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DECIPHERING THE ROLE OF PLANT MICROBIOME ON STRAWBERRY AND RASPBERRY GROWTH, RESISTANCE, FRUIT QUALITY AND AROMA

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I believe in God, only I spell it Nature.

Frank Lloyd Wrigh

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Abstract

In nature, plants live in close contact with several different microbes. Besides their own adaptation strategies, plants might exploit microbial symbionts for overcoming both biotic and abiotic stresses and increase fitness. The current scenario of rapid climate change is urgently demanding for more sustainable agricultural management practices both to prevent further changes and to enhance climate adaptation. The application of microbe-based products might be a valid alternative to synthetic pesticides and fertilizers. However, several knowledge-gaps hinder the successful application of beneficial microbes in the field. These, together with the possible use of microbes to overcome stresses exacerbated by climate change, have been reviewed in the first part of this thesis. Berry fruits are widely cultivated and appreciated for their aromatic and nutraceutical properties. This thesis is focused on the role of plant and fruit microbiome on strawberry and raspberry growth, resistance, fruit quality and aroma. Firstly, a taxonomical and functional description of the microbiome of different organs of three strawberry genotypes was performed both by traditional cultural dependent method and Next Generation Sequencing (NGS) technique. This study highlighted that both plant organs and genotype had a significant role in determining the composition of microbial communities. Additionally, a selection of bacteria native of strawberry plants were isolated and screened for their plant growth promoting (PGP) abilities, in both normal and stress conditions. In particular, the biotic stress of *Xanthomonas fragariae* infection and the abiotic stress of induced salinity were tested. The monitoring of biometric parameters on inoculated plants allowed the selection of a more restricted panel of bacterial strains, whose beneficial potential was tested in coordinated inoculations, or else with a selective application on different plant organs.

Raspberry plant was taken as a model for investigating the effect of cultivation method (Organic or Integrated Pest Management) in determining fruit microbiome, and its consequent influence of berry quality and aroma. Interestingly, the cultivation method strongly influenced fruit nutraceutical traits, aroma and epiphytic bacterial biocoenosis. In particular, changes in fruit aroma could be partly explained by volatile organic compounds (VOCs) emitted by key bacterial genera characterizing organic or IPM raspberry fruits. The involvement of the bacterial microbiota in fruit aroma determination was evaluated by performing an untargeted GC–MS analysis of VOCs occurring in control, sterile and artificially reinoculated berries and by characterizing

control and reinoculated berry microbiome by NGS. Differently treated berries showed significantly different aromatic profile, which confirms the role of bacteria in fruit aroma development.

Overall, the aim of this thesis was to extensively describe the microbiome of strawberry and raspberry plants and to examine their role in shaping plant performances, quality and aroma. The results obtained will hopefully support further application of microorganisms in the horticultural sector, both in pre- and post-harvest crop management.

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1. General Introduction

1.1 Plant-associated microbial communities and their role in plant growth

The steady increase of the global population, together with an urgent need of increasing the environmental sustainability of the entire food supply chain, requires efficient methods aimed at improving the resource use efficiency of crops production worldwide. Since the colonization of lands 450 million years ago, plants interact with microorganisms which either live around the below-ground part of the plant (rhizosphere), inside (endosphere) or on the above-ground surface (phyllosphere) of the plant (Orozco-Mosqueda et al., 2018). Microbes affect the fitness and growth of the macroorganisms they are associated with.

Thus, a deep knowledge of the interactions occurring between microbial communities and their hosts is essential for their efficient exploitation and application in agriculture.

