earlier with 100 µL *Streptomyces* sp. SA51, were placed, without the lid, in the middle. In other two boxes (FOL+ISP2 and W+ISP2), three Petri dishes, containing ISP2 media inoculated 2 days earlier with 100 µL of NaCl 0.9%, were placed, without the lid, in the middle (Figure 2.1). Sterile water was sprayed on seedlings and the boxes were covered with transparent film to avoid gas exchange with

the external environment. Once *per* week, for three weeks, sterilised water was sprayed on seedlings and Petri dishes were replaced with freshly prepared ones.

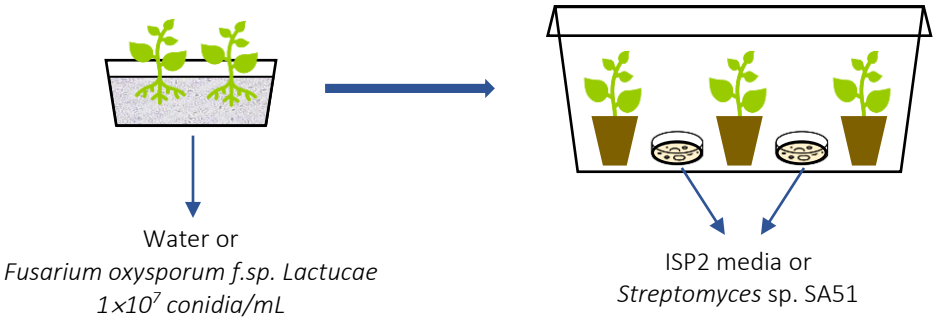


Figure 2.1: Schematic representation of the *in vivo* experiment.

2.2.6 Symptoms severity evaluation

Twenty-one days after inoculation, in order to verify the antifungal activity of VOCs produced by *Streptomyces* sp. SA51 against Fusarium wilt on lettuce plants, the severity of symptoms was assessed according to a disease rating scale ranging from 0 to 4 (Garibaldi *et al.*, 2004): 0 = healthy plant; 1 = reduction in development, initial symptoms of leaf chlorosis; 2 = reduction in development, evident leaf chlorosis, sometimes asymmetric development of the head and initial vascular browning; 3 = severe leaf chlorosis and inhibition of growth, evident deformation and vascular browning; 4 = plant strongly deformed with leaf chlorosis or completely necrotic leaves, totally wilted.
The data were expressed as McKinney index (MKI), calculated using the following formula:

$$MKI = [\sum(i * \cdot n_i) / (4 * N)] * 100.$$

where i is the rate of the scale (0–4); n_i is the number of plants with rate i, 4 is the maximum value of the scale and N is the total number of plants.

2.2.7 Morphological parameters

Twenty-one days after inoculation, morphological measurements were carried out to evaluate the effect of VOCs produced by *Streptomyces* sp. SA51 on lettuce plants size. For each plant, the epiphytic part was separated from the roots at the crown level, the fresh weight (FW) and the height of each plant were immediately measured. Plants were placed in an oven at 80° C for six days and weighed to determine the dry weight (DW).

2.2.8 Data analysis

Data were analysed with RStudio version 4.1.1. For data obtained from symptoms severity evaluation a one-way analysis of variance (ANOVA) was performed. Statistical comparison of means was carried out after the ANOVA to reveal the differences between treatments using Duncan Test ($\alpha = 0.05$).

Regarding the analysis of morphological parameters, a one-way analysis of variance (ANOVA) was performed for each parameter: height, fresh weight, and dry weight. A statistical comparison of means was carried out after to reveal the differences between treatments using Duncan Test (α = 0.05). In addition, a principal component analysis (PCA) was performed in order to evaluate the relationships between variables and parameters.

2.2.9 Volatile organic compounds analysis

VOCs were analysed by headspace solid-phase micro extraction (HS-SPME) followed by gas-chromatography/mass spectrometry (GC-MS) analysis.

Twenty-five-mL screw-cap glass vials, provided with Mininert© valves, were filled with 8 mL of ISP solid medium, which was inoculated with 20 µL of *Streptomyces* sp. SA51 cell suspension. As blank reference the medium inoculated with NaCl 0.9% was used. Two replicate vials were prepared for the *Streptomyces* sp. SA51, and two replicate vials were prepared for reference control. The vials were incubated at 27°C for 48 h. Afterwards, the VOCs were extracted from the vial's headspace and preconcentrated by SPME by using a divinylbenzene/carboxen/polydimethylsiloxane fibre (DVB/CAR/PDMS) (Supelco, Milan, Italy), mounted on a manual holder. The fibre was exposed in the headspace (HS) for 1 hour at room temperature (RT), after that the VOCs were desorbed into the GC injector port, set in splitless mode, for 3 minutes at 250 °C. Chromatographic separation was carried out by an Agilent GC-MSD (7890A/5975C, Agilent Technologies Inc., Santa Clara, CA, USA) provided with a Stabilwax-DA (0.25 mm i.d. × 30 m × 0.25 µm) capillary column (Restek, Milan, Italy). The GC oven temperature program was as follows: 50 °C for 3 minutes, raised from 50 to 160 °C at 5 °C min^{-1}, 160 °C for 2 minutes, raised from 160 to 240 at 20 °C min^{-1} and 240 °C for 2 minutes. Helium was used as carrier gas with a constant column flow rate of 1.0·mL min^{-1}. The transfer line temperature was maintained constant at 220 °C. 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 30 to 300. Peak identification was carried out by comparison with system libraries (Wiley, Nist). The relative VOCs content of each sample was reported as absolute peak area (Aprea et al., 2012). The sample unit was represented by two replicate analyses for each condition and the experiment was conducted once.

2.3 RESULTS

2.3.1 *In vitro* growth inhibition

In order to assess the antifungal effect on the mycelial growth of *F. oxysporum* f.sp. *lactucae* by the volatile compounds produced by *Streptomyces* sp. SA51, a double Petri dish assay system was set up. The effect was evaluated by measuring the pathogen radial mycelial growth at 5 and 7 days.
The results were reported as percentage of inhibition of mycelial growth (GI). The VOCs inhibited significantly the fungal mycelium by 12,6% (p-value < 0.0001) and by 8.2% (p-value = 0.0003) after 5 and 7 days, respectively, compared to the negative control (Figure 2.2 b). In addition, the morphological observation of the samples showed that the mycelium treated with bacterial VOCs was white and less compact at the external crown of the colony (Figure 2.2 a).

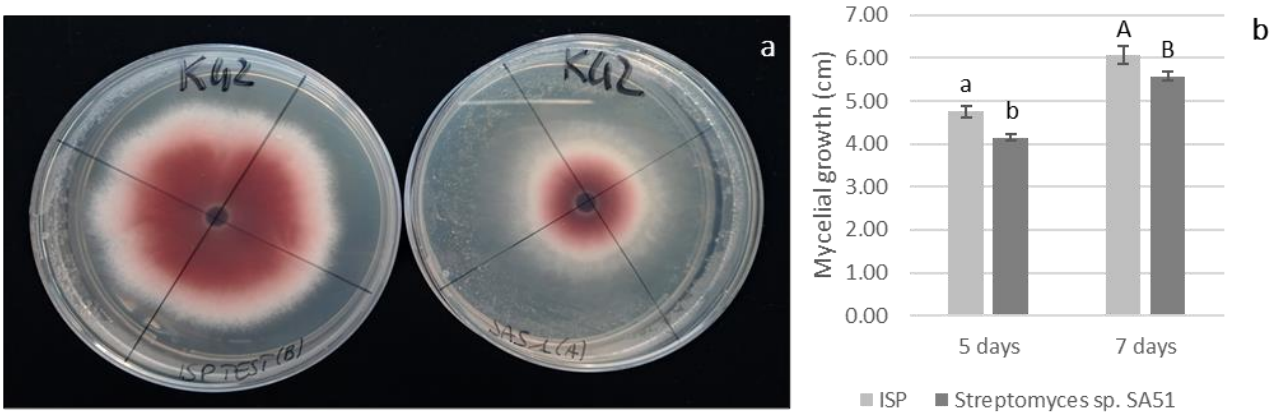
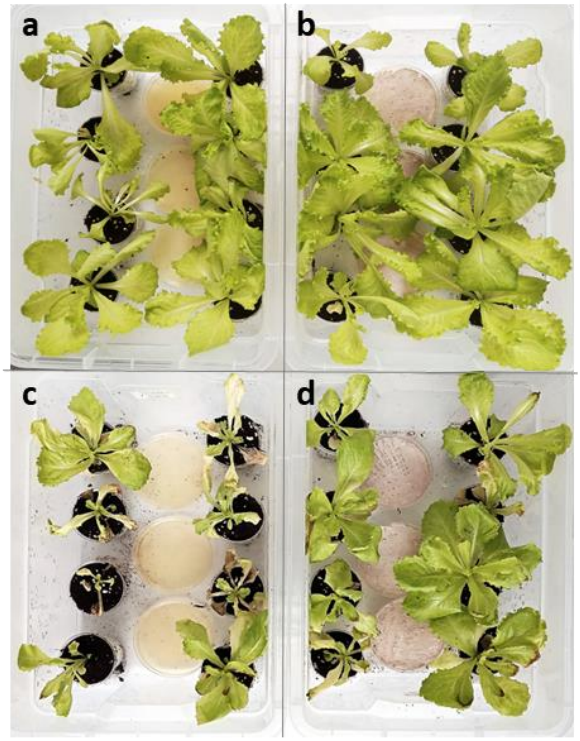
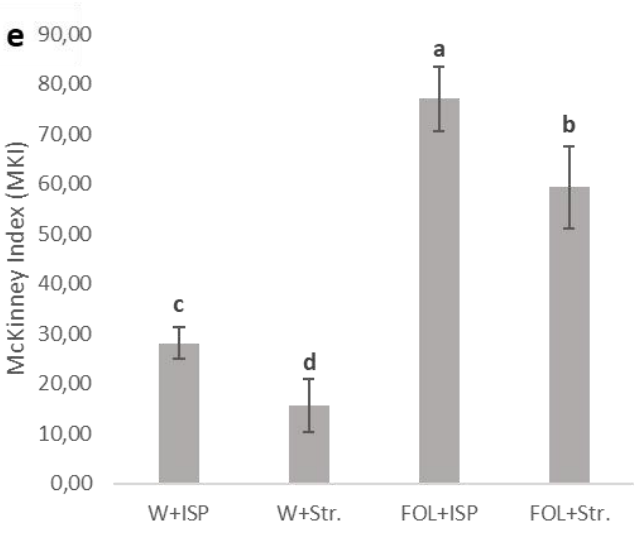


Figure 2.2: Effect of organic volatile compounds produced by *Streptomyces* sp. SA51 *in vitro*. a) Double Petri dish assay: *F. oxysporum* f.sp. *lactucae* on ISP agar media grown in presence (treatment - right) and in absence (negative control - left) of VOCs produced by *Streptomyces* sp. SA51. b) Colony diameter (cm) measured after 5 and 7 days at 27 °C. Each value is the mean of 10 plates (replicates) ± standard deviation. Above the histograms are reported the GI percentages. Different letters represent significant differences between the treatments within the time of the control according to Duncan Test (α = 0.05).

2.3.2 *In vivo* assay: effect of VOCs on fusarium wilt symptoms and on morphological traits of lettuce plants inoculated whit FOL

In order to test the antifungal efficacy of VOCs produced by *Streptomyces* sp. SA51 against FOL *in planta*, an *in vivo* assay was conducted as reported in paragraph 2.3.5. The efficacy was evaluated according to plants health condition, reported as McKinney index (MKI) value (Figure 2.3 e-f). Twenty-one days post-inoculation, the MKI of plants treated with FOL and grown in presence of VOCs produced by *Streptomyces* sp. SA51 significantly decreased by 23 % with respect to plants treated with FOL and grown in absence of VOCs (from 77.1 in FOL+ISP to 60.4 in FOL+*Str.,* p-value = 0. 008). Similarly, the MKI of plants not treated with FOL and grown in presence of VOCs produced by *Streptomyces* sp. SA51.decreased by 44.5 % with respect to plants not treated with FOL and grown in absence of VOCs (from 28.2 in W+ISP to 16.7 in *W+Str.,* p-value = 0.031). As it is possible observed in Figure 2.3 (a, b, c and d), there were clear differences in plant vigour and health conditions between treatments.



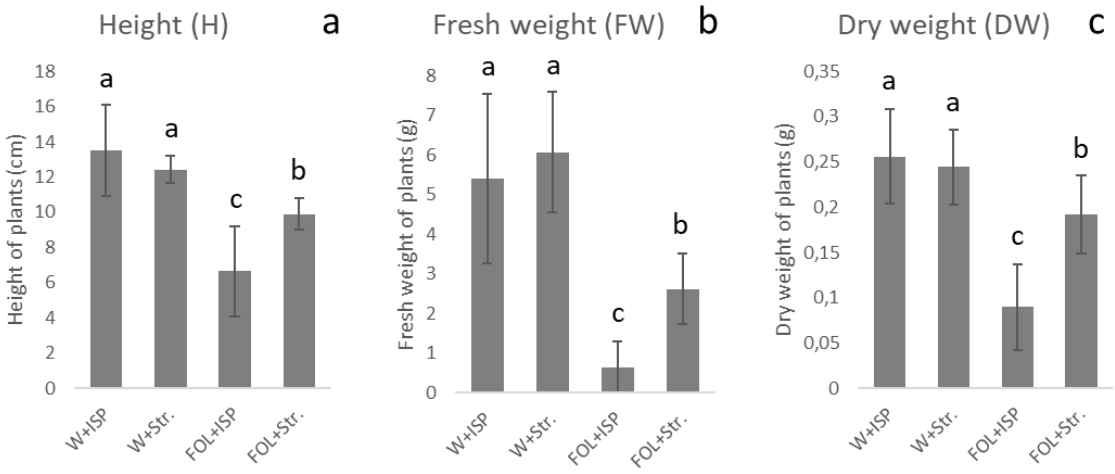


f	W+ISP	W+Str.	FOL+ISP
W+Str.	*	-	-
FOL+ISP	***	***	-
FOL+Str.	***	***	**

Figure 2.3: *In vivo* antagonistic effect of volatile compounds produced by *Streptomyces* sp. SA51 on fusarium wilt symptoms on lettuce plants 21 dpi. *a)* lettuce plants not inoculated with FOL and grown in absence of VOCs produced by *Streptomyces* sp. (W+ISP2) *b)* lettuce plants not inoculated with FOL and grown in presence of VOCs produced by *Streptomyces* sp. (W+*Str.*) *c)* lettuce plants inoculated with FOL and grown in absence of VOCs produced by *Streptomyces* sp. (FOL+ISP2) *d)* lettuce plants inoculated with FOL and grown in presence of VOCs produced by *Streptomyces* sp. (FOL+*Str.*) *e)* McKinney index (MKI) expressed as percentage for the 4 treatments. Each value is the mean of 3 replicates (8 plants each) ± standard deviation. Different letters above the histograms represent significant differences between the treatments according to Duncan Test (α = 0.05). f) The asterisks represent a significance code (0 = ***; 0.001 = **; 0.01 = *; 0.05 = .; 0.1 or > =), calculated according to Duncan Test (α = 0.05) for each couple of treatments.

In order to assess the stimulating effects of VOCs produced by *Streptomyces* sp. SA51 on the morphological parameters of lettuce plants treated or not with FOL, plant height, plant fresh weight (FW) and plant dry weight (DW) were measured.

There were significant differences between the plants treated with VOCs and inoculated with the FOL and the plants inoculated with FOL and without treatment (FOL+ISP and FOL+*Str.*). It is interesting to highlight that the presence of VOCs produced by *Streptomyces* sp. SA51 in the headspace of lettuce plants inoculated with FOL caused a remarkable increase of all parameters, in particular: the values of plant height increased by 49% with respect to the plants inoculated with FOL without the presence of VOCs produced by *Streptomyces* sp. SA51 in the headspace, the values of FW increased by 315% and the values of DW increased by 111%. On the other hand, the values of morphological parameters of plants not inoculated with FOL are equal when the plants grown in presence or in absence of VOCs. In general, the trend was similar among evaluated parameters (Figure 2.4).



d

	H			FW			DW		
	W+Str.	**FOL+ISP**	**FOL+Str.**	**W+Str.**	**FOL+ISP**	**FOL+Str.**	**W+Str.**	**FOL+ISP**	**FOL+Str.**
W+Str.	-	-	-	-	-	-	-	-	-
W+ISP		***	**		***	**		***	*
FOL+ISP	***	-	-	***	-	-	***	-	-
FOL+Str.	*	**	-	***	*	-	*	*	-

Figure 2.4: a) Average height values of lettuce plants ± standard deviations; b) Average fresh weight values of lettuce plants ± standard deviation; c) Average dry weight values of lettuce plants ± standard deviation. Different letters represent significant differences among treatments. d) The asterisks represent a significance code (0 = ***; 0.001 = **; 0.01 = *; 0.05 = .; 0.1 or > =), calculated according to Duncan Test (α = 0.05) for each parameter.

A PCA was performed on the morphological data in order to have an overall understanding of VOCs effect (Figure 2.5). The first component (Dim1) accounts for the 89.4% of the variability, while the second component (Dim2) accounts only for the 7.1%. Plant height and FW are the parameters that mainly influenced the PCA. There was a clear separation between the plants infected with FOL and the plants not infected. In addition, there was a further subdivision depending on the presence of VOCs produced by *Streptomyces* sp. SA51 during the growth, among the plants treated with FOL. On the other hand, it is not possible to observe the same subdivision among the plants not treated with FOL.

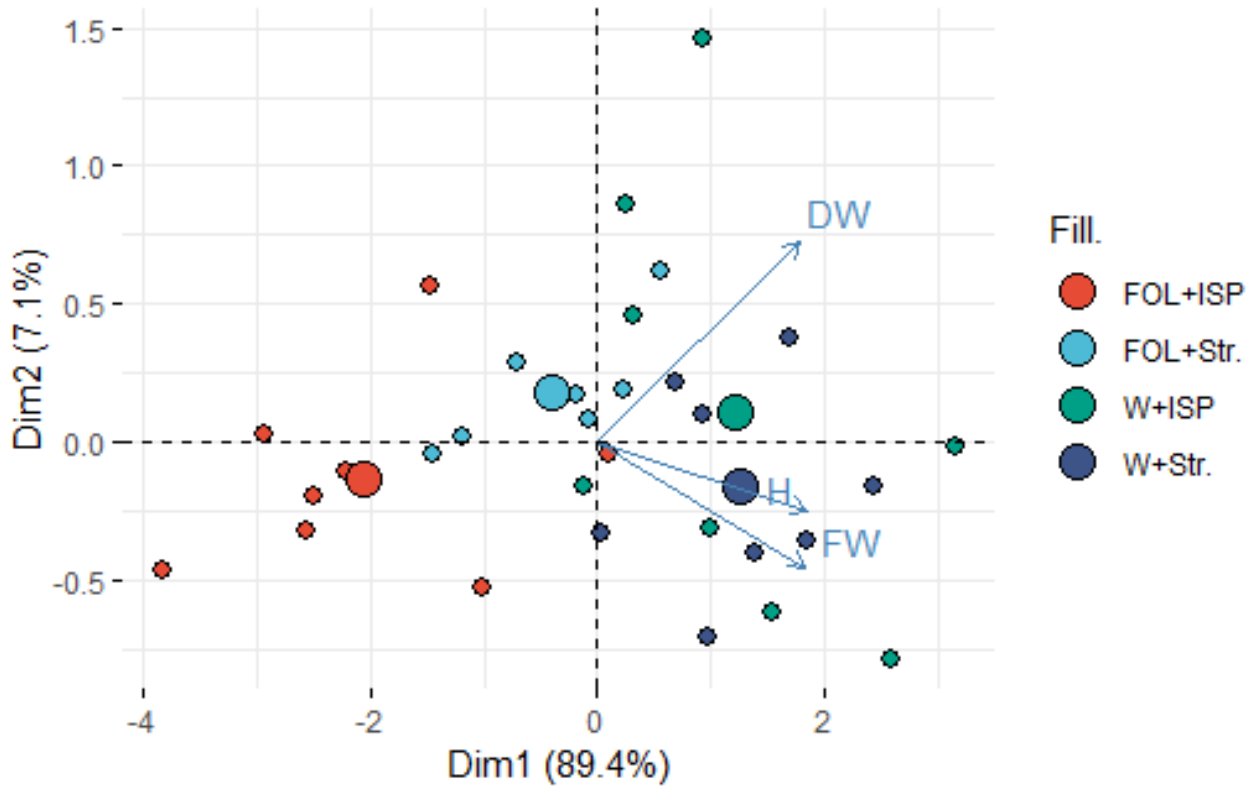
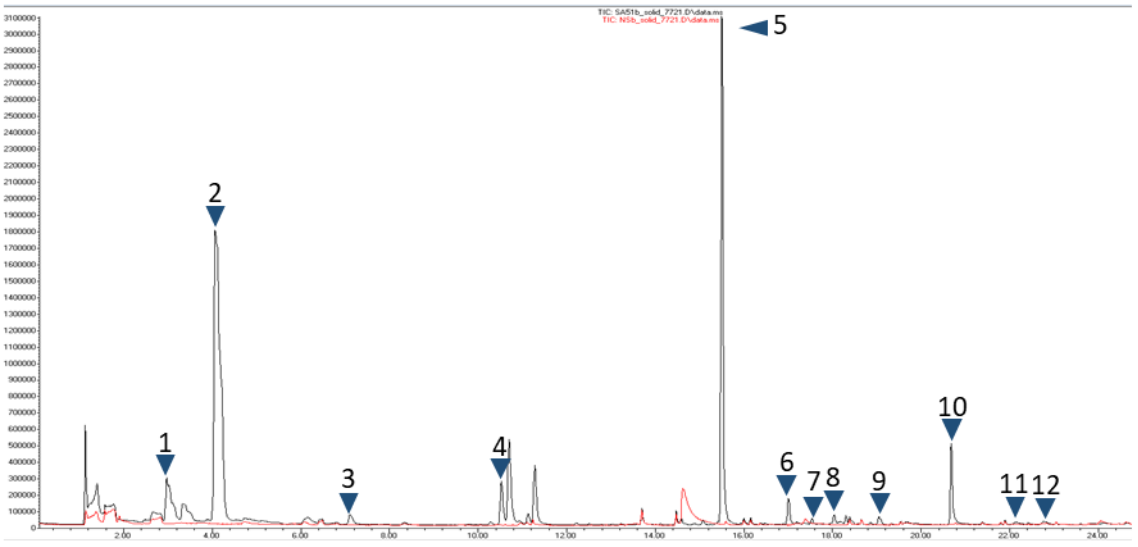


Figure 2.5: PCA plot representing *in vivo* effect of volatile compounds produced by *Streptomyces* sp. SA51 on morphological traits of lettuce plants inoculated with FOL. H, FW and DW arrows represent the original variables. The biggest dot for each colour, represents the average values for each treatment.

2.3.3 Identification of VOCs Produced by *Streptomyces* sp. SA51

Volatile organic compounds (VOCs) were extracted with HS-SPME technique for both the biological replicates of *Streptomyces* sp. SA51 and for the blank reference. Subsequently, they were analysed by GC-MS. From the chromatogram generated following the analysis of the VOCs emitted by *Streptomyces* sp. SA51 (about 60 peaks corresponding to 60 volatile compounds), were considered only the peaks, and therefore the VOCs, confirmed on both the biological replicates and which were not present in the chromatogram of the blank reference. Among these peaks, those identified with a match quality >65% against the system libraries, were further accounted. Thus, in total, 12 compounds were extrapolated (Figure 2.6).

Most of the VOCs identified, 7 out of 11, belonged to the terpenoid group, representing more than 91% of the total area related to the identified peaks. While, the classes of alcohols, polycyclic hydrocarbons, imines, trace amines and aromatic alcohols had one representative each.
It was not possible to determine the nature of the compound whose peak correspond to retention time (RT) = 17.01, and whose area accounted for 2.02% of the total.



	RT[a]	% Area (Ab*s)[b]	Name[c]	Chemical group/class[d]
1	3.28	3.65	2-Methylenebornane	Terpenoid
2	4.11	53.92	2-Methyl-2-bornene	Terpenoid
3	7.11	1.22	Benzenemethanol	Alcohol
4	10.54	3.70	1H-Indene	Polycyclic hydrocarbon
5	15.50	31.38	2-methylisoborneol	Terpenoid
6	17.01	2.02	Not identified	Not identified
7	17.54	0.38	γ-Muurolene	Terpenoid
8	18.04	0.75	Germacrene D	Terpenoid
9	19.64	0.48	Oxime-. methoxy-phenyl-	Imines
10	20.68	1.15	Geosmin	Terpenoid
11	22.12	0.87	Phenylethanolamine	Trace amine
12	22.75	0.48	Phenylethyl Alcohol	Aromatic alcohol

Figure 2.6: GC profile of bacterial volatiles: the red chromatogram refers to the blank (ISP2-medium), the black one refers to the *Streptomyces* sp. SA51 culture. The triangles highlight the relevant VOCs produced by *Streptomyces* sp. SA51 and are numbered according to the annexed identification table. The table reports: a) Retention time (RT) of the peak; b) Relative area (%) of the peak (Ab*s); c) Compound name according to International Union of Pure and Applied Chemistry (IUPAC); d) Chemical group/class of the compound.

2.4 DISCUSSION

Currently, four different races of *F. oxysporum* f.sp. *lactucae* are widespread in the main countries where lettuce is grown (such as Japan, United States of America, Iran, Taiwan, Brazil, Italy, Portugal, the Netherlands, France, etc) causing serious economic losses (Gilardi et al., 2017). The exploitation of bacteria naturally occurring in agricultural soil as potential BCA to find an environmentally friendly alternative for the control of FOL is a stimulating challenge of modern agriculture. Many bacteria belonging to *Streptomyces* genus are used in agriculture as BCAs. Studies on the use of *Streptomyces* for the control of FOL on lettuce plants in greenhouse conditions are limited and are mainly focused on the direct application of the bacterial suspensions on lettuce plants (Yadav et al., 2021). In a previous work, the effectiveness of *Streptomyces* sp. SA51 as BCA in controlling *Xanthomonas vesicatoria* was proven on potted tomato plants (Vurukonda et al., 2021). In the present work the attention was focused on the identification and the effects of VOCs produced by *Streptomyces* sp. SA51 against FOL *in vitro* and *in vivo*.

The choice to evaluate VOCs was motivated by the need to explore new possible strategies for the application of BCAs. VOCs, in fact, have the advantage of being effective even at low concentration and at a distance. In this way the direct contact between the antagonistic bacteria and the plant host is avoided, limiting the risks related to potential allergenic substances produced by BCAs. Furthermore, lettuce plants, as well as many other vegetable and fruit species, are mostly grown in greenhouses and VOCs could be easily spread in the environment by airtight containers as it happens, for example, for pheromones (Wang et al., 2013).

Studies on VOCs produced by several species of the genus *Streptomyces*, such as *S. globisporus* JK-1 (Q. Li et al., 2012), *S. coelicolor* (Danaei et al., 2013), *S. philanthi* RM-1-138 (Boukaew et al., 2013), *S. alboflavus* TD-1 (Wang et al., 2013), showed that these compounds can inhibit the mycelial growth, spore formation and germination of different plant pathogens. Whereas the VOCs produced by *S. angustmyceticus* NR8-2 (Wonglom et al., 2019) and *S. lavendulae* SPS-33 (X. Li et al., 2020) can reduce fungal disease symptoms by *in vivo* assay.

The results of the antifungal activity of VOCs *in vitro*, in which the pathogen and the antagonist share the HS without getting in contact with each other, demonstrated that, after 5 days, VOCs produced by *Streptomyces* sp. SA51 are able to interfere with the life cycle of FOL reducing both mycelial growth and pink pigmentation, but not showing high percentages of inhibition of the mycelial growth.

The comparison among the MKI values calculated for the four treatments (Figure 2.3) displayed a better activity of SA51 on lettuce plants against FOL *in vivo*, allowing to make some interesting considerations. Although the FOL strain used for the experiment had a strong virulence to lettuce plants, the exposure of plants infected with FOL to VOCs by *Streptomyces* sp. SA51 resulted in a significant reduction of symptoms severity and an increase of plant biomass and vigour. These results showed that these volatile compounds are able to interact directly with the pathogen, contrasting its development. On the other hand, when only the VOCs were applied, there was not a promotion of plant growth, as reported by Vurukonda et al. (2021) in tomato plants, in which an increase of root and shoot length and dry root biomass was observed after *Streptomyces* sp. SA51 inoculation. These

results could imply that a direct interaction between *Streptomyces* sp. SA51 and the host plant is necessary to trigger the plant growth stimulation. However, also the quantity of volatiles produced can influence the positive response of plants (Garbeva & Weisskopf, 2020). Although a significant promotion of plant growth and biomass was not found, PCA and MKI values differentiated plants treated with VOCs and not inoculated from plants nor treated or inoculated, suggesting some positive effects of volatile compounds on lettuce plants.

Overall, the *in planta* and *in vitro* experiments revealed that the VOCs emitted by *Streptomyces* sp. SA51 were clearly responsible for the antifungal activity against FOL. GC/MS analysis was necessary to understand which volatile compounds were involved in antifungal activity. According to our results, 5 compounds were identified as VOCs with a potential role in the antagonistic interaction between *Streptomyces* sp. SA51 and FOL.

One of the most promising antifungal compounds found in this study was the phenylethyl alcohol (PEA). PEA is a volatile organic compound with a rose-like odour that occurs widely in nature (Zhu et al., 2011). PEA has shown to have antifungal properties in many *in vitro* studies. Already in the 1960s, Lester (1960) revealed that PEA can inhibit the synthesis of RNA, DNA and consequently of proteins in *Neurospora crassa,* compromising the mycelial growth. Angel et al. (2016) reported that, when tested as diffusible metabolite using the bilayer plate technique, PEA showed a strong antifungal activity against *Ganoderma boninense,* until reaching 100% inhibition at concentration of 1.6 mg/ml or higher. A fumigation with PEA was also able to kill more than 50% of conidia of *A. flavus* and *A. parasiticus* and to reduce their production of aflatoxins with an exposure of only 6 hours and a gradually strengthened of activity with extension of exposure (Boukaew & Prasertsan, 2018).

PEA has also been proved to be effective in post-harvest conditions controlling *Botrytis cinerea* on strawberries, and *Penicillium digitatum* and *P. italicum* on citrus fruits and increasing the shelf life of products (Liu et al., 2014; Mo & Sung, 2007).

Recently, Intana et al. (2021) reported that the antifungal activity of PEA against *Fusarium incarnatum* can be increased in combination with other VOCs released by *Trichoderma asperellum* T76-14. This result represents an interesting suggestion for further studies. The remarkable efficacy of VOCs produced by *Streptomyces* sp. SA51 against FOL is, most likely, due to a combination of several volatile compounds rather than just one. Understanding and optimizing the composition of this blend is essential to achieve a precise and effective application.

The most abundant class of VOCs resulted from GC/MS analysis, both in terms of number of compounds and in terms of concentrations, were the classes of terpenoids, which includes monoterpenes and sesquiterpenes. Terpenoids are the largest class of plant secondary metabolites that interfere in plant-fungal interactions showing antifungal properties. They can be volatile, such as monoterpenes and sesquiterpenes, or not volatile. The latters can intervene only after pathogen contamination, preventing further invasion (de Oliveira-Júnior et al., 2018).

Over the past decades, many studies have shown an increase of volatile terpenoid emission in plants after fungal infection (Lee et al., 2016; Vuorinen et al., 2007). For example, recently, He et al. (2022) observed a negative correlation between the area of lesions caused by A*lternaria tenuissima* in susceptible *cultivars* of chrysanthemum, and the concentration of terpenoids in the leaves, including

γ-muurolene. However, it is more and more clear that bacteria also have a great potential as source of terpenoids with useful properties (Cane & Ikeda, 2012; Wang et al., 2018).
Germacrene D is a widely spread monocyclic sesquiterpenes and an important precursor of many other sesquiterpenes, including γ-muurolene (Bülow & König, 2000). It also has a variety of bioactivities such as insecticidal activity, mosquito repellent, anti-aphid and antitick activities (Birkett et al., 2008; Bruce et al., 2005; Ravi Kiran & Sita Devi, 2007). To our knowledge, no bacterial germacrene D has been identified so far, but it has been frequently detected as common component of essential oils of many plants, showing a significant antibacterial and antifungal activity. For example, in *Origanum vulgare* essential oil, where germacrene D has been detected as the second main component (Sahin et al., 2004). The essential oil of *Parentucellia latifolia* is also rich in germacrene D, accounting for 59.2% of the total oil. The essential oil demonstrated to be effective against bacteria and fungi frequently affecting cellulosic objects and infesting historical-artistic craftsmanship (Badalamenti et al., 2022). In our study, germacrene D represents only 0.75% of the total amount of VOCs produced by *Streptomyces* sp. SA51 that have been identified, however its antifungal potential may have been a determining factor for the overall effect.

Geosmin is a terpenoid typically produced by bacteria belonging to the *Streptomyces* genera and it is responsible for the typical smell of wet earth. As a result of the ubiquitous diffusion of these microorganisms, geosmin is a widespread compound (Gerber & Lechevalier, 1965). The saprophytic bacteria of the *phylum Actinobacteria*, to which the genus *Streptomyces* belongs (Kurtböke, 2017), are together with the myxobacteria and cyanobacteria the main producers of geosmin (Zaroubi et al., 2022). The high occurrence of geosmin synthase genes in a wide range of unrelated bacterial groups, suggests that geosmin plays a key role in the fitness of these microorganisms.
Geosmin was also the major terpenoid found in our study among the VOCs produced by *Streptomyces* sp. SA51.
Usually the geosmin-producing microorganisms synthetize also another odorous terpenoid, the 2-methylisoborneol (2-MIB) (Yamada et al., 2015)suggesting that the two compounds either synergize or are detected by a non-overlapping spectrum of targets. Therefore, as expected, *Streptomyces* sp. SA51 is also able to synthesize the terpenoid 2-methylisoborneol (2-MIB), which is the second most abundant VOC among those identified, covering 31.38% of the total area of the peaks of all VOCs. Even though geosmin is so widely distributed, its functions are still unclear. Zaroubi et al. (2022)proposed that geosmin may act as a warning signal advertising the production of toxic secondary metabolites. The authors tested the influence of both geosmin and 2-MIB on nematodes *Caenorhabditis elegans*, and the two compounds appear to work similarly altering the movement of nematodes and reducing their predation activity.
Recently, Boukaew & Prasertsan (2020) observed that geosmin had a severe effect on the growth of two fungal strains of the species *A. parasiticus* and *A. flavus,* with an inhibition ranging between 79.3% and 90.8% respectively, and 100% depending on the concentration: 1.0 µL/L the lowest and 100 µL/L the highest. The low concentration at which geosmin proved effective in this work, opens the possibility that the amount of geosmin emitted by *Streptomyces* sp. SA51 is sufficient to play a key role in the observed antifungal effect.

In the past decades, a number of studies have been conducted to test the effect of VOCs produced by different *Streptomyces* strains, on an equal number of fungal phytopathogens, both *in vitro* and *in planta*. *Streptomyces* VOCs have been tested against, just to name a few: *B. cinerea* (Ayed et al., 2021), *Rhizoctonia solani* on rice leaves (Boukaew et al., 2013), *Colletotrichum gloeosporioides* in post-harvest chili fruits (Boukaew et al., 2018, 2021), *A. flavus* and *A. parasiticus* on peanuts kernels (Lyu et al., 2020), *Ceratocystis fimbriata* on sweet potato (Gong et al., 2022), *P. italicum* on Philippine Lime (Q. Li et al., 2010) and many others. In most cases the results are in line with what was observed in the present work. The VOCs emitted by *Streptomyces* strains can be valid allies to counteract plant diseases caused by fungi. It is therefore logical to ask whether the VOCs identified by other authors, and consequently responsible for the inhibitory effects observed, are the same ones identified in our study. The presence of geosmin is always confirmed by all authors, but the general composition of VOCs emitted by the different *Streptomyces* strains is strongly variable. However, some of the compounds that we hypothesized to be responsible for the antagonistic effect on FOL, have also been identified by other authors as volatile compounds produced by other *Streptomyces* antagonists of pathogenic fungi. For example, among the VOCs identified by Lyu et al. (2020) the presence of 2-Methyl-2-bornene (2-M2B), 2-Methylisoborneol (2-MIB) and γ-muurolene, are confirmed. Gong et al. (2022), similarly to us, identified the γ-muurolene and Li et al. (2010) found the phenylethyl alcohol.

An interesting experiment was carried out by Cheng et al. (2020), who analyzed the volatile compounds emitted by nine *Streptomyces* strains belonging to nine different species. They were able to identify 33 volatile compounds, 16 of which were not previously reported in *Streptomyces*. The nine strains analysed share seven volatile compounds with the *Streptomyces* sp. SA51: geosmin, phenylethyl alcohol (PEA), 2-Methyl-2-bornene (2-M2B), 2-methylisoborneol (2-MB), germacrene D, γ-muurolene and 1H-Indene.

The growth conditions of the bacteria and above all the composition of the culture medium can significantly affect the quantity and the quality of VOCs produced by microorganisms (Gotor-Vila et al., 2017). Thus, our results are linked to the use of ISP2 media, a nutritionally rich media specific for the growth and maintenance of *Streptomyces*. Tryptone soya agar medium (TSA), due to its amino acid content, was proved to be the best medium to maximise the quantity and the quality of VOCs with antimicrobial activities (Gotor-Vila et al., 2017). Therefore, in the next future, culture media with different amino acids compositions and TSA should be evaluated with the aim of improving the antagonistic activities of VOCs produced by *Streptomyces* SA51. Furthermore, to better investigate which VOCs are actually responsible for the antifungal effect, an experiment both *in vitro* and *in vivo*, with the related pure synthetic compounds, should be evaluated.

2.5 CONCLUSIONS

In the present study, we described the ability of VOCs emitted by *Streptomyces* sp. SA51 in contrasting the lettuce pathogen *F. oxysporum* f.sp. *lactucae* (FOL) both *in vitro* and *in vivo.*

We can assess that the *Streptomyces* sp. SA51 is able to emit a relevant number of VOCs that may have a toxic effect against fungal plant pathogens. This antagonistic effect was found to be particularly evident when it was exerted against the development of symptoms of Fusarium wilt of lettuce, with respect to the effect exerted on mycelial growth *in vitro*. Therefore, we propose an effective approach to manage the Fusarium wilt of lettuce showing how microbial VOCs may be a promising, safety and environment-friendly tool for agronomic progress. In the next future, further experiments need to be conducted to understand which compounds are valuable of being applied, for example, as biofumigants in lettuce cultivation or in other situations, such as postharvest preservation.