1.1.1 Plant-associated microbial communities: concepts definition

Several evidences have underlined that microbial communities and their host interact and perform in a concerted manner (Zilber-Rosenberg, 2008; Sessitsch et al., 2019), giving birth to the **holobiont** concept. In this view, the plant cannot be seen as a separate and self-enclosed entity, but rather as a complex biological unit where the plant-associated microbial community is pivotal in delivering multiple functions, such as plant growth promotion and increased stress resilience. Plant-associated microbial communities can be referred to as microbiomes, microbiotas or metagenomes, depending on the entities, environments and activities considered (Berg et al., 2020). **Microbiota** is defined as the assemblage of living microorganisms present in a defined environment (Marchesi and Ravel, 2015), thus bacteria, fungi, actinomycetes and protists (excluding viruses, plasmids, prions, viroids and free DNA) (Duprè and O'Malley, 2013). On the other hand, when the set of microorganisms living, thriving and interacting with a particular plant organ accomplishes several activities, it can be defined as **microbiome** (Orozco-Mosqueda et al., 2018). According to this definition, the microbiome includes molecules, metabolites and mobile genetic elements produced by the microbiota, but also by its host (Berg et al., 2020). The collection of genomes and genes of the components of the microbiota are designated as metagenome (Marchesi and Ravel, 2015).

Plant microbiome composition is mostly influenced by plant compartments, environmental conditions and host phylogeny (Bouffaud et al., 2014). Bulk soil shows the highest microbial species richness, followed by the rhizosphere and the endophytic compartment (Rodriguez et al., 2019). Similarly, different communities might colonize the epiphytic surface of flowers and leaves of the same plant (Junker et al., 2011). Phyllospheric and floral environments are characterized by similar conditions, being exposed to UV radiation, desiccation and uneven nutrient availability and thus microbial colonization is limited in both organs (Vannette, 2020). Traditionally, microbiome studies focused on the taxonomy of plant-associated microbial communities. In this regard, indices of alpha, beta and gamma-diversity are widely used (Table 1.1).

Table 1.1. Definition of ecological diversity indexes.

Index	Definition
Alpha diversity	Microbial diversity within a particular area/ecosystem. It is referred also as species richness, i.e. number of species in that ecosystem
Beta diversity	Change in species diversity between different ecosystems, i.e. total number of unique species of a given ecosystem
Gamma diversity	It measures the overall diversity for different ecosystems within a region

In the last decades, **functional diversity** has received prime consideration in the context of sustainable agriculture as it has been recognized as the best predictor of ecosystem processes and properties (Johnson and Pomati, 2020). Being plants sessile entities, functionalities provided by the microbiota are essential for supporting plants in coping with adverse environmental conditions (Lemanceau et al., 2017). Understanding what microorganisms are doing and how they respond to stresses is pivotal for their successful application in agriculture. Similarly to taxonomic diversity, functional diversity is also largely determined by the plant niche (Bai et al. 2015), although biochemical functions are more uniformly distributed among organs than taxa (Lemanceau et al., 2017). Potential functions expressed by associated microbial communities might be examined both by a culture-dependent and -independent approach, by *in vitro* screening of microbial traits and by applying computational techniques, such as Tax4fun, respectively.

The complexity of the interactions occurring in plant-associated microbiomes poses a challenge for the elucidation of the specific functionals microbes affecting plant growth and fitness (Mueller and Sachs, 2015). The definition of a **core microbiome** is essential for focusing on stable microbial populations providing beneficial features to the host, excluding transient associations (Busby et al., 2017). The core microbiome can be defined based either on the taxonomy or on the functionalities of microbes. Several definitions have been proposed, depending on the ecological scale defined. The core can be, for example, the microorganism taxa recurring in different samples of the same plant species in the same environment or in different conditions. Unravelling networks and microbial hubs existing in microbial communities is essential for mining functionalities and assembly rules (van der Heijden and Hartmann, 2016). These are pivotal knowledges for efficient agricultural manipulation of the microbiota (Busby et al., 2017).

1.1.2 Interactions of the microbial communities with the host and the ecosystem

Microbes are known to interact with all plant organs, both epi- and endophytically. Endophytic microbes present several advantages over surface-colonizing microorganisms, being less affected by external predators, by fluctuating environmental conditions and probably communicating in a more efficient way within plant tissues (Coutinho et al., 2015). This might be due to the lower concentrations of bacterial metabolites needed for the elicitation of biological effects, with respect to an open environment, such as the rhizosphere (Santoyo et al., 2017).