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Chapter 3

Use of beneficial microbes to increase the polyphenolic content of fresh-cut vegetables

ABSTRACT

Polyphenols constitute a large group of bioactive compounds naturally present in plant-based foods. They can be constitutively synthesized by plants to carry out essential functions such as attraction of pollinators, plant stability or modulating of the respiration and photosynthesis rate. However, they can also be modulated, to protect plant in case of biotic or abiotic stresses. It has been demonstrated that plant growth promoting bacteria (PGPB) can improve plants polyphenolic content both in case of presence or absence of stress conditions.

This study aims to test the ability of three bacterial endophytes (*Streptomyces* sp. SA51, *Pseudomonas synxantha* DLS65 and *Pantoea* sp. S1) to increase the polyphenolic content of three vegetable cultivars destined to the baby-leaf market (red mustard, lettuce, and bull's blood).

For each fresh-cut vegetable species, seeds were treated separately with three bacterial suspensions, or with sodium chloride as control, and sowed. After 5 weeks growth parameters (height and fresh weight) were evaluated, then the plants immediately ground under liquid nitrogen and lyophilized. Phenolic compounds were extracted from freeze-dried powder. The total phenolic content was estimated for each treatment with the Folin-Ciocâlteu colorimetric assay, and the antioxidant activity was evaluated with the ABTS assay. Red mustard plants were significantly higher when treated with all the bacteria, while the fresh weight increased when treated just with *P.synxantha* DLS65 and *Pantoea* sp. S1. Lettuce and bull's blood did not account any significant variation. Significant increases of total phenolic compounds and antioxidant activity were observed in red mustard treated with *Pantoea* sp. S1, while lettuce in combination with *Streptomyces* sp. SA51 and *Pantoea* sp. S1 showed an increase of total phenolic compounds content. An LC-ESI-IT-MS/MS analysis was performed to assess the qualitative and quantitative modifications on the phenolic compound profiles for each treatment, the polyphenols identified were grouped in five classes HCA, HBA, anthocyanins, flavones, and flavonols.

Pantoea sp. S1 stimulated the production of anthocyanins in red mustard, of HCA, HBA, and flavonols in lettuce, and of HCA and anthocyanins in bull's blood. *Streptomyces* sp. SA51 treatment increased HCA, HBA, and flavonols in lettuce, while *P. synxantha* DLS65 did not stimulate any positive variation.

Based on the results, it is possible to conclude that endophytic bacteria can modify the phenolic content of plants, both from a qualitative and quantitative point of view. Therefore, their use may find a possible application for improving the nutraceutical value of vegetables.

3.1 INTRODUCTION

3.1.1 Polyphenols

Plant-based foods naturally contain polyphenols, which constitute a large group of bioactive compounds, *i.e.*, phytochemicals involved in protecting human health. Indeed, they play a role in contrasting cardiovascular disease, osteoporosis, neurogenerative diseases, cancer, and diabetes mellitus and other degenerative diseases (D'Archivio et al., 2007; Scalbert et al., 2005).

Polyphenols have an aromatic ring with at least one hydroxyl group as basic monomer. Based on their structure, which can vary from a simple molecule to a complex polymer with high molecular weight (El Gharras, 2009), they can be divided into classes. There is disagreement regarding their classification, the most used involves a subdivision in two main groups: flavonoids, including anthocyanins, flavones and flavonols, and non-flavonoid polyphenols, including phenolic acids, stiblins, phenolic alcohols, and lignans (Abbas et al., 2016; Durazzo et al., 2019) (Figure 3.1).

Polyphenols can be included in the diet through the intake of food of plant origin, such as fruit, vegetables, cereals, and coffee (Dragovicuzelac et al., 2007). More than 8,000 different phenolic compounds have been identified in the Plant Kingdom, representing one of the largest and most widely distributed groups of plant secondary metabolites (Durazzo et al., 2019).

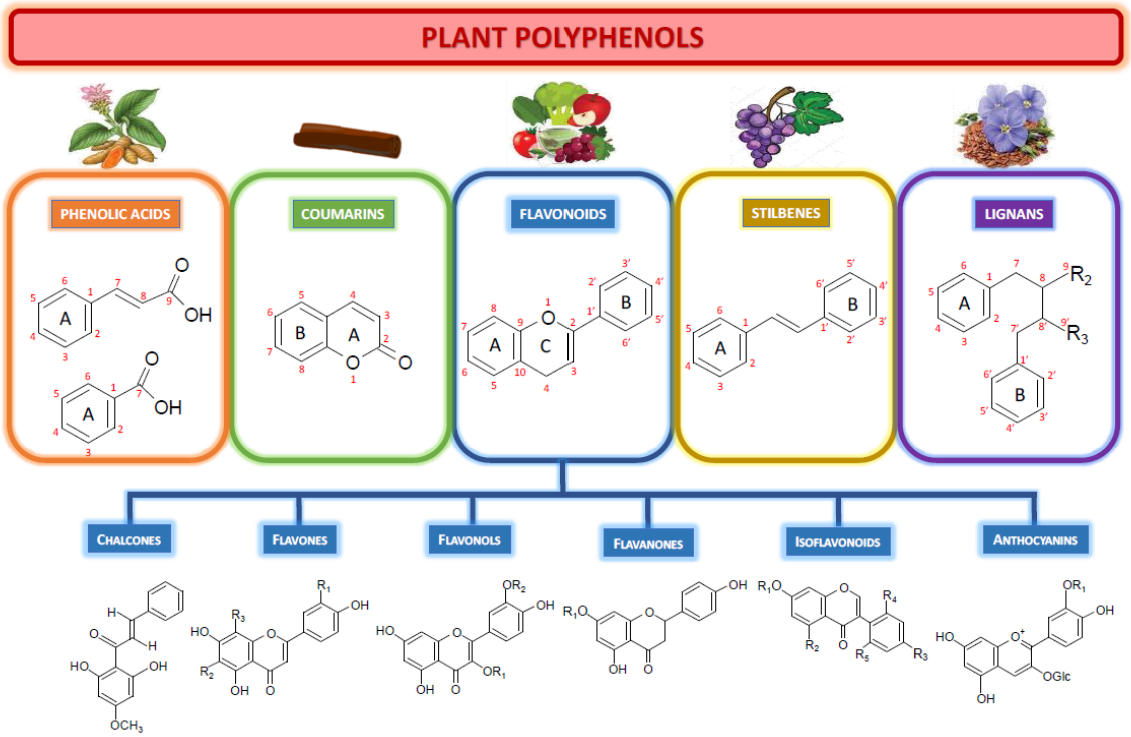


Figure 3.1: Polyphenol classification including phenolic acids, coumarins, flavonoids and their subgroups, stilbenes, and lignans (Hano & Tungmunnithum, 2020)

3.1.2 Classification of polyphenols

3.1.2.1 Phenolic acids

Hydroxycinnamic (HCA) and hydroxybenzoic (HBA) acids are the two classes into which phenolic acids (non-flavonoid polyphenols) are subdivided: they are characterized by a carboxyl group linked to a benzene ring (Lafay & Gil-Izquierdo, 2008) and are derived respectively from cinnamic and benzoic acids. The most common phenolic acids in plants are gallic acid, tannic acid, and capsaicin (Roche et al., 2017). These compounds can bring various benefits to human health,for example, ellagic acid, abundant in berries, has been shown to decrease blood pressure and cholesterol, to exert anti-inflammatory activities and even to reduce skin wrinkles due to solar radiation. Another example is gallic acid, which can be found in tea, mango, rhubarb, and soy and which is known for its antioxidant property (Roche et al., 2017).

Free phenolic acids are mainly present in fruits and vegetables, while cereals and derivatives are characterised by the presence of bound phenolic acids (Chandrasekara & Shahidi, 2010; Dueñas et al., 2016; Evoli et al., 2016; Stuper-Szablewska & Perkowski, 2019).

Hydroxycinnamic acids are present at high concentrations in many food products, including fruits (especially the red ones), vegetables, tea, cocoa, wine, coffee, and whole grains (el Gharras, 2009; El-Seedi et al., 2012; Santana-Gálvez et al., 2017). The strong interest in phenolic acids is associated with their potential applications in food preservation and in therapeutic field (Białecka-Florjańczyk et al., 2019).

Studies concerning hydroxycinnamic acids have evaluated their possible beneficial effects in fighting neurodegenerative diseases, thanks to their anti-inflammatory, antioxidant, and neuroprotective actions (M. Zhang et al., 2019). Moreover, they have shown to be able to protect endothelial functions through the attenuation of oxidative stress, the improvement of the bioavailability of nitric oxide and the decrease of the expression of E-selectin, ICAM-1, and VCAM-1, which play a role in cell adhesion to the vascular endothelium, and may have a part in tumour cell dissemination (Fuentes & Palomo, 2014; O'Hanlon et al., 2002). Furthermore, the association between the intake of phenolic acids and the reduction of blood pressure and triglycerides was observed (Grosso et al., 2018).

Hydroxybenzoic acids are mainly known for their antioxidant properties, which exert a potential beneficial effect in counteracting chronic diseases. Epidemiological studies revealed an inverse association between hydroxybenzoic acid intake and the risk of developing cardiovascular diseases and obesity (Guo et al., 2017; Tresserra-Rimbau et al., 2014a; Tresserra-Rimbau et al., 2014b).

3.1.2.2 Flavonoids

Flavonoids are phenolic compounds having a phenylbenzopyran chemical structure with a carbon skeleton of a C6-C3-C6 bound to a chroman ring (Pereira et al., 2009). Generally, three or more-hydroxyl groups are linked to the support structure (H. Zhang & Tsao, 2016). Flavonoids can occur as aglycones or as conjugated to sugars and/or organic acids (Khoddami et al., 2013; Kumar & Pandey, 2013). A large variety of pharmacological activities are attributed to flavonoids, thanks to their antioxidant, antibacterial, hepatoprotective, anti-inflammatory, and antihyperlipidemic properties (Abenavoli et al., 2018; Farhadi et al., 2019; Farhat et al., 2017; Iriti et al., 2017; Ninfali et al., 2017;

Riccio et al., 2018). The large and heterogeneous class of flavonoids can be divided into further subclasses.

3.1.2.3 Anthocyanins

Anthocyanins are pigments, whose colour varies from red-orange to blue-violet, and are responsible for the colour of many fruits and flowers. The basic structural unit of anthocyanins is the flavylium ion (2 - phenylchromenylium) (Pervaiz et al., 2017). Most of the anthocyanins occur as acylated by organic acids (p - coumaric, sinapic, caffeic, ferulic, or sinapic acids) via ester bonds (Zhao et al., 2017). The most common anthocyanins are cyanidin, delphinidin, malvidin, and peonidin. Some examples of anthocyanin-rich foods are black currants, red raspberry, elderberries, chokeberries, or strawberry, plums, pomegranates, blood orange, beans, cabbage, and red onions, are examples of anthocyanins sources (Albuquerque et al., 2018; Weber & Larsen, 2017). Anthocyanins have antioxidant and anti - inflammatory properties and their relevance for human health have been extensively explored through numerous studies. These compounds have shown promise in contrasting cardiovascular diseases and associated complications, cognitive outcomes, and cancer (Guo et al., 2016; Kimble et al., 2019; Lin et al., 2007).

3.1.2.4 Flavonols

Many fruits and vegetables, such as onions, apples, persimmon, saffron, berries, broccoli, lettuce, tea, and red wine, are good sources of flavonols (Bataglion et al., 2015; Durazzo et al., 2014; Hoffmann-Ribani et al., 2009; Sultana & Anwar, 2008). The most common flavanols are quercetin and kaempferol. Quercetin is found primarily in onions, apples, and berries; it is known for the antioxidant and anti-inflammatory activities, which play a key role in its many clinical effects, such as contrasting cancer, chronic inflammation, aging, and cardiovascular diseases (Durazzo et al., 2019). These properties are so recognised that, recently, several patents have been reported on quercetin derivatives used for therapeutic applications (Sharma et al., 2018). Additionally, there is clinical evidence that quercetin improves exercise endurance (Kressler et al., 2011). Kaempferol is present in various edible plants such as tea, broccoli, cabbage, kale, beans, endive, leek, tomato, strawberries, and grapes. Similarly to quercetin, it is known to possess anti-inflammatory, anticancer, and cardiovascular protective properties (Devi et al., 2015; Rajendran et al., 2014).

3.1.2.5 Flavones

The basic chemical structure of flavones contains two benzene rings connected through a heterocyclic pyrone ring. The main flavones found in foods are luteolin and apigenin, most commonly in their glycosidic forms (Durazzo et al., 2019). Dietary intake of flavones may promote health and exert therapeutic effects. These compounds, for example, may help to reduce weight gain (Adriouch et al., 2018), or they may contrast diabetes, amnesia, Alzheimer's disease, depression, insomnia, and cancer related to apigenin (Salehi et al., 2019). Furthermore, a negative correlation has been observed between the incidence of ovarian cancer and the intake of kaempferol and luteolin (Mohammadi et al., 2016). Flavones are present in many foods such as acerola, apricot, cashew, bean, cabbage, cardon, dandelion, apple, artichoke, mango, papaya, and onion (Bataglion et al., 2015; Colla et al., 2013; Hussain et al., 2013; Siriamornpun & Kaewseejan, 2017).

3.1.3 Functions of polyphenols in plants

Plants synthesize some phenolic derivatives constitutively and they carry out essential functions. For example, anthocyanins play a role as visual cues for the attraction of pollinators and for other animals which help in the seed dispersal (Mol et al., 1998), or they can contribute to the stability and robustness of plant tissues like lignin, suberin, or tannins, (Treutter, 2010). Polyphenols also take part in the modulation of physiologically essential processes such as signal transduction, transcriptional regulation, vesicle trafficking, and membrane permeability or they can decrease or increase the respiration and photosynthesis rates (Bidel et al., 2010).
However, plant growth and development are often disturbed by a wide range of environmental stresses that can be classified under two broad categories, abiotic and biotic.
Abiotic and biotic stresses have become major risks for food security (Zandalinas et al., 2021) and accounts for 50% and 30% of losses in agricultural productivity worldwide, respectively (Kumar & Verma, 2018). Phenolic compounds act as protective companion to various types of abiotic stresses, viz. salinity, frost, chilling, flood, drought, light, heavy metals, temperature, and nutrient deficiency as these compounds possess strong antioxidant properties. Antioxidant properties of these compounds help the plant to scavenge the reactive oxygen species (ROS) generated during the abiotic stress. A large number of researchers have reported that there is an accumulation of phenolic compounds whenever plant faces stress conditions (Kumar et al., 2020). Various biotic stresses like viruses, bacteria, fungi, insects, nematodes, etc. have a huge negative impact on agriculture (Dresselhaus & Hückelhoven, 2018). Phenolic compounds are synthesized by the phenylpropanoid pathway and concentrated in subepidermal layer of plant tissue when plants are attacked by the pathogens. Additionally, phenolics are also found covalently associated with plant cell wall and other types of phenolics present in waxes or on the peripheral surfaces of plant organs (Vishwanath et al., 2015).

3.1.4 Effect of Plant growth promoting bacteria (PGPB) on polyphenols in plants

One of the main functions of polyphenols in plants is to protect them from adverse effects of stresses through their antioxidant action (Hernández et al., 2009). Polyphenols have proven effective in counteracting oxidative stress induced by heavy metals. Due to their ability to donate electrons or hydrogen atoms, they act as metal chelators and ROS scavenger (Michalak, 2006; Psotová et al., 2003).
Numerous studies, over the last decades, have confirmed that plant growth promoting bacteria (PGPB) can support plant defence by improving their polyphenolic content.
Two strains of PGPRs belonging to the *Proteus mirabilis* species increased phenol levels in corn (*Zea mays*) under Cr-induced stress condition. Exercising their role as natural chelators, phenols have contributed to the overall health of plants (Islam et al., 2016).
Khanna et al. (2019) investigated the role of two PGPRs, *Pseudomonas aeruginosa* and *Burkholderia gladioli* on *Solanum lycopersicum* seedlings subjected to Cd stress. The presence of Cd triggered an increase of total phenols, flavonoids, and anthocyanin content by 30.23, 92.72, and 59.51% respectively. However, supplementation with *P. aeruginosa* and *B. gladioli* further improved the levels of these compounds. Furthermore, the already significant increase of total polyphenols

induced by Cd (368.7%) was further enhanced with the supplementation of *P. aeruginosa* (105.1%) and *B. gladioli* (52.3%). The study showed augmented levels of numerous polyphenols (catechin, caffeic acid, chlorogenic acid, umbelliferone, rutin, ellagic acid, gallic acid, quercetin, coumaric acid, epicatechin, and kaempferol. Catechin, rutin, gallic acid, kaempferol, caffeic acid, umbelliferone, ellagic acid, and coumaric acid) in all seedlings treated with Cd, *P. aeruginosa* and *B. gladioli*.
The rhizospheric bacterium *Enterobacter cloacae* PM23 has been shown to relieve salinity stress by enhancing radical scavenging capacity, relative water content, soluble sugars, proteins, total phenolic and flavonoid content in corn (Ali et al., 2022).
PGPB can influence the content of phenolic compounds in plants even in absence of stress. Cappellari et al., (2013) evaluated the effects of single inoculation and co-inoculation of two PGPRs (*Pseudomonas fluorescens*, *Azospirillum brasilense*) in marigold (*Tagetes minuta*). They observed that the total phenolic content was 2-fold higher in both singly and co-inoculated plants with respect to the control.

This study aims to test the ability of three bacterial endophytes (*Streptomyces* sp. SA51, *P. synxantha* DLS65, and *Pantoea* sp. S1) to modify or increase the polyphenolic content of three vegetable species produced for the baby-leaf market (red mustard, lettuce and bull's blood). The different species were chosen because they were expected to have different polyphenolic profiles: they belong to different families and have different colours. The purpose was to select bacteria that can be used in the production of vegetables richer in polyphenols, which therefore can constitute a functional food beneficial to consumers health, and, at the same time, a plant more resistant to biotic and abiotic stresses.

3.2 MATERIALS AND METHODS

3.2.1 Microorganisms and plants

Three bacterial strains were used for the experiment. The strain *Streptomyces* sp. SA51, isolated from the rhizosphere of an olive tree, was stored at -80°C on liquid ISP2 (International Streptomyces Project-2 Medium) and glycerol 30%. The strain *P. synxantha* DLS65 isolated from actinidia trees (*Actinidia deliciosa*) and the strain *Pantoea* sp. S1 isolated from ryegrass (*Lolium multiflorum*) seeds, were both stored at -80°C on liquid NSA (Nutrient Sucrose Agar) and glycerol 30%.
For the experiments, three plant cultivars destined for the baby-leaf market were used: Red mustard (*Brassica juncea),* lettuce *(Lactuca sativa*) and bull's blood (*Beta vulgaris*) (Figure 3.2). Seeds were provided by 'Azienda Agricola Gambaro Barbara e Paolo & C.s.s' (Noale, Italy) and stored at 4°C, until the use.

Figure 3.2: Plants species involved in the experiment: A) Red mustard (*Brassica juncea*); B) Lettuce (*Lactuca sativa*); C) Bull's blood (*Beta vulgaris*).

3.2.2 Bacterial cell suspensions

Five days before inoculation, a fresh culture of each bacterial strains was prepared: on ISP2 agar medium (for 1 L of water:4 g of yeast extract, 10 g of malt extract, 4 g of dextrose and 20 g of agar) for *Streptomyces* sp. SA51 and on NSA medium (for 1 L of water: 50 g of sucrose, 8 g of nutrient broth and 15 g of agar) for *P. synxantha* DLS65 and *Pantoea* sp. S1. The cultures were incubated at 27°C for 48 h. A single colony of each strain was picked up and inoculated in 250 mL of ISP2 liquid medium or NSB respectively and incubated at 27°C and 120 rpm in the dark. After 48 h, the liquid culture was centrifuged at 4,000 g for 15 minutes and the pellet was suspended in NaCl 0.9% to obtain an OD_{600} of 1.

3.2.3 Plant experiment

The seeds of the three plant species were surface sterilised by stirring for 2 min in 2% hypochlorite and then 5 times in sterile MilliQ water for 1 min. The seeds were soaked separately for 2 h in the three previously prepared bacterial cell suspensions or in 0.9% sodium chloride solution for the control. Therefore, for each plant species four treatments were set up: three treatments with bacterial strains suspension and the control.
Subsequently, the liquid was gently discarded, and the seeds were distributed in Petri dishes containing a layer of filter paper and 5 mL of sterile water. The seeds were incubated in the dark at

27 °C for 48 h. For each treatment, 30 germinated seeds were transferred in three trays of ten wells (replicates), obtained from a plug trays (60 wells/tray) filled with sterile peat (Potgrond H, Klasmann-Deilmann GmbH, Geeste, Germany) (features of peat: pH (in H_2O): 6; electric conductibility: 0.45 dS/m; dry bulk density: 160 kg/m^3; porosity: 85% v/v.) and placed in growth chamber with 16/8 hours day/night at 24 °C and 20 °C, respectively.
Thirty-five days (5 weeks) after sowing, morphological measurements were carried out to evaluate the effect of the three bacterial strains on plants size of the three cultivars. For each plant, the epiphytic part was separated from the roots at the crown level, the fresh weight (FW) and the height of each plant were measured, then it was immediately milled into fine powder in a mortar under liquid nitrogen. The powder was freeze-dried under vacuum at −56 C for 5 days, in a Heto Power Dry LL 3000 lyophilizer, Thermo Electron Corporation (USA). The freeze-dried powder was stored in the dark at room temperature.

3.2.4 Extraction of phenolic compounds

Forty mg of lyophilized plant material were added to 2 mL of water/methanol/formic acid (19:80:1, v/v/v) solution and homogenized by Vortex for 2 min. The suspension was then subjected to six cycles of sonication (Sonorex Super RK 255 H, Bandelin) at room temperature for 15 min, interspersed with 1 min of ice bath, after that the suspension was centrifuged at 4000g for 20 min. The supernatant was collected in a fresh tube, while the pellet was resuspended by Vortex in 1 mL of water/methanol/formic acid (19:80:1, v/v/v) solution. The suspension was again subjected to three sonication-ice bath cycles and then centrifuged under the same conditions. The two supernatants were combined.

3.2.5 Total phenolic compounds and antioxidant activity assays

The concentration of total phenolic compounds in the different plant species treated with the three bacterial strains, or with NaCl 0.9% for the control, was estimated through the spectrophotometric Folin–Ciocâlteu assay (Singleton et al., 1999) performed on the plant extracts. Gallic acid was used for the calibration curve, and the data were expressed as µmol of gallic acid per 100 g of fresh plant material.
The antioxidant activity of the different plant species treated with the three bacterial strains, or with NaCl 0.9% for the control, was determined with the ABTS (2,2’ -azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) assay, which was carried out according to Re et al. (1999) on the plant extracts. The results were expressed as µmol of trolox equivalent per 100 g of fresh plant material.

3.2.6 Identification and quantification of phenolic compounds by liquid chromatography electrospray ionization ion trap mass spectrometer (LC-ESI-IT-MS/MS)

Plant extracts were analysed on a HPLC Agilent 1200 Series system equipped with a C18 column (HxSil C18 Reversed phase, 250 × 4.6 mm, 5 µm particle size, Hamilton Company, Reno, Nevada, USA). The mobile phase consisted of (A) H_2O/formic acid (99:1, v/v) and (B) acetonitrile/formic acid (99:1, v/v). The gradient started at 4% B for 0.5 min then linearly ramped up to 30% B in 60 min. The mobile

phase composition was raised up to 100% B in 1 min and maintained for 5 min in order to wash the column before returning to the initial condition. The flow rate was set at 1 mL/min. After passing through the column, the eluate was split, and 0.3 mL/min was directed to an Agilent 6300 ion trap mass spectrometer. Two MS experiments were performed, one in ESI negative ion mode and one using positive ESI ionization (for anthocyanins), under the same chromatographic conditions. ESI-MS parameters were as follows: potential of the ESI source, 4 kV; capillary temperature, 400 °C (Del Rio et al., 2004).

Identification of phenolic compounds was carried out using full scan, data dependent MS^2 scanning from *m/z* 100 to 800. Anthocyanins were quantified in cyanidin 3-*O*-glucoside equivalents. Flavonols and flavones were quantified as quercetin-3-*O*-glucoside or kaempferol equivalents. Hydroxybenzoic acids were quantified as gallic acid or protocatechuic acid equivalents, whereas hydroxycinnamic acids as caffeic acid or coumaric acid or ferulic acid equivalents. Quantitative results were expressed as mg of compounds per 100 g of dry plant material. The limits of detection (LOD) were 0.1 ng for anthocyanins, 0.27 ng for hydroxybenzoic acids, 0.15 ng for hydroxycinnamic acids, and 0.19 ng for flavonols and other flavonoids. The limits of quantification (LOQ), defined as the lowest quantifiable concentration, were 0.6 ng for anthocyanins, 1.9 ng for hydroxybenzoic acids, 0.39 ng for hydroxycinnamic acids, and 2.1 ng for flavonols and other flavonoids.

3.2.7 Statistics

All data are presented as mean ± SD for three replicates for each prepared sample. One-way analysis of variance (ANOVA) was performed with RStudio version 4.1.1. Statistical comparison of means was carried out after the ANOVA to reveal the differences between treatments with the Tukey HSD post-hoc test, differences were considered significant when p-value ≤ 0.05.

3.3 RESULTS

3.3.1 Effect of bacterial treatments on plant growth

In order to verify the effects of treatment with the three bacterial strains (*Streptomyces* sp. SA51, *P. synxantha* DLS65 and *Pantoea* sp. S1) on the growth of the three plant cultivars (red mustard, lettuce, and bull's blood), two morphological parameters, height (H) and fresh weight (FW), were evaluated. Among the treatments only the red mustard plants significantly benefited from the use of microorganisms. Compared to the control, the height of red mustard plants increased by 13.7% when treated with *Streptomyces* sp. SA51 (p-value 0.009), by 22.6% when treated with *Pseudomonas synxantha* sp. DLS65 (p-value $7.2*10^{-5}$) and by 17.4% when treated with *Pantoea* sp. S1 (p-value 0.001) (Figure 3.3 A). While fresh weight increased by 25.4% when treated with *Pseudomonas* sp. DLS65 (p-value 0.016) and by 42.24% when treated with *Pantoea* sp. S1 (p-value 0.0001). In all the other cases, the treatments did not significantly affect the growth of plants (Figure 3.3 B).

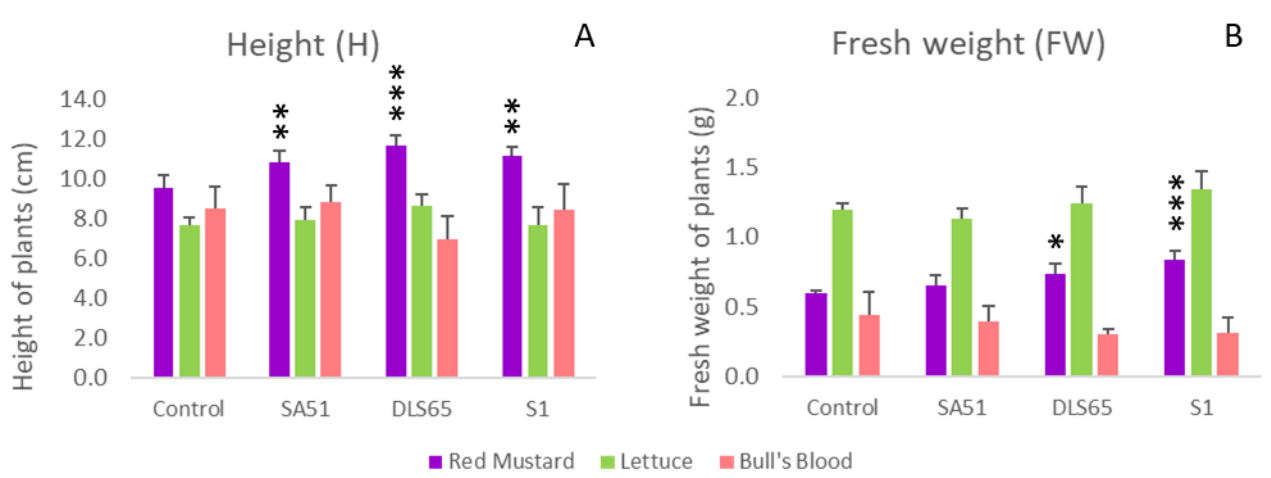


Figure 3.3: A) Average height values of plants ± standard deviations; b) Average fresh weight values of plants ± standard deviation. The asterisks above the histograms indicate significant differences among treatments and represent a significance code (0 = ***; 0.001 = **; 0.01 = *; 0.05 = .; 0.1 or > =), calculated according to Tukey HSD Test (α = 0.05) for each parameter.

3.3.2 Effect of bacteria on total polyphenolic content and antioxidant activity of plants

The variation of the total polyphenolic content of the three plant species (red mustard, lettuce, and bull's blood) after the inoculation with the three bacterial strains, was evaluated in comparison with a control, with the Folin–Ciocâlteu assay (Figure 3.4 A).

The total polyphenolic content was significantly improved in lettuce, both by *Streptomyces* p. SA51 and *Pantoea* sp. S1, with a recorded increase of 16.8% (p-value 0.0005) and 32.0% (p-value $4.6*10^{-6}$), respectively. *Pantoea* sp. S1 had a positive effect also on red mustard, where the gallic acid equivalent increase by 13.8% (p-value 0.0019). Bull's blood, on the other hand, register a negative impact of *P. synxantha* sp. DLS65, with a significant reduction of 16.6% (p-value 0.0035). The plant species with the highest polyphenolic content was red mustard (108.9 ± 4.3 µmol gallic acid /100 g fresh plant weight) followed by lettuce (117.5 ± 14.2 µmol gallic acid /100 g fresh plant weight) and then by bull's blood (91.9 ± 8.5 µmol gallic acid /100 g fresh plant weight).

In order to study how the antioxidant activity of the three plant species (red mustard, lettuce, and bull's blood) was modified by the treatment with the three bacterial strains, the ABTS assays was performed (Figure 3.4 B).
The antioxidant activity was only enhanced in red mustard inoculated with *Pantoea* sp. S1, where a 7.6% increase of µmol of trolox equivalent was estimated (p-value 0.005). Bull's blood, on the other hand, is negatively affected by treatment with all the bacterial strains, which cause a significant decrease of antioxidant activity. Red mustard resulted to be the plant species with the highest antioxidant activity (1206.4 ± 58.4 µmol trolox/100 g fresh plant weight) followed by bull's blood (603.4 ± 54.2 µmol trolox/100 g fresh plant weight) and lastly by lettuce (337.4 ± 51.8 µmol trolox/100 g fresh plant weight).

Red mustard resulted the plant species with the highest polyphenolic content and antioxidant activity, and both these values were positively affected by *Pantoea* sp. S1. Bull's blood registered a negative impact with all the bacteria used for the treatments, especially for the antioxidant activity. Lettuce was positively affected by *Streptomyces* p. SA51 and *Pantoea* sp. S1 regarding the polyphenolic content, but no effect was observed for the antioxidant activity (Figure 3.4 A and B).

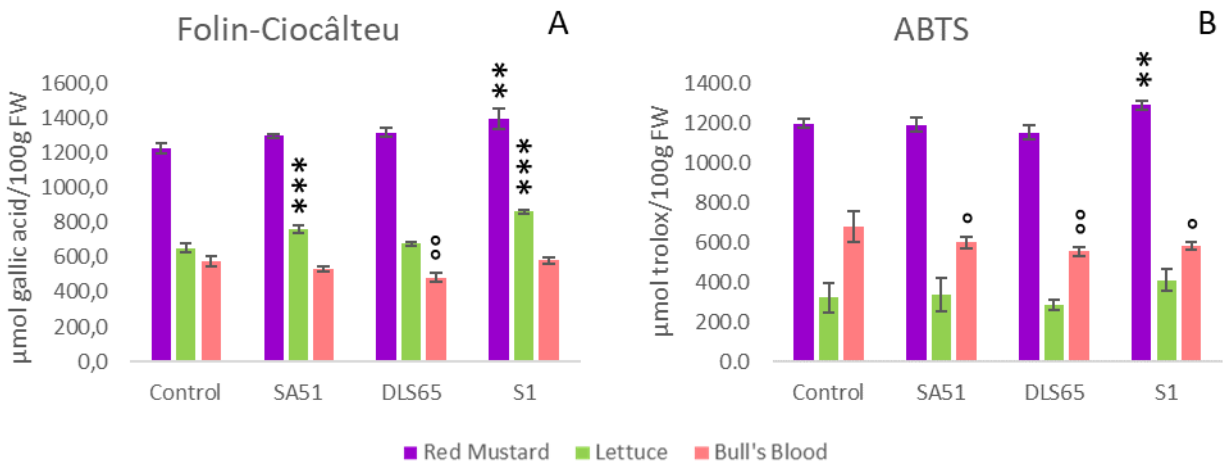


Figure 3.4: A) Total phenolic content, determined with Folin–Ciocâlteu assay of the three cultivars (red mustard, lettuce, and bull's blood), treated with three bacterial strains (*Streptomyces* sp. SA51, *Pseudomonas synxantha* DLS65 and *Pantoea* sp. S1), expressed as mg of gallic acid equivalent/100 g of fresh plant material ± standard deviations. B) Antioxidant activity determined with ABTS assay of the three cultivars (red mustard, lettuce, and bull's blood), treated with three bacterial strains (*Streptomyces* sp. SA51, *Pseudomonas synxantha* DLS65 and *Pantoea* sp. S1), expressed as mg of trolox acid equivalent/100 g of fresh plant material ± standard deviations. The asterisks and the circles above the histograms indicate positive and negative significant differences respectively, between treatments and control. Significance code (0 = ***; 0.001 = **; 0.01 = *; 0.05 = .; 0.1 or > =), calculated according to Tukey HSD Test (α = 0.05).

3.3.3 Effect of treatment with bacteria on classes of phenolic compounds

In this study, the phenolic profile and content of red mustard, lettuce, and bull's blood plants were analysed after the treatment with three bacterial strains The phenolic composition of the plants was investigated through LC-ESI-MS/MS analysis.

This approach allowed the characterization of the phenolic fraction of the plants, therefore the identification and quantification of phenolic compounds with respect to the reference standards. Fifty-four phenolic compounds were identified in the extract from red mustard, 29 in lettuce and 18 in bull's blood. After identification, the phenolic compounds were grouped by class (Figure 3.5 A and B).

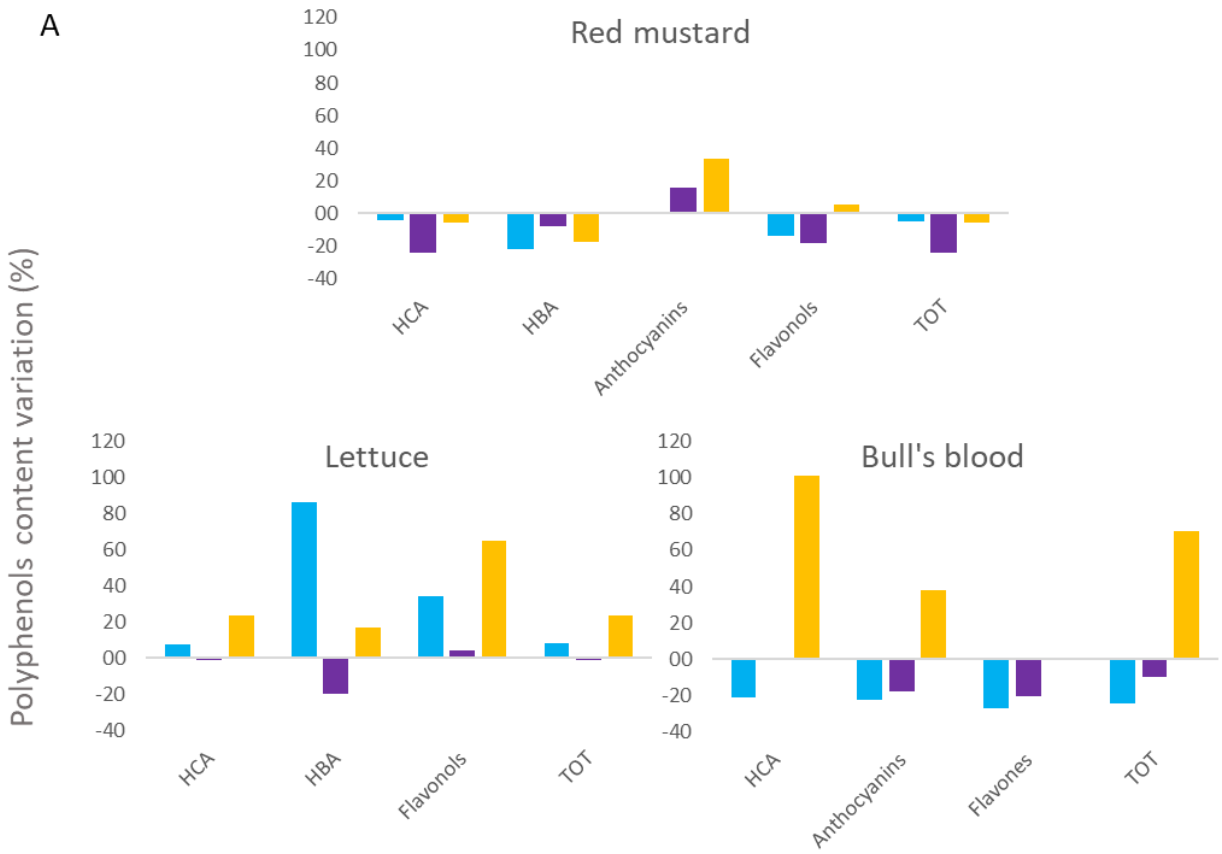
Red mustard resulted the richest cultivar both in the number of compounds, which were divided into four classes (HCA, HBA, anthocyanins and flavonols), and in the overall amount of these compounds. In red mustard, however, the only significant increase was observed for anthocyanins when the plants were treated with *Pantoea* sp. S1.

Lettuce benefited from the inoculation with both *Streptomyces* sp. SA51, and *Pantoea* sp. S1, in fact these bacteria were able to increase the content of all classes of polyphenols. In the bull's blood plants, the treatment with *Pantoea* sp. S1 drastically increased HCA and anthocyanins.

Therefore, it is possible to state that *Pantoea* sp. S1 represents the bacterial strain which more than the others was able to increase the polyphenolic content in the tested plants. The treatment with *Pantoea* sp. S1 improved the content of anthocyanins in red mustard, all classes of polyphenols in lettuce, and HCA and anthocyanins in bull's blood. *Streptomyces* sp. SA51 had a positive impact in combination with lettuce, where it was able to significantly increase all classes of polyphenols. *Pseudomonas synxantha* DLS65, on the other hand, did not show any positive effect in combination with the three cultivars.