Traditionally, microbial communities residing in the rhizospheric compartment received more attention from the scientific community. However, the pivotal role of the phyllospheric microbiome in delivering beneficial functionalities to the plant has been suggested for years (Orozco-Mosqueda et al., 2018).

Plant-associated microbes are characterized by several traits which grant their successful establishment on/in the host. They should be able to use nutrients, metabolites and polysaccharides provided by the plant. N-acyl homoserine lactones (AHL) are among the best characterized molecules playing a key role in bacterial signalling and quorum sensing. Additionally, microbes are able to react to environmental stresses by implementing several strategies, such as the production of siderophores, formation of biofilms, and the mobilization of nutrients from the soil or the associated plants. Altogether, the microbial mechanisms mentioned above might facilitate the promotion of plant growth and enhance plant capability to cope with

biotic and abiotic stresses. Here, the main beneficial functions expressed by microbes will be briefly described.

1.1.3 Phytohormones modulation

Beside regulating basic physiological processes, phytohormones are fundamental for the modulation of plant responses to various environmental stimuli. Microbes, as well, are able to produce phytohormones to establish plant–microbe interactions, such as pathogenesis and phytostimulation. Thus, several phytohormone-synthesizing microorganisms may have a potential for agricultural application, for instance as biofertilizers or biostimulants.

Auxins regulate essential plant processes, such as cell elongation, root growth induction, flower and fruit development and leaf senescence (Vanneste and Friml, 2009). They are known to be produced both by the plant and by microbes (Spaepen and Vanderleyden, 2011). Indole-3-acetic acid (IAA) is the most studied and well characterized auxin, which is assumed to be synthetized by over 80% of rhizospheric bacteria (Patten and Glick, 1996). In microorganisms, its biosynthesis occurs both via tryptophan-dependent and independent pathways, being the first pathway the most common one. Exogenous auxins of microbial origin might interfere with the regular development of plants both in positive or negative ways. Indeed, pathogenic bacteria, such as *Agrobacterium* spp., as well as plant growth promoting bacteria (PGPB), such as *Azospirillum* spp., are known to synthetize IAA (Spaepen and Vanderleyden, 2011). Since several decades, auxins and cytokinins are known because their *in vitro* application to undifferentiated callus cultures leading to the regeneration of plant organs. The type of organs regenerated depends on the auxin/cytokinin ratio. If the ratio is high, hormones application triggers root formation; on the contrary, low ratios lead to shoot formation (Skoog and Miller, 1957). In this sense, cytokinins and auxins acts as antagonists to each other. Microbial production of cytokinins has been described. However, its role in plant growth stimulation needs to be fully understood. Indeed, cytokinins in microbes have been often only described *in silico*. Application of *Bacillus megaterium* on *Arabidopsis thaliana* resulted in shorter tap roots and highly branched lateral roots attributable to the synthesis of cytokinins (Ortiz-Castro et al., 2008).

Gibberellins affect cell division and elongation and are involved in several plant developmental processes, including seed germination, stem elongation, flowering, fruit setting, delay of senescence and root growth promotion in different organs and

plant species (MacMillan, 2002). Gibberellins have been found to be synthesized by several endophytes and root-associated bacteria (*Azotobacter*, *Arthrobacter*, *Azospirillum*, *Pseudomonas*, *Bacillus*, *Acinetobacter*, *Flavobacterium*, *Micrococcus*, *Agrobacterium*, *Clostridium*, *Rhizobium*, *Burkholderia*, and *Xanthomonas*) and to be associated to their plant growth promotion mechanism (Gamalero and Glick, 2011) .

Abscisic acid (ABA) biosynthesis is known to be induced by drought stress, resulting in stomata closure. Although ABA metabolism in microorganisms is not well investigated yet, several rhizobacteria have been observed to produce ABA in vitro under stressful conditions (Dodd et al., 2010).