Based on the trend of the phenolic compounds detected in this analysis, the plant species that most benefited from the inoculation with the bacteria was lettuce, followed by bull's blood and red mustard.

A



B	HCA	HBA	Anthocyanins	Flavonols	Flavones	TOT
RM_Control	16256.5±361.6	455.0±8.8	23.4±0.2	41.0±0.6	-	16775.9±208.3
RM_SA51	15573.9±1360	356.6±19.8	23.6±0.3	35.3±1.2	-	15989.4±1375.3
RM_DLS65	12286.4±278.1	418.0±6.4	27.1±1.4	33.6±0.7	-	12765.1±71.7
RM_S1	15321.0±506.1	375.8±7.3	**31.3±0.6****	43.2±1.0	-	15771.2±437.2
L_Control	8037.7±45.1	91.8±0.71	-	11.8±0.3	-	8141.4±15.5
L_SA51	**8630.3±61.0****	**171.4±3.4*****	-	**15.9±0.2*****	-	**8817.6±45.5****
L_DLS65	7954.0±37.9	73.6±1.3	-	12.3±0.1	-	8039.9±8.4
L_S1	**9953.6±115.4*****	**107.0±1.7****	-	**19.5±0.1*****	-	**10080.2±82.1*****
BB_Control	1211.9±14.8	-	633.6±3.9	-	239.9±4.6	2085.4±14.9
BB_SA51	957.8±11.9	-	488.5±8.1	-	174.4±2.3	1620.7±1.1
BB_DLS65	1201.2±26.9	-	521.8±9.3	-	190.5±4.2	1913.0±7.4
BB_S1	**2439.4±7.3*****	-	**876.0±8.6*****	-	238.9±2.1	**3554.4±5.8*****

Figure 3.5: A) Percentage content variations, with respect to the control, of each class of polyphenols according to the bacterial strain used for the inoculation (*Streptomyces* sp. SA51, *Pseudomonas synxantha* DLS65 and *Pantoea* sp. S1. B) Average value of the content of each class of each class of polyphenols, expressed as mg /100 g of dry plant material ± standard deviations. Values in bold represent a positive significant difference indicate positive significant differences between treatments and control. The asterisks indicate positive significant differences between treatments and control. (RM=red mustard, L=lettuce, BB= bull's blood). Significance code (0 = ***; 0.001 = **; 0.01 = *; 0.05 = .; 0.1 or > =), calculated according to Tukey HSD Test (α = 0.05).

3.4 DISCUSSION

Climate change ongoing in recent years is forcing the agricultural sector to deal with new threats and is bringing new challenges for scientists. It places considerable limits on the growth and development of plants because it introduces new stresses such as drought or floods and rising temperatures. In turn, the changes in environmental conditions in a certain area may create a favorable environment for the development and proliferation of pathogenic microorganisms which were absent up to that moment in that area, which means that farmers are generally unprepared to deal with them (Gilardi et al., 2019; Scholar et al., 2020; Shabani et al., 2014). Abiotic stresses such as the increasing of atmospheric CO_2 concentration, temperature, drought and salinity also pose a major threat to crop productivity and food security (Chaudhry & Sidhu, 2022). Furthermore, anthropogenic activities have caused over the past decades a sharp increase of heavy metal pollution such as Cd, Zn, Cu, Pb, Hg (Jacob et al., 2018; Zandalinas et al., 2021).

One of the main strategies implemented by plants to deal with all these stresses is the increasing of polyphenols content (Dresselhaus & Hückelhoven, 2018; M. Kumar et al., 2020; Vishwanath et al., 2015). In addition, polyphenols also have several beneficial effects on human health such as contrasting cardiovascular disease, osteoporosis, neurogenerative disease, cancer, and diabetes mellitus (D'Archivio et al., 2007; Scalbert et al., 2005). Some studies have demonstrated that PGPs endophytes can contribute to stimulate plants to produce phenolic compounds (Cappellari et al., 2013; Khanna et al., 2019). Therefore, the present work arose from this awareness, and aimed to test some bacterial endophytes to evaluate their ability to stimulate the production of polyphenols by plants. This study intended to evaluate the possibility of obtaining plants biofortified in terms of polyphenols content and, at the same time, more resistant to stresses.

First, height and fresh weight of the three baby-leaf plant cultivars subjected to the various treatments were measured. The intent was to assess the impact that bacteria had on growth and development of lettuce plants. As regards to *Streptomyces* sp. SA51, it was observed that the treatment did not have a statistically significant impact on plant growth of lettuce and bull's blood, but it increased the height of red mustard. This is consistent with what is reported in chapter 2 of this thesis. In that case, it was observed that VOCs produced by *Streptomyces* sp. SA51, while having a beneficial effect on plants health enough to reduce the MKI by 44.5% in absence of *Fusarium oxysporum* f.sp. *lactucae* infection, did not alter the height and fresh weight of lettuce plants. However, the effect of the same bacterium on the growth of red mustard was different. The *P. synxantha* DLS65 strain is already known for its biocontrol activity (Aiello et al., 2019; Tontou et al., 2016), but it has never been tested before as PGP. The strain showed only a positive effect on height and fresh weight only in red mustard, as well as *Pantoea* sp. S1, which was never tested before nor as PGP neither as biocontrol agent.

The average total phenolic content (Folin–Ciocâlteu assay) and the average antioxidant activity (ABTS assay) among all the treatment was much higher in red mustard than in lettuce (total phenolic content +77%, antioxidant activity + 258%) and bull's blood (total phenolic content +141%, antioxidant activity + 100%). Despite the dark red colour of red mustard, anthocyanins account for only about the 0.2% of the total phenolic compounds detected by LC-ESI-MS/MS. Therefore, the presence of anthocyanins cannot explain this difference in the total phenolic content among species.

The most abundant class of polyphenols in red mustard was the hydroxycinnamic acids (HCA), which account for about 96% of the total phenolic content. HCA ensure the integrity, the maintenance of the shape and the defence against pathogens of plant cell walls (Faulds & Williamson, 1999). Moreover, it has been demonstrated that the synthesis of HCA is up regulated, at transcript expression and enzymatic activity levels, under abiotic stress conditions (Martinez et al., 2016).
Carotenoids are a class of ubiquitous pigments that give to many plants, animals, and microorganisms a pigmentation that can take on different shades of yellow, orange, red and purple. Carotenoids are very important for their antioxidant activity even though they are not polyphenols. The central structural element of carotenoids is a polyene backbone consisting of a series of conjugated C=C bonds. This structure is responsible for both the pigmenting properties and their ability to interact with free radicals and singlet oxygen and thus act as effective antioxidants (Young & Lowe, 2018). All the three species, red mustard, bull's blood (Xiao et al., 2015) and lettuce (Ferrón-Carrillo et al., 2021)contain carotenoids in different amounts. This could be one explanation why, despite having a lower phenolic content than lettuce, bull's blood has a higher antioxidant activity.
Xiao et al., (2015) in their study, correlated the total phenolic content of six species of microgreens, included red mustard and bull's blood, with some flavour attributes, such as sourness, astringency, and bitterness. It is well known that the astringency of food is due to the presence of phenolic compounds, in fact in the study consumers who were subjected to a panel test indicated red mustard as significantly more astringent than bull's blood. This data correlates with the total phenolic content measured in the two species, both in the study conducted by Xiao et al. (2015) and in our work. In both cases, this value is more than doubled for red mustard.
Our study partially confirms what has been observed in other works. In fact, even though in our experiment, in some cases (red mustard + *Pantoea* sp. S1, lettuce +*Streptomyces* sp. SA51 and lettuce + *Pantoea* sp. S1), the increase of phenolic content and/or of antioxidant activity was statistically significant. Other studies showed an increase of the total phenolic content much greater: 2-fold higher after treatment with PGPs bacteria (Cappellari et al., 2013; Lavania et al., 2006).
However, Khanna et al., (2019) showed how the already positive effect of two PGPR strains, *P. aeruginosa* and *B. gladioli,* on the total phenolic content of *S. lycopersicum,* was strongly strengthened by the introduction of an abiotic stress such as Cd supplementation. Therefore, it could be an interesting future perspective, to verify what is the effect of *Streptomyces* sp. SA51, *P. synxantha* DLS65 and *Pantoea* sp. S1 on phenolic content of the three vegetable species object of this study, in stress condition. In light of what was observed in chapter 2 of this thesis, it could be useful to investigate what could be the outcome of a combined inoculation of *F. oxysporum* f.sp. *lactucae* and *Streptomyces* sp. SA51 in lettuce seedbed, in order to have a more complete understanding of the mechanisms of antagonism exerted by BCA.
As regards the subdivision of phenolic compounds by classes, it is interesting to note that the same bacterial strain had a different impact on the same class of polyphenols in different plant species (Figure 3.5). To cite just a few examples, hydroxybenzoic acid (HBA) were strongly potentiated by *Streptomyces* sp. SA51 in lettuce, while they were reduced by the same bacterium in red mustard. Hydroxycinnamic acids (HCA) were doubled by treatment with *Pantoea* sp. S1 in bull's blood, were increased by 23% in lettuce, and decreased by 16% in red mustard. Therefore, based on these results, it is not possible to predict what impact a certain bacterial strain will have on a certain plant species

without specific trials. Therefore, further investigations involving more bacterial strains and more plant species will allow to identify which is the optimal microorganism or combination of microorganisms with which to treat the different plant species in order to induce a greater production of phenolic compounds. In this way it will be possible to maximize the positive effects for plants and for humans, such as greater resistance to biotic and abiotic stresses, and healthier food with a greater nutritional value for humans.

3.5 CONCLUSIONS

In the present study, we described the effect of three bacterial strains *Streptomyces* sp. SA51, *P. synxantha* DLS65 and *Pantoea* sp. S1 on the growth and the polyphenolic content of three vegetable cultivars destined for the baby-leaf market (red mustard, lettuce and bull's blood).

Based on the results of the obtained results, it is possible to conclude that the impact of the bacteria was different according to the cultivar inoculated, however some positive effect has been observed in all the cultivar after treatment with *Streptomyces* sp. SA51 and *Pantoea* sp. S1. Further studies are needed, this work opens to new investigations about the possibility of using these strains individually or in combination to improve plants polyphenolic content, and consequently to the improvement of their resistance to stresses, health conditions and nutraceutical content.

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Chapter 4

Food quality improvement through vitamin B_{12}-producing bacteria

ABSTRACT

Vitamin B_{12} (cobalamin) plays a crucial role in animal and human health, and its insufficient intake can cause many health issues, such as megaloblastic anaemia. Vitamin B_{12} can be produced *de novo* only by certain bacteria and archaea. Humans can obtain this vitamin exclusively through diet, mainly from animal products. Vitamin B_{12} is not synthesised by plants, and consequently, vegetarians, vegans, and people with poor diets are at risk of vitamin B_{12} deficiency.

Nevertheless, due to the symbiosis between plants and certain vitamin B_{12}-producing bacteria, vitamin B_{12} can be found in small amounts in a few plants. Endophytic bacteria are already widely exploited in agriculture for enriching plant food with different nutrients and micronutrients, such as mineral elements, antioxidants, secondary metabolites, and vitamins. This process is known as biofortification.

The aim of this study was to identify endophytic bacterial strains able to synthesise vitamin B_{12} *de novo* and verify whether these strains can enrich edible plants with the vitamin.

First, an *in-silico* genome analysis was carried out on 69 genomes of isolates from the AIT strain collection and the genome of the reference strain *Pseudomonas denitrificans* ATCC 13867, already known for its ability to produce vitamin B_{12}. The genomes were analysed with the RAST server, and the results obtained were compared with the information in the MetaCyc database. In this way, the presence of the vitamin B_{12} metabolic pathway in the genomes was verified. Based on the completeness of their metabolic pathways, 38 strains were selected for further testing.

The effective ability of the strains to produce vitamin B_{12} was verified with an HPLC-DAD analysis of pure culture extracts. Out of the 38 strains, 11 were proved to be capable of producing detectable amounts of vitamin B_{12} under tested conditions.

The best candidates were further tested to assess their efficacy in producing vitamin B_{12} in lettuce under sterile conditions. Lettuce seeds were soaked in bacterial cell suspensions and then sowed in MS media with and without cobalt chloride. After 25 days, the vitamin B_{12} was extracted from the edible parts of the plants and purified with immunoaffinity columns. Vitamin B_{12} detection and quantification were performed using HPLC-DAD analysis. *Methylobacterium* sp. P1-11 was proved capable of producing vitamin B_{12} *in planta*: 1.654 and 2.559 µg of vitamin B_{12} per g of dry weight were measured on MS medium and MS medium supplemented with cobalt chloride, respectively.

Finally, the colonization by *Methylobacterium* sp. P1-11 of lettuce plants was confirmed by the isolation of microorganisms from lettuce leaves followed by IGS RFLP analysis.

4.1 INTRODUCTION

Cobalamin (Cbl) exists in different analogue forms belonging to a family of complex cofactors, also known as vitamin B_{12}. Their structure includes a cyclic tetrapyrrolidine (corrinoid ring) with a cobalt atom in the centre. The lower ligand in the α-position, the 5,6-dimethylbenzimidazole (DMB), is common to all the forms of cobalamin. In the β-position, four different upper ligands can be found, forming methylcobalamin (MeCbl), adenosylcobalamin (AdoCbl), hydroxocobalamin (OHCbl) or cyanocobalamin (CNCbl) (Acevedo-Rocha et al., 2019) (Figure 4.1).

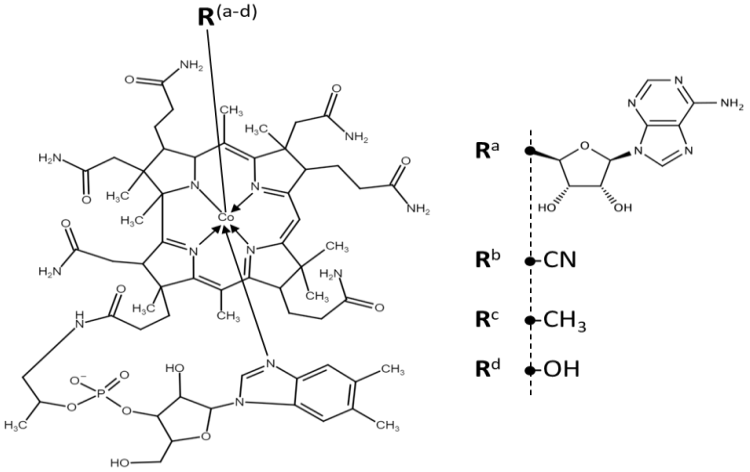

Figure 4.1: Structure of cobalamin (vitamin B_{12}). R^a = deoxyadenosine which forms adenosylcobalamin (AdoCbl); R^b = cyano group which forms cyanocobalamin (CNCbl); R^c = deoxyadenosine which forms methylcobalamin (MeCbl), R^d = hoxidrile group which forms hydroxycobalamin (OHCbl). Designed with www.reaxys.com.

Only some prokaryotes can produce cobalamin *de novo* by aerobic or anaerobic metabolic pathways, while some prokaryotes and eukaryotes can take up an extracellular cobinamide and convert it to adenosylcobalamin by contracted salvage pathway (Bryant et al., 2020; Danchin & Braham, 2017; Lu et al., 2019; Shelton et al., 2018). The *de novo* pathways can be divided into three major stages: (1) production of uroporphyrinogen III (UroIII), the first macrocyclic intermediate in tetrapyrrole synthesis; (2) transformation of UroIII into cobinamide (Cbi), i.e., formation and adenylation of the corrin ring; (3) nucleotide loop assembly, i.e., synthesis of a lower ligand and the attachment to the corrin ring (Balabanova et al., 2021).

The genes/enzymes of the aerobic and anaerobic pathways are defined as *cob*/Cob and *cbi*/Cbi, respectively. Many enzymes in the two pathways are homologues or orthologues, but some are pathway specific. The aerobic pathway has been found and described in *Pseudomonas denitrificans*, a well-known producer of vitamin B_{12} also exploited in industrial processes (Nguyen-Vo et al., 2018). The anaerobic pathway has been found and described in *Salmonella enterica* and other prokaryotes (Caspi et al., 2020; Fang et al., 2017; Roth et al., 1993). Finally, salvage pathways are initiated by absorbing cobinamide produced by bacteria living in the environment. The cobinamide, which has an incomplete corrinoid structure, is then mostly converted into adenosylcobalamin (Bryant et al., 2020; Fang et al., 2017).

Gene clusters for adenosylcobalamin (AdoCbl) synthesis have been identified in numerous prokaryotic genomes and may contain hem-cob operons for the aerobic pathway or the hem-cbi-pdu-etu operons for the anaerobic pathway (Rodionov et al., 2003; Shelton et al., 2018). The two metabolic pathways are distinguished based on the timing of cobalt insertion and the oxygen requirement, and they separate starting from the precorrin-2 stage (Fang et al., 2017). In the formation step of adenosylcobyrinic acid a,c-diamide, the aerobic and anaerobic pathways combine again and continue to be fulfilled by structurally similar enzymes (Balabanova et al., 2021)(Figure 4.2). In the biosynthesis of AdoCbl, the cob(I)yrinic acid a,c-diamide is adenosylated to the cobalt ion, by the corrinoid adenosyltransferases CobA, which is orthologous to CobO (EC 2.5.1.17), resulting in the adenosylcobyrinic acid a,c-diamide (Bryant et al., 2020; Caspi et al., 2020).
The adenosylcobyric acid synthase CobQ/CbiP, with glutamine amidotransferase domain (EC 6.3.5.10), catalyses the four-step amidation sequence from adenosylcobyrinic acid a,c-diamide into adenosylcobyric acid. The adenosylcobinamide-phosphate synthase CobD/CbiB (EC 6.3.1.10) binds an aminopropanol ligand to the free carboxylic acid to obtain the adenosylcobinamide phosphate (Bryant et al., 2020; Caspi et al., 2020). Aminopropanol is derived from L-threonine O-phosphate which is decarboxylated by the L-threonine-phosphate decarboxylase CobC (EC 4.1.1.81) to form (R)-1-amino-2-propanol O-2-phosphate (Shelton et al., 2018; Tavares et al., 2018; A. C. Torres et al., 2018). The genes cobP/cobU/cobY encode for the adenosylcobinamide-phosphate guanylyltransferases, which converts the adenosylcobinamide phosphate to adenosylcobinamide -GDP (EC 2.7.7.62) (Caspi et al., 2020). Adenosylcobalamin 5'-phosphate synthase CobV/CobS (EC 2.7.8.26) substitutes the GDP in adenosylcobinamide -GDP with 5'-phosphate derived from α-ribazole 5'-phosphate. The α-ribazole 5'-phosphate is generated by linking the 5,6-dimethylbenzimidazole to nicotinamide mononucleotide, and the reaction is catalysed by a nicotinate-nucleotide-dimethylbenzimidazole CobU/CobT (EC 2.4.2.21). Finally, the phosphate is removed by the action of adenosylcobalamin 5'-phosphate phosphatase CobC (EC 3.1.3.73), obtaining the adenosylcobalamin (Caspi et al., 2020).
The MetaCyc Metabolic Pathway Database provides, for each enzyme, the related biosynthetic reactions at a detailed level (Caspi et al., 2020).