Ethylene is a hormone involved in several plant growth aspects, such as germination, root development, senescence and fruit ripening, and in stress regulation and tolerance (Bleecker and Anthony, 2000). The pathway for ethylene biosynthesis universally encountered in plants is initiated by adenosyl methionine (SAM), which is converted into aminocyclopropane-1-carboxylate (ACC) by mean of ACC synthase (ACS) and then to ethylene via the induction of the ACC oxidase enzyme (ACO). In bacteria, other ethylene-producing pathways use 2-oxoglutarate or 2-ketomethylthiobutyrate as the precursors (Zhang et al., 2017; North et al., 2017).

Bacteria might contribute to plant ethylene regulation by 1-aminocyclopropane-1-carboxylate deaminase (AcdS) production (Glick, 2014). Indeed, after plant colonization by AcdS-producing bacteria, ACC is shunted from ethylene to ammonium and alpha-ketobutyrate, lowering ethylene production in plants. A detailed overview of the role of AcdS-producing bacteria in helping plants overcoming biotic and abiotic stresses can be found in chapter 3.

1.1.4 Nitrogen fixation, siderophore production, phosphorus solubilization: bacteria help plants in nutrient acquisition

Nitrogen (N) is one of the essential nutrients for ensuring plant growth and productivity. In agriculture it might become a limiting factor and therefore external application is generally needed. N-fixing microorganisms able to convert the atmospheric N_2 into accessible forms for plants belong to phyla Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Firmicutes, and Cyanobacteria (Pathania et al., 2020). N_2 can be fixed by microorganisms either symbiotically or non-symbiotically.

Phosphorus (P) is one of the most limiting nutrients in plant cultivation and it is essential for cell division and new tissue generation (Dissanayaka et al., 2018). Uptake

of P from plants is only possible in the form of mono- and dibasic phosphates. Thus, Phosphate Solubilizing Bacteria, able to transform insoluble P into the soluble form, might be applied to increase plant growth and yield (Jha and Saraf, 2015). Bacterial genera capable of phosphate solubilization are *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Enterobacter* and *Streptomyces* (Singh and Satyanarayana, 2011).

Plant growth and metabolism can be severely compromised by an iron (Fe) deficiency, which might result in poor yields and low crop quality. Fe has a key role in chlorophyll synthesis and is fundamental for chloroplast maintenance, however it can be assimilated by plants only as Fe^{2+} (Neilands, 2014). Fe^{3+} is the most abundant Fe ion in the soil, and it can be chelated by bacterial siderophores (Ghosh et al., 2020). Once the chelated complex has been formed, it is transported into the cell. Here, it is released by reduction as Fe^{2+}. Assimilation of Fe^{2+} by plants can occur both by direct uptake of the siderophore complex or via an exchange reaction using an appropriate ligand (Sadaghiani et al., 2014). Besides being involved in plant growth promotion, siderophores can also play a key role in plant protection (Robin et al., 2008). Siderophores secreted by PGPB may reduce the growth of phytopathogens by reducing the iron availability for plant pathogens (Ghosh et al., 2020).

1.1.5 Antibiotics and lytic enzymes production

Decrease in crop productivity can be determined by losses due to pests and pathogens. Besides promoting plant growth through hormone production or via enhancement of nutrients acquisition, microorganisms can produce antibiotics and secrete lytic enzymes that help plants in coping with pathogens attack. Production of these secondary metabolites results in competitive advantage, enhancing the colonization ability of microorganisms (Zaidi and Khan, 2017). 2,4- diacetylphloroglucinol (DAPG) and hydrogen cyanide are among the most well studied bacterial antibiotics. They are mainly produced by *Pseudomonas* spp. (Mishra and Arora, 2018; Blumer et al., 2000), providing biological control against several diseases affecting diverse crops worldwide. Chitinases, glucanases and lipases are among the best studied extracellular enzymes secreted by microbes, being a desirable trait in biocontrol agents, having a key role in weakening and degrading the cell walls of plant pathogenic fungi (Veliz et al., 2017). For instance, *Bacillus pumilus* MCB-7 was able to inhibit the growth of plant pathogenic bacteria and of the rice pest *Scirpophaga incertulas* due to its high chitinolytic activity (Rishad et al., 2017). Halophylic bacteria,

besides chitinase production, displayed the ability to produce β-1,3-glucanase, being effective in controlling grey mould in strawberry (Essghaier et al., 2009).