As described before, cobalamin in nature is available in many chemical forms, but the biologically active ones for humans are only methylcobalamin and adenosylcobalamin. However, the most stable form is cyanocobalamin, which is, therefore, the form primarily used in pharmaceutical products, supplements or for food fortification. During metabolism, ileal enterocytes convert cyanocobalamin to methylcobalamin (Marley et al., 2009).
Vitamin B_{12} is essential to mammals because it is a cofactor for two enzymes. The first one is methionine synthase, crucial for the synthesis of purines and pyrimidines. This enzyme depends on methylcobalamin and folate: it transfers the methyl group of methyltetrahydrofolate to homocysteine to form methionine and tetrahydrofolate. A deficiency of vitamin B_{12} or folate leads to the development of megaloblastic anaemia (O'leary & Samman, 2010). The second enzyme, for which vitamin B_{12} is a cofactor, is methylmalonyl-CoA mutase (MCM), which is involved in the degradation of the amino acids valine, isoleucine, methionine, and threonine, odd-chain fatty acids, and cholesterol (Takahashi-Iñiguez et al., 2012). Vitamin B_{12} is therefore required for the development,

myelination, and normal functioning of the central nervous system; for normal red blood cells formation, and for methyl group translocation in DNA synthesis (Froese et al., 2019; Nouri et al., 2018).

Recommended Dietary Allowance (RDA) ranges from 0.4 µg to 1.8 µg for children aged 0 to 14, is 2.4 µg for adults and increases up to 2.6 µg and 2.8 µg for pregnant and breastfeeding women, respectively (Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes and its Panel on Folate, 1998).

Vitamin B_{12} deficiency is rare in healthy humans as it is the only water-soluble vitamin that can be stored in the liver for many years (Carmel, 1996). However, vitamin B_{12} deficiency may develop due to several causes, such as pernicious anaemia (Hernandez & Oo, 2015) and other gastrointestinal problems, or to a strict vegetarian diet (Langan & Goodbred, 2017; Pawlak et al., 2012). Vitamin B_{12} deficiency can lead to megaloblastic anaemia (abnormal blood cell growth), symptoms of which include excessive tiredness, listlessness, breathlessness, and poor resistance to infections. Prolonged deficiency can lead to nerve degeneration and irreversible neurological damages (Pawlak et al., 2012).

Hence, although *de novo* synthesis of cobalamin appears to be limited only to certain bacteria and archaea, it is an essential nutritional requirement for animals, including humans, and protists, which are also unable to synthesize it (Burgess et al., 2009).

The microorganisms that synthesise vitamin B_{12} are, therefore, the source of B_{12} compounds that we find in foods. The cobalamin synthesised by bacteria accumulates in animal tissues, and, sometimes also in some plant tissues. For example, ruminants acquire this essential nutrient through a symbiotic relationship with the microflora inside their stomachs, as long as they have a sufficient supply of cobalt in their diet. Consequently, ruminants' meat and milk are good sources of vitamin B_{12} for humans (González-Montaña et al., 2020). In aquatic environments, phytoplankton acquires vitamin B_{12} through a symbiotic relationship with bacteria. Then, it becomes food for fish and bivalves, which are also a rich source of vitamin B_{12} for humans (Croft et al., 2005; Helliwell, 2017) (Figure 4.2).

Figure 4.2: Vitamin B_{12} content in food products per serving. From the highest to the lowest: beef liver (70 µg in 85 g), clams (17 µg in 85 g), tuna (9.3 µg in 85 g), salmon (2.6 µg in 85 g), beef (2.4 µg in 85 g), milk (1.3 µg in 240 mL), cheese (0.5 µg in 42 g), eggs (0.5 µg per egg), turkey (0.3 µg in 85 g), mushrooms (0.04 in 85 g) (https://fdc.nal.usda.gov).

Thus, vitamin B_{12} is naturally present in foods of animal origin, including fish, meat, poultry, eggs, and dairy products (Marriott et al., 2020). However, its bioavailability varies depending on the type of food source. The bioavailability of cobalamin in dairy products, for example, appears to be about three times higher than in meat, fish, and poultry (Matte et al., 2012).

Some edible species of mushrooms, including black trumpet (*Craterellus cornucopioides*) and golden chanterelle (*Cantharellus cibarius*), contain considerable amounts of vitamin B_{12}: 1.09–2.65 µg/100 g of dry weight (Watanabe et al., 2012, 2013), even though they cannot synthesise it. A high amount of vitamin B_{12} has been detected in mushrooms grown in the culture medium containing vitamin B_{12} synthesising bacteria, suggesting that cobalamin found in mushroom fruiting bodies was derived from bacterial concomitants (Teng et al., 2015). Some widely consumed edible algae, such as green (*Enteromorpha* sp.) and purple (*Porphyra* sp.) lavers, also contain considerable amounts of vitamin B_{12} analogues: 133 µg/100 g of dry weight. However, the biological activity of these algal-derived corrinoids in the human body remains unclear (Miyamoto et al., 2009; Watanabe et al., 2002).
As already mentioned, plants neither synthesise nor require vitamin B_{12} (Burgess et al., 2009). For this reason, plant-based food is not a rich source of vitamin B_{12} (Kumar et al., 2010; Pawlak et al., 2013). Although plants do not need vitamin B_{12} for their functions. As already seen for mushrooms, this is due to the symbiosis between plants and certain vitamin B_{12}-producing bacteria (Nakos et al., 2017). One of these is nitrogen-fixing actinobacterium *Frankia alni*, which forms endophytic nodules in woody trees and shrubs (Wall, 2000). Thus, actinorhizal plants, such as sea buckthorn (*Hippophae rhamnoides*), couch grass (*Elymus repens*), elecampane (*Inula helenium*) or black mustard (*Brassica nigra*), which form a symbiosis with actinobacterium *F. alni*, can contain a considerable amount of vitamin B_{12}. For example, vitamin B_{12} concentration in sea buckthorn (*Hippophae rhamnoides*) can reach about 37 µg/100 g of dry weight (Nakos et al., 2017).

Some plant growth-promoting bacteria (PGPB) are already widely exploited in agriculture for their ability to improve the quality and growth of host plants.
Endophytic microorganisms (endophytes), residing within plants have the advantage of being protected from adverse environmental conditions such as temperature, osmotic potentials, and UV radiation, which may strengthen their efficacy (Senthilkumar et al., 2011). Endophytes can stimulate plant growth in multiple ways, either through direct contribution or indirect support. Direct contributions include the provision of nutrients (e.g., N_2-fixation) and support in their uptake (e.g., synthesis of enzymes, peptides or siderophores that mediate uptake of phosphorous, potassium or zinc). Furthermore, microorganisms can produce phytohormones (auxins, cytokinin and gibberellic acid) to promote plant growth such as 1-aminocyclopropane-1-carboxylate (ACC; inhibitor of ethylene biosynthesis) to alleviate the stress response of plants or hydrogen cyanide to support plant resistance against pathogens. In addition, endophytes can indirectly enhance the tolerance against abiotic stresses and antagonism to pathogenic organisms, by activating mechanisms such as competition for nutrients, production of antibiotics, siderophores, hydrolytic enzymes (such as *β*-1, 3-glucanase, chitinases), volatile organic compounds (VOCs) and HCN, or by improving plant polyphenolic content (Cappellari et al., 2013; Rana et al., 2020; Verma et al., 2016; Yadav et al., 2017). Consequently, endophytes are good candidates for consideration in biofortification strategies, that aim to enhance the bioavailable concentration of nutrients and micronutrients in edible portions of food crops through agronomic interventions or genetic selections (Singh et al., 2016; White & Broadley, 2009). Endophytes can help plants in the acquisition of mineral elements, and, at the same time, they can improve the biosynthesis of secondary metabolites. Biofortification through microorganisms is important because it is an effective and environmentally friendly approach to

overcoming nutrient deficiency by growing food crops, especially staple food crops such as cereals, with higher levels of bioavailable nutrients and minerals (Hussain et al., 2018).

Numerous cases of biofortification targeting mineral elements, most commonly lacking in human diets: iron (Fe), zinc (Zn), copper (Cu), manganese (Mn), calcium (Ca), magnesium (Mg) and selenium (Se) can be found in the literature (Gopalakrishnan et al., 2016; Jha & Warkentin, 2020; Kaur et al., 2020). For example, Fe and Zn were successfully increased by up to 50% in different wheat genotypes through inoculation of siderophore-producing (*Arthrobacter sulfonivorans* DS-68 and *Enterococcus hirae* DS-163) and Zn-solubilising endophytes (*Bacillus subtilis* DS-178, *Arthrobacter* sp. DS-179), respectively (D. Singh et al., 2017, 2018). The PGPBs and cyanobacteria were combined for micronutrient enrichment in rice. Inoculation with *Providencia* sp., *Brevundimonas diminuta* and *Ochrobactrum anthropic* resulted in 13%–16% higher Fe, Zn, Cu, and Mn concentrations in rice grains. In wheat, *Providencia* sp. inoculation yielded a significantly higher content of Fe and Cu (44%–45%) (Rana et al., 2015). Putative Se-tolerant, endophytic PGPB from genera *Paenibacillus*, *Klebsiella*, *Bacillus* and *Acinetobacter* were useful for the biofortification of Se in wheat (Durán et al., 2014). In another study, wheat plants were amended with Se-tolerant bacteria, such as *Stenotrophomonas* sp., *Bacillus* sp., *Enterobacter* sp. and *Pseudomonas* sp., and the results showed that these bacteria could make Se available for plant uptake under greenhouse conditions (Acuña et al., 2013).

Minerals are not the only nutrients that can be targeted through biofortification. In plant-endophyte interactions, significant changes in the secondary metabolism of the symbionts frequently occur. For example, Khanna et al. (2019) observed that *Pseudomonas aeruginosa* and *Burkholderia gladioli* elevated the levels of phenolic compound, osmolytes (carbohydrates, reducing sugars, trehalose, glycine betaine and proline) and low molecular weight organic acids (fumaric acid, malic acid, succinic acid, and citric acid) on *Solanum lycopersicum* seedlings subjected to Cd stress.

Plants colonised by beneficial endophytes are often less prone to abiotic stresses than those not colonised (Saikkonen et al., 2010). This could be explained by the fact that endophytes may benefit their host in two ways: endophytes may adopt molecular strategies to stimulate host stress-responsive genes, phytohormones production, generation of antioxidants, promoter elements and transcription factors (Lata et al., 2018). They can themselves produce antioxidants (Pang & Wang, 2010) and secondary metabolites that help to counteract reactive oxygen species (ROS) that may escape the plant defence (Pang & Wang, 2010; Torres et al., 2012).

Another example of secondary metabolism modification was studied by Liu et al. (2020). The authors identified 14 bacterial endophytes capable of increasing the accumulation of five Amaryllidaceae alkaloids (narciclasine, lycorine, galanthamine, lycoramine and tazettine) in leaves, in the bulbs or in the roots of *L. radiata* plants. Furthermore, they observed that several bacterial endophytes that have been shown to increase alkaloid concentration can also synthesise indole acetic acid (IAA). IAA mediates root development and nutrient uptake of host plants. Therefore, by benefiting the plant's primary metabolism, the endophytes provide the basis to support the synthesis of plant secondary metabolites (Zhou et al., 2018).

The endophytic bacteria *Luteibacter* spp. isolated from the tea plant, can produce theanine, which is the main non-protein amino acid in tea plants and plays an important role in tea quality (Sun et al., 2019).

Furthermore, the vitamin content of plants can also increase through the presence and activity of endophytes, as evidenced by the almost doubled amount of vitamin C found in strawberry fruits produced by plants inoculated with *Phyllobacterium* sp. PEPV15 compared to the fruits of uninoculated plants. Overall, the inoculation with the strain PEPV15 resulted in increased yield, quality, and functionality of strawberry fruits (Flores-Félix et al., 2015).

Thus, the aim of this study was to identify, through genome and biochemical analysis, endophytic bacterial strains able to synthesise vitamin B_{12} *de novo*, and then attest whether these strains can be exploited to enrich edible plants with this essential nutrient.

4.2 MATERIALS AND METHODS

4.2.1 Genome analysis

The RAST (Rapid Annotation using Subsystem Technology) annotation server (https://rast.nmpdr.org/rast.cgi) was used to annotate the genomes of 68 endophytes from the AIT Austrian Institute of Technology, Bioresources unit, strain collection. As a reference, the *P. denitrificans* ATCC 13867, known from the literature as a vitamin B_{12} producer (Ainala et al., 2013) and used for industrial production, was included. Its genome was obtained from GeneBank (accession number: GCA_000349845.1).

The MetaCyc Metabolic Pathway Database (https://metacyc.org/) and the information on the vitamin B_{12} metabolic pathways (Balabanova et al., 2021) were used to compare the genes detected by RAST in the different genomes to the enzymes involved in the cobalamin metabolic pathway. Considering that the aerobic and the anaerobic metabolic pathway for vitamin B_{12} synthesis have in common the last eight enzymes, from the cob(I)yrinic acid a,c-diamide to the adenosylcobalamin (Balabanova et al., 2021), the presence of shared genes was investigated. Then, based on the presence and completeness of those genes, the most promising strains were selected for further analysis.

4.2.2 Bacterial culture

Bacterial cultures of the selected strains were prepared in order to verify, through HPLC-DAD analysis, their effective ability to produce vitamin B_{12} *de novo*. As a reference, *P. denitrificans* ATCC 13867, purchased from the German Collection of Microorganisms and Cell Cultures GmbH (www.dsmz.de), was used. Cryopreserved bacterial stocks were transferred to 10% Tryptic Soy Agar (TSA, Merck) medium and incubated at 27°C for 48 h. Then a single colony of each strain was sub-cultured in Luria-Bertani Broth (LB Broth, Merck) medium supplemented with 0.018 g/L cobalt chloride ($CoCl_2$), the pH was adjusted to 6.5 before sterilisation (121°C, 15 min). The bacterial cultures were incubated in the dark for 48 h at 27°C and 180 rpm.

4.2.3 Vitamin B_{12} extraction from pure bacterial culture

Cobalamin (vitamin B_{12}) present in bacterial cells was extracted as described by Chamlagain et al., (2015). Briefly, for each bacterial strain, 1 g of bacterial cell pellet was resuspended in 10 mL of extraction buffer (8.3 mM sodium hydroxide, and 20.7 mM acetic acid, pH 4.5). Subsequently, 100 µL of 1% (w/v) potassium cyanide (KCN) solution was added to convert all vitamin B_{12} analogues to the more stable cyanocobalamin. The suspension was mixed using Vortex for 30 sec and extracted in a boiling water bath for 30 min, then the sample was cooled in an ice-bath for 10 min, and finally centrifuged at 6,000 rcf for 10 min. The supernatant was collected in a fresh tube, and the residual pellet was mixed using Vortex once again with 5 mL extraction buffer (pH 6.2, adjusted from the pH 4.5 extraction buffer with 3% sodium hydroxide) and centrifuged using the same conditions. The supernatants were combined and filtered through Whatman™ filter paper (Grade 1: 11 µm, VWR). The volume was adjusted to 20 mL with pH 6.2 extraction buffer and filtered again via syringe filter

(RC 0.45 µm, Macherey-Nagel). The entire extraction process was carried out under subdued light conditions to protect the vitamin B_{12} from light degradation.

4.2.4. HPLC-DAD analysis of pure cultures extract

The analysis of vitamin B_{12} content in the bacterial cells extract was performed with an HPLC-DAD (Agilent 1100 series©, Agilent Technologies Inc.) consisting of a solvent degasser G1379A, a quaternary pump G1311A, an autosampler G1313A and a thermostated column compartment G1316A. A Waters 2996 photodiode-array detector (DAD) (Zellik, Belgium) was used for detection at 361 nm and full spectra were recorded in the range 330-390 nm with detection every 2 nm. Chromatographic separation was achieved using an Agilent ZORBAX Eclipse Plus C-18 analytical column (5 µm, 4.6 mm × 150 mm i.d.). Equipment control, data acquisition and integration were performed with Agilent Chem Station HPLC software (Copyright C Agilent Technologies 2001-2005).
The mobile phase consisted of a mixture of water acidified with 0.025% trifluoroacetic acid (pH 2.6) and acetonitrile. The initial setting was 100% water for 0.21 min, which was linearly decreased to 85% water over 2.59 min and then decreased further to 75% over the next 2.4 min. After that, the percentage of water was increased linearly to 90% over 0.24 min and then again to 100% in the next 1.36 min. These conditions were maintained for 4.2 min. The total runtime was 11 min. The flow rate was set to 1 mL/min, the injection volume was 50 µL and each sample was injected twice. The experiment was carried out at 30°C.
Vitamin B_{12} in cells extracts was quantified with an external calibration curve obtained by injecting a set of cyanocobalamin (Merck) standards eluted in vitamin B_{12} extraction buffer (pH 6.2) at concentrations of 0, 0.1, 0.2, 0.3, 0.5, 0.75, 1, 1.5 ng/µL. The cobalamin content in each sample was quantified with the calibration curve after measuring the area integrated from the peaks with the corresponding RT of cobalamin.

4.2.5 Plant experiment

The three best *in vitro* vitamin B_{12} producers, possessing all eight considered metabolic pathway genes (strains 1489, P1-11, C8BA17), also including the reference strain *P. denitrificans* ATCC 13867, were evaluated in producing vitamin B_{12} in lettuce plants.
Strains were grown in the dark for 48 h at 27°C in continuous agitation (180 rpm) on LB Broth enriched with 0.018 g/L of cobalt chloride, then the cell suspensions were centrifuged and resuspended in 0.9% sodium chloride (NaCl) to obtain an OD_{600} of 1.
The seeds of the lettuce *cultivar* "Chiara" (ISI sementi S.p.a., Italy), used for the fresh-cut leaves market, were surface sterilised by stirring for 2 min in 70% ethanol, for 5 min in 5% hypochlorite and then 5 times in sterile MilliQ water for 1 min. The seeds were left to dry on filter paper under sterile conditions for 30 min and were subsequently soaked separately for 2 h in the previously prepared bacterial cell suspensions or 0.9% sodium chloride solution for the negative control.
Subsequently, the suspension was gently discarded, and the seeds were sowed in 80 mL glass culture tubes previously filled with 15 mL of MS medium (10 g sucrose, 8 g Duchefa Daishin Agar in 1 L MilliQ water, pH 5.8) or MS medium enriched with 0.018 g/L of cobalt chloride. The tubes were placed in plant growth chamber SE-41E2T5 (Pervicial) with 14/10 h light/dark and 24 and 20°C, respectively.

Light intensity 650 µmol/m^2s^1, light spectrum ca.360 nm-780 nm. For each treatment 2 replicates of 18 plants were prepared. After 25 days, the epiphytic part of 10 plants for each replicate were weighted and immediately frozen in liquid nitrogen, then freeze-dried with lyophilizer Alpha 2-4 LSCplus (Christ) and milled into fine powder with MIXER MILL MM 400 (Retsch). The weights were statistically analysed with RStudio version 4.1.1 using a t-test between each treatment and the control. The remaining plants were used for bacterial isolation in order to confirm the bacterial colonisation of plants.

4.2.6 Vitamin B_{12} extraction from plant

The cobalamin (vitamin B_{12}) from plant matrices was extracted as described by (Chamlagain et al., 2015) with some modifications. Briefly, for each replicate, 0.1 g of freeze-dried and milled plant material was resuspended by vortex with 10 mL of extraction buffer (8.3 mM sodium hydroxide, and 20.7 mM acetic acid, pH 4.5) supplemented with 1% (w/v) pepsin and 0.5% (w/v) diastase. Subsequently, 100 µL of 1% (w/v) potassium cyanide was added. The suspension was sonicated (Sonorex Super RK 255 H, Bandelin) for 15 min at room temperature, shaken at 37°C at 200 rpm for 2 h (Digital Orbital Shaker, Heathrow Scientific) and extracted in a boiling water bath for 30 min. Then it was cooled in ice-bath for 10 min and centrifuged at 6,000 rcf for 10 min. The supernatant was filtrated through Whatman™ filter paper (Grade 1: 11 µm, VWR), the volume was adjusted to 10 mL with the extraction buffer and then filtered again using a syringe filter (RC 0.45 µm, Macherey-Nagel, Germany).

4.2.7 Vitamin B_{12} purification

In order to purify and concentrate the vitamin B_{12}, the plant extract was cleansed using EASI-EXTRACT® VITAMIN B_{12} immunoaffinity column (R-Biopharm) according to the manufacturer's instructions. Briefly, the buffer in the immunoaffinity column was drained, after which 20 mL of plant extract for each treatment (10 mL from each one of the two replicates) was passed throw the column. The column was washed with 10 mL of MilliQ water and dried by passing air. Vitamin B_{12} was then eluted with 3 mL of methanol. The eluate was evaporated overnight at 65°C, and the residue was reconstituted in 300 µL of MilliQ water acidified with 0.025% trifluoroacetic acid (pH 2.6).

4.2.8 HPLC-DAD analysis of plant extract

The analysis of vitamin B_{12} content in plant matrix extract was performed with the same method used for bacterial cells with few modifications. For each replicate, the volume was 100 µL and was injected twice. The cyanocobalamin standards were eluted in MilliQ water acidified with 0.025% trifluoroacetic acid (pH 2.6) and the concentrations for setting the calibration curve were 0, 0.01, 0.02, 0.03, 0.05, 0.1, 0.2, 0.3 ng/µL.

4.2.9 Bacterial persistence in lettuce and DNA extraction

A cultivation-based approach was used to confirm the colonisation of lettuce plants by vitamin B_{12}-producing endophytic bacteria. For each replicate, the seedlings were gently removed from the MS

medium, and surface sterilised by stirring them for 1 minute in 70% ethanol, followed by 2 min in 2% hypochlorite and then rinsed 3 times in sterile MilliQ water for 1 minute. Then 1 g of plant material and 9 mL of 0.9% sodium chloride were mixed and homogenised with an Ultra-Turrax homogeniser for 1 min. Each extract was diluted 1:100, 100 µl were inoculated on TSA plates, which were incubated at 27°C for 48 h. Then, for each replicate, 10 colonies presenting a similar morphology to the original strain used for seed treatment were selected and grown overnight in Tryptic Soy Broth (TSB, Merck) medium. Subsequently, DNA isolation was performed using Nexttec™ 1^{-Step} DNA Isolation Kit for bacteria.

4.2.10 Restriction fragment length polymorphism analysis (RFLP) of the 16S-23S rRNA intergenic spacer (IGS) region

The 16S-23S IGS region of rDNA was amplified using the primers pHr (5'- TGCGGCTGGATCACCTCCTT -3') and P23SR01 (5'- GGCTGCTTCTAAGCCAAC -3') (Massol-Deya et al., 1995).
Each amplification reaction was conducted in a final volume of 50 µL containing: 1x Reaction Buffer BD (Solis BioDyne), 2.5 mM $MgCl_2$ (Solis BioDyne), 0.2 mM dNTPs (Thermo Scientific), 0.3 mM of each primer, 2 U FIREPol® DNA Polymerase (Solis BioDyne) and 4 µL of gDNA. The thermal cycling parameters were as follows: an initial denaturation step at 94°C for 5 min, followed by 30 cycles of 45 s at 95°C, 60 s at 54°C and 2 min at 72°C, and a final step of 10 min at 72°C. The amplified DNA fragments were analysed in 1% (w/v) agarose gels and visualised under UV light in comparison with DNA Marker Lambda/Hind III (A&A Biotechnology).
Aliquots of 5 µL of PCR products were individually subjected, without further purification, to the restriction fragment length polymorphism (RFLP) analysis. The digestion was performed for 3 h at 37°C in a final volume of 15 µL containing 1x Tango buffer (Thermo Scientific) and 5 U of restriction endonuclease *Alu*I or *Hha*I (Thermo Scientific) with recognising sequences 5′-AG↓CT-3′ and 5′-GCG↓C-3′ respectively. The restriction fragments were separated by electrophoresis on 2.5% (w/v) agarose gels and visualised under UV light in comparison with GeneRuler DNA Ladder Mix (Thermo Scientific). The RFLP patterns obtained from bacteria isolated from lettuce were compared to those obtained from the parent strains. Thus, it was possible to establish whether the microorganisms belonged to the same strain.

4.3 RESULTS

4.3.1 Genome analysis

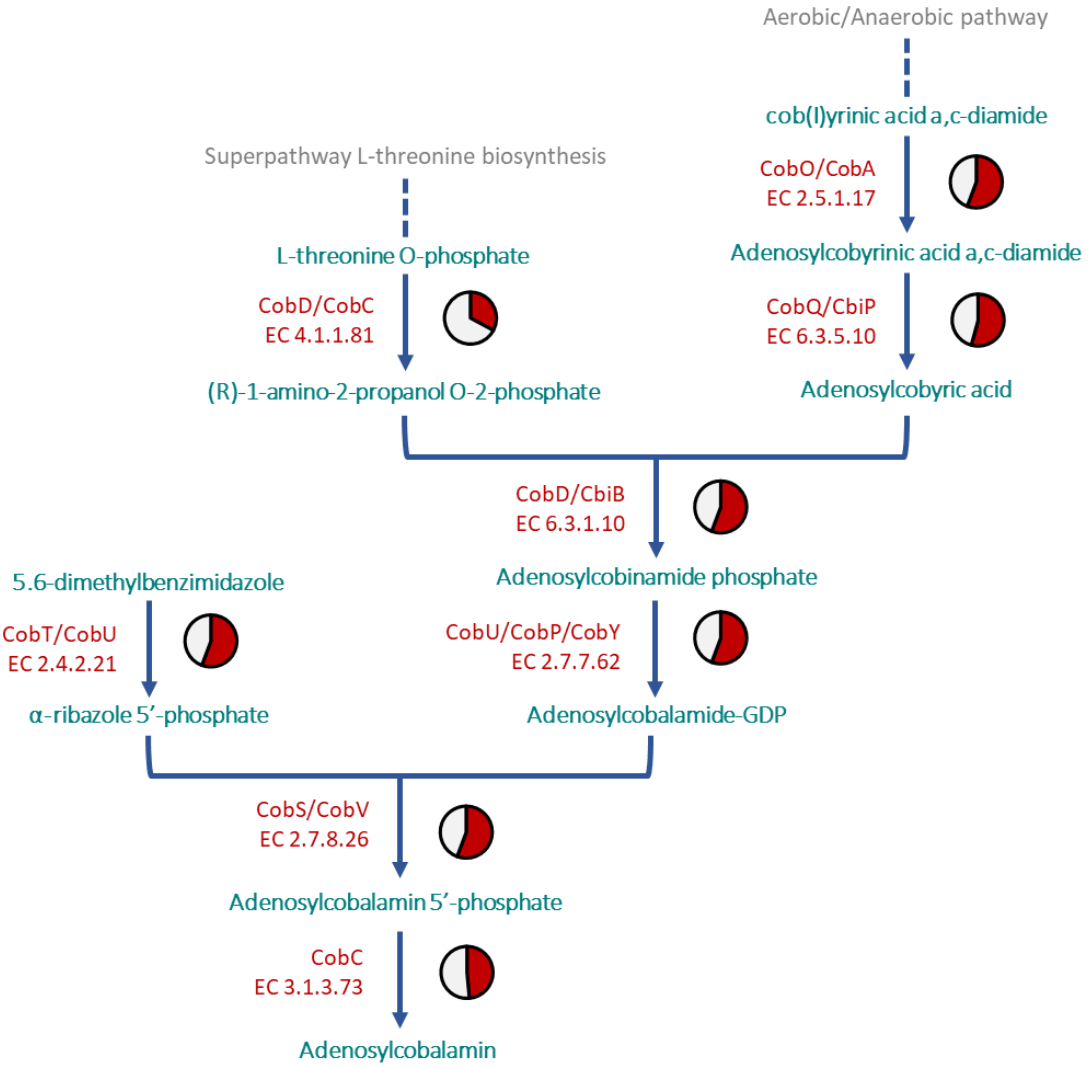

Figure 4.3: Portion of vitamin B_{12} metabolic pathway, from the cob(II)yrinic acid a,c-diamide to the adenosylcobalamin, including the last eight enzymes shared between aerobic and anaerobic pathways. In the pie charts the percentage of strains (out of 69 analysed) possessing that enzyme (white – gene absent, red – gene present).

The RAST server was used for the annotation of 69 endophyte genomes from the AIT strain collection, including the reference genome of *P. denitrificans* ATCC13867 strain. After the annotation, the presence and completeness of the vitamin B_{12} metabolic pathway were evaluated for each genome. It was observed that 38 strains, including the ATCC13867, had the cobalamin pathway in their genome with different degrees of completeness. Of the eight genes considered (Figure 4.3), 22 strains had all of them, including the reference strain ATCC13867, 11 strains had seven genes, and five strains had six genes. In the remaining strains, no genes were detected. The least frequent gene was the corrinoid adenosyltransferase (EC 2.5.1.17), present in only 23 genomes. The enzyme

adenosylcobalamin-5-P phosphatase (EC 3.1.3.73), the last enzyme of the pathway that transforms the adenosylcobalamin 5'-phosphate into adenosylcobalamin, was present in 33 genomes. The enzyme threonine-phosphate decarboxylase (EC 4.1.1.81) was found in 37 genomes, while the other five genes (EC 6.3.5.10, EC 6.3.1.10, EC 2.7.7.62, EC 2.4.2.21, EC 2.7.8.26) were present in 38 genomes. These 38 strains were selected for further testing.

4.3.2 HPLC analysis on pure bacterial culture

The vitamin B_{12} production of the 38 strains selected through the genome analysis was tested by HPLC-DAD analysis of extracts obtained from pure bacterial cultures grown on LB Broth supplemented with 0.018 g/L cobalt chloride (Table 4.1). It was possible to detect and quantify vitamin B_{12} in 11 of the 38 strains tested. Production ranged from 1.067 to 6.438 µg of cyanocobalamin per g of bacterial cells. The reference strain, *P. denitrificans* ATCC 13867, was the least productive under tested conditions. For all the peaks identified, the 330-390 nm spectra corresponded to that of the standard (Figure 4.4), confirming the specificity of the detected signal. The 11 producing strains belong to six different genera, and in all of them, with the exception of *Paenibacillus* sp. 2136, all eight genes of the metabolic pathway have been detected.

Strain	Genera	µg B12/g cells	Number of genes
ATCC 13867	***Pseudomanas***	**1.067**	**8**
1489	*Pseudomanas*	6.438	8
P1-11	*Methylobacterium*	4.300	8
2136	*Paenibacillus*	3.990	7
C8BA17	*Aneurinibacillus*	3.537	8
A3-14	*Bacillus*	2.485	8
C13BA17	*Lysinibacillus*	2.071	8
1390	*Bacillus*	1.985	8
G66BA17	*Bacillus*	1.797	8
1173	*Pseudomanas*	1.447	8
G65BA17	*Bacillus*	1.184	8

Table 4.1: Bacterial strains showing vitamin B_{12} production and respective concentration expressed as µg of vitamin B_{12} for gram of bacterial cell. For each strain, the genus and the number of genes belonging to the common portion of the vitamin B_{12} metabolic pathway (Figure 4.3, eight genes) detected in the genome of the strain are reported.

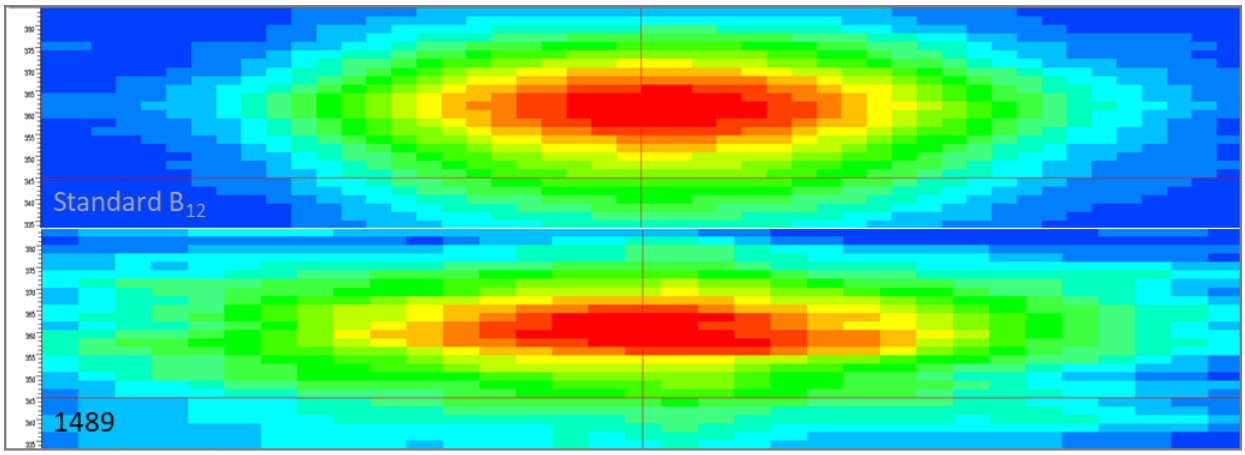

Figure 4.4: Isoplots representing the 330-390 nm spectra from the pure vitamin B_{12} standard (top) and from the extract of the bacterial strain *Pseudomonas* sp. 1489 (bottom). The isoplots depict the intensity of the peak according to the time (x) and the wavelength(y).

4.3.3 Plant growth evaluation and HPLC-DAD analysis

Pseudomonas sp. 1489, *Methylobacterium* sp. P1-11 and *Aneurinibacillus* sp. C8BA17 and the reference strain *Pseudomonas denitrificans* ATCC 13867, were selected to evaluate their efficacy in producing vitamin B_{12} in lettuce plants. Before proceeding with vitamin B_{12} extraction, the epiphytic part of the lettuce seedlings was weighted to evaluate the effect of bacteria on plant growth. In most cases, the bacteria did not show any significant effect on plant growth, with the only exceptions of *Pseudomonas* sp. 1489, which caused an average decrease in weight of 62.5 % (p-value 0.022), and *Methylobacterium* sp. P1-11, which caused an average weight gain of 57.5 % (p-value 0.016) (Figure 4.5). In both cases, the difference was detected in plants grown on MS medium without the cobalt chloride supplementation.

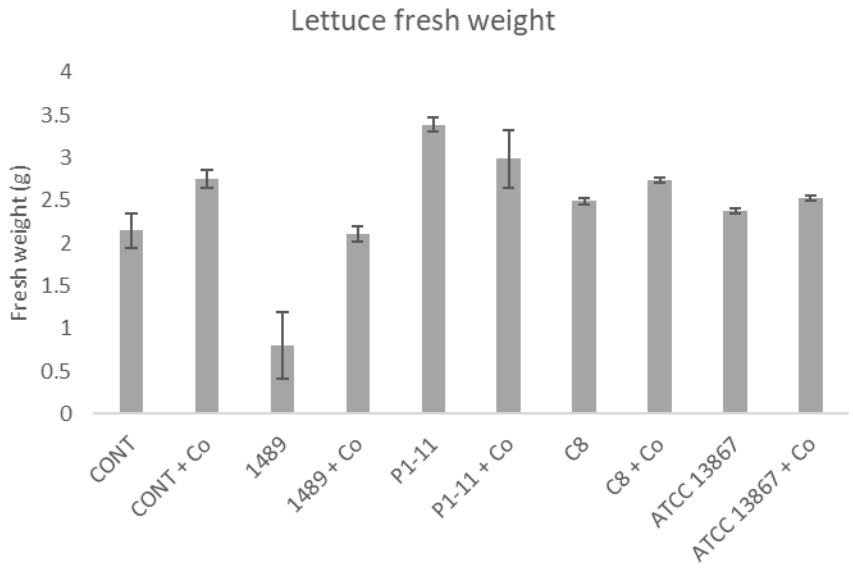

Figure 4.5: Average fresh weight values of lettuce plants ± standard deviation, different letters above histograms represent significant differences among treatments. +Co indicates that the plants were grown on MS medium supplemented with cobalt chloride. CONT – uninoculated control, 1498 – plants inoculated with *Pseudomonas sp. 1489*, P1-11 – plants inoculated with *Methylobacterium sp. P1-11*, C8 – plants inoculated with *Aneurinibacillus sp. C8BA17*, ATCC13867 – plants inoculated with *Pseudomonas denitrificans* ATCC13867.

Different letters represent significant differences between the treatments within the time of the control according to Duncan Test (α = 0.05).

The vitamin B_{12} extract was analysed by HPLC-DAD after purification with immunoaffinity columns and was expressed as µg of vitamin B_{12} per g of dry weight (DW) of lettuce leaves. With the used technique, it was possible to detect vitamin B_{12} only in lettuce plants treated with *Methylobacterium* sp. P1-11 grown both on MS medium and MS medium supplemented with cobalt chloride. The amounts of vitamin B_{12} detected were 1.654 and 2.559 µg/g, respectively.

4.3.4 Colonisation confirmation: IGS-PCR and RFLP

To confirm the colonisation of lettuce plants by vitamin B_{12}-producing endophytes, bacterial isolation from lettuce leaves was performed, followed by gDNA extraction, IGS PCR amplification and RFLP digestion with two different restriction enzymes (*Alu*I and *Hha*I). In general, the enzymatic digestion of the IGS-PCR amplification product provides a restriction pattern for each endonuclease that is conserved among the strains of the same species (Quirós et al., 2006). Bacteria isolated from lettuce gave an identical pattern to the inoculated strain with both enzymes: *Alu*I and *Hha*I (Figure 4.6), except for *Aneurinibacillus* sp. C8BA17 inoculated samples, from which inoculated strain could not be retrieved under applied experimental conditions. Therefore, the test enabled us to confirm that the bacteria *Pseudomonas* sp. 1489, *Methylobacterium* sp. P1-11 and *Pseudomonas denitrificans* ATCC 13867 colonized lettuce plants. It is, therefore, plausible to hypothesize that the vitamin B_{12} present in lettuce plants treated with *Methylobacterium* sp. P1-11 was actually produced by the inoculated bacteria.

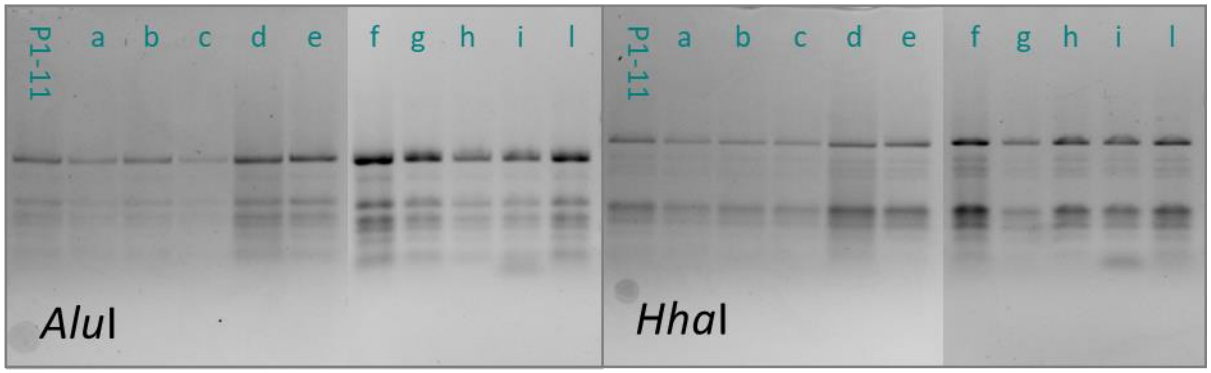

Figure 4.6: 16S rRNA-RFLP patterns (agarose gel 2.5%) obtained from *Methylobacterium* sp. P1-11 and from bacteria isolated from lettuce (a - l) treated with the same strain. The patterns were obtained using the endonucleases *Alu*I (left)and *Hha*I (right).

4.4 DISCUSSION

In recent years, people have become increasingly attentive to the problems caused by ongoing climate change, the challenges related to global population growth and the role that food production plays within this framework. Food production and distribution systems contribute to the consumption of natural resources (e.g., land, water, energy) and endanger biodiversity and environmental balances in many regions of the Earth (Aiking, 2014; Campbell et al., 2017; Ritchie et al., 2018). The production of meat and foods of animal origin generates large emissions of greenhouse gases and consequently contributes to global warming. Therefore, there is a broad consensus that food sustainability can benefit from a transition towards greater dependence on plant-based foods (Aiking & de Boer, 2020; Clark & Tilman, 2017; Godfray et al., 2018; Poore & Nemecek, 2018; Willett et al., 2019)

Additionally, many authors have outlined numerous health advantages promoted by a plant-based diet (Appleby et al., 2011; Barnard et al., 2005; Fraser, 2009; Tonstad et al., 2009), such as the reduction of various risks related to chronic diseases and an overall increase in health and longevity. The major health-promoting components include lower intakes of fat and cholesterol and higher intake of antioxidants, fibres, and protective nutrients such as vitamin C, vitamin E, folate, provitamin A, copper, potassium, and magnesium (Dyett et al., 2013).

However, vitamin B_{12} deficiency poses a risk to people who follow a strictly plant-based diet because foods of plant origin, unlike products of animal origin such as meat, milk, and liver of ruminants, are not a source of vitamin B_{12} (Kumar et al., 2010; Pawlak et al., 2013). This is due to the fact that plants neither synthesise nor need this vitamin (Burgess et al., 2009). Therefore, the lack of vitamin B_{12} in plant food represents an obstacle to the transition to a more sustainable diet, as it might influence the health and well-being of consumers.

A solution to this problem could be offered through biofortification promoted by beneficial vitamin B_{12}-producing bacteria. Endophytes are already known to be capable of increasing the content of minerals or other nutrients in plants, such as polyphenols, vitamins (C and B complex), saponines or alkaloids (Durán et al., 2014; Gopalakrishnan et al., 2016; Jha & Warkentin, 2020; Kaur et al., 2020; Ku et al., 2019; Liu et al., 2020;Rana et al., 2015;Singh et al., 2017, 2018; Sun et al., 2019). Therefore, it is feasible to investigate the possibility of extending microbial applications for plant biofortification with additional nutraceutical compounds.

Next-generation sequencing (NGS) has been responsible for a significant reduction in the cost and time required for genome sequencing. This has led to a rapid increase in the availability of genetic information (Zhang et al., 2011), including a large number of sequenced bacterial genomes (Barbosa et al., 2014), available to everyone via online databases like GenBank (https://www.ncbi.nlm.nih.gov/genbank/). Analysing genomes and predicting metabolic pathways have also become relatively simple procedures to perform, thanks to online bioinformatics tools such as Kyoto Encyclopaedia of Genes and Genomes (KEGG) and MetaCyc (Shrestha et al., 2022). These bioinformatics approaches can constitute an interesting starting point for screening a large number of bacteria to select those capable of producing molecules of interest and, eventually, to identify potential biofortification agents.

In order to screen the 69 genomes used in this study, the annotation obtained with the RAST server was compared with the prediction of enzymes involved in the metabolic pathways provided by the MetaCyc database and by Balabanova et al. (2021). RAST was developed in 2008 to annotate bacterial and archaeal genomes. It supplies a standard pipeline for the identification and annotation of genomic features such as protein-coding genes and RNA (Brettin et al., 2015). The MetaCyc database and(Balabanova et al., 2021), however, were used to determine which genes belonging to cobalamin pathway should be considered. It was decided to search each genome for only the last eight genes involved in the cobalamin pathway since they are common to both the aerobic and the anaerobic cobalamin pathways.

More than half of the genomes analysed (38 out of 69) had at least some genes involved in the cobalamin pathway, yet only 22 of them had all eight genes investigated. Nevertheless, all 38 strains were selected for the *in vitro* experiments because sometimes the metabolic pathways lack some genes or have unknown alternative genes (Shelton et al., 2018; A. C. Torres et al., 2018).

Our screening also included great biodiversity. The 69 analysed strains belonged to ten different genera, *Bacillus* (34), *Paenibacillus* (16), *Pseudomonas* (10, including the reference strain), *Pantoea* (4), *Lysinibacillus* (2), *Methylobacterium* (1), *Aneurinibacillus* (1) and *Tardiphaga* (1). The strains that were confirmed able to produce vitamin B_{12} *de novo* through HPLC-DAD analysis belonged to genera *Bacillus* (4), *Paenibacillus* (1), *Pseudomonas* (3, including the reference strain), *Lysinibacillus* (1), *Methylobacterium* (1) and *Aneurinibacillus* (1).

Of the 11 bacterial strains that were shown to be able to synthesize cobalamin *de novo*, ten had all eight genes of the cobalamin metabolic pathway considered in the analysis of the genomes, while the strain *Paenibacillus* sp. 2136, only had seven. The gene missing in *Paenibacillus* sp. 2136, which was also missing in all the other 15 *Paenibacillus* strains analysed, was the gene encoding for the enzyme corrinoid adenosyltransferases (CobA/CobO, EC 2.5.1.17). This is the first of the eight genes considered in the metabolic pathway (Figure 4.3), and its function is the adenosylation of cob(I)yrinic acid a,c-diamide to cobalt ion, resulting in adenosylcobyrinic acid a,c-diamide (Bryant et al., 2020; Caspi et al., 2020). CobA and CobO are two orthologous forms of the same enzyme, the former was found in *S. enterica* subsp. *Enterica* serovar Typhimurium, and the latter was found in *P. denitrificans* (https://metacyc.org/). Thus, a possible explanation for this missing gene could be that while most strains present the *cobA* form, *Paenibacillus* strains have the orthologous form *cobO*.
We also found differences in the potential to produce vitamin B_{12} among the different genera. Remarkably, even though we analyzed only two strains of *Lysinibacillus* spp., one of *Methylobacterium* sp. and one of *Aneurinibacillus* sp. among these, the proportion of strains capable of producing vitamin B_{12} was of 50%, 100%, and 100%, respectively. These genera appear to be a promising source of vitamin B_{12}-producing bacteria, as was partially reported before. *Methylobacteria* spp. have been known for a long time to be vitamin B_{12} producers (Toraya et al., 1975), and there are some studies demonstrating that *Lysinibacillus* strains have the essential genes for cobalamin synthesis, but it has not been experimentally proven if they can produce the vitamin B_{12} *de novo* (Lukáčová et al., 2022; Rodionov et al., 2019). No studies were found regarding the ability of *Aneurinibacillus* strains to synthesise cobalamin, however, bacterial strains belonging to this

species have been reported to be endophytes (Hosmani et al., 2021; Syed et al., 2019). Therefore, it would be interesting to screen more bacterial strains of these genera, both *in silico* and *in vitro*.

The proportion of vitamin B_{12}-producing *Pseudomonas* strains was also quite considerable (30%). However, this outcome is not surprising because *Pseudomonas* strains are already known vitamin B_{12} producers and are widely exploited in the industry for its production (Balabanova et al., 2021).

On the other hand, many strains (27 out of 38) did not show the ability to produce vitamin B_{12} under tested conditions. This could be explained by the fact that in the genomic analysis, only the last eight genes of the metabolic pathway were considered, which are shared by both the aerobic and the anaerobic pathways. Therefore, it could be hypothesized that the strains that have the eight shared genes but have not produced vitamin B_{12}, may be anaerobic producers, and consequently, the aerobic experimental conditions did not allow them to produce cobalamin *de novo.* This could be the case for the three *Bacillus* strains. In general, *Bacillus* strains are known to be cobalamin producers and are used for its industrial production. However, *Bacillus* sp. can be facultative anaerobes (Gudiña & Teixeira, 2022) and were previously reported to be anaerobic vitamin B_{12} producers (Mohammed et al., 2014).

Regarding the *Paenibacillus* strains analyzed *in silico*, 11 had seven genes, four had six genes, and one had none of the genes considered. Only one of them, out of 11 strains tested *in vitro*, (*Paenibacillus* 2136) was able to produce vitamin B_{12} under tested experimental conditions, as discussed above. Previous studies have demonstrated that *Paenibacillus* strains have the genes essential for cobalamin synthesis. However, it has not been experimentally proven whether and under which conditions they can produce the vitamin B_{12}(Rodionov et al., 2019; Wu et al., 2015; Zhu et al., 2015). Moreover, *Paenibacillus* strains have been reported to be facultative anaerobic (Chhe et al., 2021), thus they may need anaerobic conditions for vitamin B_{12} production, which could be an explanation for the observed variability in the vitamin B_{12} production in our study.

Even though the oxygen effect provides the most straightforward way to explain the observed differences between the genomic potential and actual, observed vitamin B_{12} production, it is not the only one. This becomes evident when reflecting on the genus *Pseudomonas* and considering seven strains that did not produce a detectable amount of cobalamin, despite having all eight genes considered, although the industrial production of vitamin B_{12} through *Pseudomonas* strains always takes place under aerobic conditions (Arasu et al., 2013; Balabanova et al., 2021; Xia et al., 2015). Vitamins are complex molecules with intricate synthetic pathways that are highly regulated and co-depend on many external factors; thus, optimised growth conditions are essential for efficient biosynthesis (Acevedo-Rocha et al., 2019). Indeed, the optimal conditions used in the industrial process for aerobic cobalamin production through *P. denitrificans,* are significantly more customised than those applied in our study. Briefly, the industrial process takes place at 30 °C, pH of 6.0–7.0 in 120 m^3 fermenters and lasts about 6–7 days. Sucrose is used as a carbon and yeast extract as a nitrogen source, and mineral salts are also added (Martens et al., 2002). Fermentation begins with a high level of dissolved oxygen concentration (8–10%) followed by its reduction to 2–5% (49–106 h) and further below 2% (107–168 h) (Li et al., 2008; Peng et al., 2014). This multi-stage dissolved oxygen concentration (DOC) control strategy increases the vitamin B_{12} yield by approximately 20% (70 mg/L). At the beginning of the culture growth, the medium is supplemented with 10–25 mg/L of 5,6-

dimethylbenzimidazole (DMB), a precursor of cobalamin, and 40–200 mg/L cobalt-nitrate (Marie Sych et al., 2016). As previously described, our experimental conditions were drastically different. For example, we did not design a multi-stage DOC control strategy and any cobalamin precursor to the medium was added. This choice was made because our objective was not to look for a producer of vitamin B_{12} to be included in an industrial production process but a strain capable of producing vitamin B_{12} in a completely independent way, which could therefore have a better chance of producing cobalamin in plants. Nevertheless, considering that all our producers showed higher yields of vitamin B_{12} than the reference strain, their exploitation for industrial production of vitamin B_{12} could be further explored.

Vitamin B_{12} extracted from lettuce was quantified by HPLC-DAD and was expressed as µg of vitamin B_{12} per g of dry weight (DW) of lettuce leaves. Our experiments showed that for lettuce 1 g of dry weight corresponds to about 20 g of fresh weight. Therefore, considering the amounts of vitamin B_{12} detected in lettuce treated with bacterial strain *Methylobacterium* sp. P1-11 were 1.654 µg/g for lettuce grown on MS medium and 2.559 µg/g for lettuce grown on MS medium supplemented with cobalt chloride, we can assess that a normal portion of 80 g of lettuce grown under our experimental conditions is sufficient to reach the vitamin B_{12} RDA (2.4 µg per day). However, we have no further information on the bioavailability of the cobalamin detected in lettuce nor do we know which forms of cobalamin were produced by *Methylobacterium* sp. P1-11, because our extraction method utilized KCN, which brings all the cobalamin analogues to the more stable cyanocobalamin. Thus, in future this aspect could be explored more in detail. It should also be checked that any dangerous residues or antibiotic compounds produced by bacteria are not released into the food.

Considering the other strains tested in lettuce, we could not detect any synthesise activity *in planta*, even though the endophytic presence was confirmed through a cultivation-based approach combined with IGS-RFLP typing for all except *Aneurinibacillus* sp. C8BA17. The interactions between endophytes and their host plants are complex and not fully understood, further research is needed to explore the multidimensional network between plants and endophytes, that involve also the soil and the environment. This approach in future will help to better exploit the potential of these microorganisms for a sustainable agriculture or for producing host metabolites, which can be utilised on a large scale for potential use in diverse areas (Compant et al., 2016; Khare et al., 2018). Therefore, a successful colonisation is only the first step towards establishing potentially beneficial symbiosis.

From a future perspective, we could consider different crops to test our bacteria. For example, bulbs, roots, and tuber crops, such as onion, beets, potatoes, and carrots, store high amounts of water and carbohydrates, as well as proteins and vitamins. To give some examples, onions store proteins, carbohydrates, sugars, vitamin C, β-carotenes, minerals (Ca, Fe, S) and some flavonoids and polyphenols (Sami et al., 2021). Sweet potatoes have high levels of carbohydrates, proteins, and fibres and are also a good source of vitamin C, vitamin E, pantothenic acid, and provitamin A (beta-carotene), which gives them the typical orange colour. Potato tubers are rich in carbohydrates, proteins, minerals, and vitamins B_6 and C (Zierer et al., 2021). Therefore, we can hypothesise that these reserve organs may also store vitamin B_{12}. Besides, some of these crops are already commonly enriched with nutrients through various biofortification approaches. Indeed, biofortification of staple foods such as potatoes and sweet potatoes is widely recognised as promising approach to alleviate

hidden hunger (Pfeiffer & McClafferty, 2007). In potatoes, for example, mineral biofortification (Fe and Zn) comes with different agronomic practices such as tuber priming (Vergara Carmona et al., 2019), and foliar or soil application of fertilizers (Kromann et al., 2017; White et al., 2017). While β-carotene enriched orange-fleshed sweet potatoes are mostly produced by conventional breeding and transgenic biofortification (Siwela et al., 2020). Moreover, roots and tubers have a high bacterial density, which ranges from 10^5 to 10^7 CFU g^{-1} of fresh weight, compared to leaves and stems, where it ranges from 10^3 to 10^4 CFU g^{-1} of fresh weight (Compant et al., 2010). Thus, a higher density of vitamin B_{12}-producing bacteria could lead to the accumulation of a higher concentration of the vitamin in the roots. Therefore, in the future it would be interesting to test the vitamin B_{12}-producing bacterial strains identified in this study, on crops having an underground edible organ.

4.5 CONCLUSION

In summary, in this study, we identified several bacteria strains capable of synthesising cobalamin, one of which produced vitamin B_{12} in lettuce plants under defined experimental conditions. To our knowledge, this is the first time that a bacterial endophyte was used for this purpose. Therefore, this work provides evidence that bacterial endophytes could be utilised to enhance the nutraceutical values of plant-based foods.

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Chapter 5

Conclusion

In the present research work, the possibility of exploiting bacterial endophytes to improve the state of health and to enhance the nutraceutical qualities of food plants was studied.
Regarding the possible application of endophytes as BCAs, the VOCs emitted by *Streptomyces* sp. SA51 were tested *in vitro* against the mycelial growth of *F. oxysporum* f.sp. *lactucae* (FOL). and in lettuce plants The VOCs emitted by *Streptomyces* sp. SA51 proved to be less effective in contrasting the mycelial growth *in vitro* (-8%) and more in relieving Fusarium wilt symptoms on lettuce seedlings. The evaluation of the health conditions of lettuce plants was carried out through the calculation of the MKI, which reported a 25% decrease when the infected lettuce seedlings were grown in the presence of bacterial VOCs, while uninfected plants also benefited from the presence of the VOCs, showing a 48% decrease. Therefore, VOCs produced by *Streptomyces* sp. SA51 demonstrated excellent potential both as biocontrol agents against FOL, one of the most concerning lettuce pathogens, and as a biostimulant for the growth of lettuce.
The study of the VOCs profile of *Streptomyces* sp. SA51, extracted by HS-SPME and analyzed by GC-MS, allowed to identify several compounds with presumed or accredited antifungal properties: phenylethyl alcohol (PEA), γ-muurolene, germacrene D, 2-methylisoborneol (2-MIB) and geosmin.
Although further investigations are needed to further characterize the volatile compound, or most likely the compounds, responsible for the significant observed results, this work opens interesting possibilities that could lead to the application of bacterial VOCs as a tool to improve the quality of plant food in an environmentally friendly perspective.

Endophytes have also been tested as a tool to increase the polyphenol content of plants for the baby-leaf market, with a consequent improvement in nutraceutical qualities and plant resistance to biotic and abiotic stresses. The seeds of three vegetable cultivars (red mustard, lettuce, and bull's blood) were treated separately with *Streptomyces* sp. SA51, *Pseudomonas synxantha* DLS65 and *Pantoea* sp. S1. The treatments showed an improvement of the morphological parameters (height and fresh weight) of the red mustard. Total phenolic compounds (measured by Folin-Ciocâlteu assay) and antioxidant activity (measured by ABTS assay) were found to be higher in red mustard treated with *Pantoea* sp. S1, while lettuce showed an increase of total phenolic compounds content both when treated with *Streptomyces* sp. SA51 and *Pantoea* sp. S1. From the identification and quantification of the extracted phenolic compounds (through LC-ESI-IT-MS/MS analysis) it was observed that *Streptomyces* sp. SA51 and *Pantoea* sp. S1 were able to increase some classes of polyphenols in all the *cultivars,* while *P. synxantha* DLS65 did not cause any positive variation.
Therefore, it is possible to conclude that endophytic bacteria can modify the phenolic content of plants, both from a qualitative and quantitative point of view, and consequently also the antioxidant potential. Although further studies are needed in the future in order to identify the best endophytes to combine with different plant species, this work represents a starting point towards new possibilities of obtaining healthier plants richer in nutraceuticals.
Finally, based on the presence of the vitamin B_{12} metabolic pathway in their genomes, 38 bacterial strains were selected and tested to verify their ability to synthesise vitamin B_{12} *de novo.* Among the 38 strains, 11 were shown to produce *de novo* the vitamin B_{12} and the three best candidates were further tested to assess whether they could be used to enrich lettuce plants with the vitamin. The experiment was carried out in sterile conditions on lettuce plants. The bacterial strain

Methylobacterium sp. P1-11 was shown to produce vitamin B_{12} *in planta*: 1.654 and 2.559 µg of vitamin B_{12} per g of dry weight measured on MS medium and on MS medium supplemented with cobalt chloride, respectively. Our results showed that the P1-11 strain is able to produce a satisfactory amounts of vitamin B_{12} with respect to the RDA. This study opens new possibilities to produce biofortified food through the application of vitamin B_{12}-producing bacteria and new perspective for people who follow a plant-based diet that, and who, therefore, cannot get the RDA of vitamin B_{12} from food of animal origin.

This work demonstrated the possibility of using endophytic bacteria, which act as promoters of plant growth directly or indirectly, to improve the nutraceutical content and the health of plants destined for the baby-leaf market. It has been proved that this approach can be a response to the needs of consumers who ask for healthy food produced in an environmentally friendly way, for all the consumers, whose diet could incur in nutritional deficiencies.