1.1.6 The role of microbial volatiles in plant-microbe interaction

Volatile organic compounds (**VOCs**) are small molecules (<300 Da; <C20), characterized by high vapour pressure, low boiling point and high lipophilicity which promote their evaporation and diffusion. VOCs are secondary metabolites synthesized both by micro- and macroorganisms, playing a key role in intra- and interkingdom interaction and communication. The term volatilome refers to the totality of VOCs emitted by a defined entity (Weisskopf et al., 2021). Bacteria generally emit VOCs belonging to alkene, alcohol, ketone, terpene, benzenoid, pyrazine, acid and ester classes whereas fungal VOCs are typically alcohols, benzenoids, aldehydes, alkenes, acids, esters and ketones (Piechulla and Degenhardt, 2014). mVOC 2.0 (http://bioinformatics.charite.de/mvoc/) is a database that comprises more than 2000 identified VOCs produced by bacteria, fungi and yeasts (Lemfack et al., 2014).

Microbial VOCs (mVOCs) act as signal molecules between microbes and with plants. mVOCs have been reported to increase plant tolerance to both biotic and abiotic stresses (Figure 1; Cellini et al., 2021). In 2004, Ryu et al., firstly discovered that the volatile 2-3-butanediol produced by *Bacillus amylolyquefaciens* and *B. subtilis* triggered induced systemic resistance (ISR) in *Arabidopsis* seedlings. From then on, the different effects that mVOCs might exert either directly or indirectly on plant performance have been deeply investigated. mVOCs might interplay with plant hormones stimulating plant growth. This occurs, for example, with indole produced by *Proteus vulgaris*, which interacts with auxin, cytokinin, and brassinosteroid pathways (Bhattacharyya et al., 2015). Similarly, acetoin and 2,3-butaendiol produced by *B. amyloliquefaciens* FZB42 induced, via the activation of the salicylic and abscisic acid pathways, the stomata closure of *Arabidopsis thaliana* and *Nicotiana benthamiana* leaves (Wu et al., 2018).

Interestingly, mVOCs can also influence the uptake or availability of specific nutritive elements (Weisskopf et al., 2021). VOCs emitted by *B. amyloliquefaciens* GB03, for example, promoted *Arabidopsis* growth via different mechanisms: rhizosphere acidification that leads to a higher iron availability; induction of the upregulation of an extracellular ferric reductase that reduced Fe^{3+} to Fe^{2+}, induction of the overexpression of plant iron transporters (Zhang et al., 2009).

mVOCs can also increase plant ability to cope with biotic and abiotic stresses. mVOCs can increase plant tolerance to pest and pathogens attack either by inducing systemic plant defenses (ISR) or by hindering the growth of the pest/pathogen (as in the cases of ammonia, cyanide, and sulfur-containing metabolites). Remarkably, the first mechanism occurs systemically at low emissions rates and persists after the removal of the emitter whereas direct pest/pathogen inhibition requires high and continuous emissions of mVOCs. Occasionally, ISR and direct inhibition mechanisms might coexist, as it is the case of dimethyl disulfide and benzothiazole (Cellini et al., 2021). Beyond increasing plant tolerance to biotic stresses, mVOCs have been demonstrated to be able to relieve plants from drought and salinity, by leading to the accumulation of compatible solutes, or lowering sodium accumulation (Cho et al., 2008; Bhattacharyya et al., 2015). VOCs produced by microbes might also trigger an enhancement of crop quality when it is directly associated to essential oils and aroma diversity and quantity (Cellini et al., 2021). Indeed, the production of these compounds might be increased in response to VOCs emitted by defense-inducing microbes. The role of the bacterial microbiome in shaping berry fruit aroma will be studied in details and proved in chapter 6 and 7 of this work.

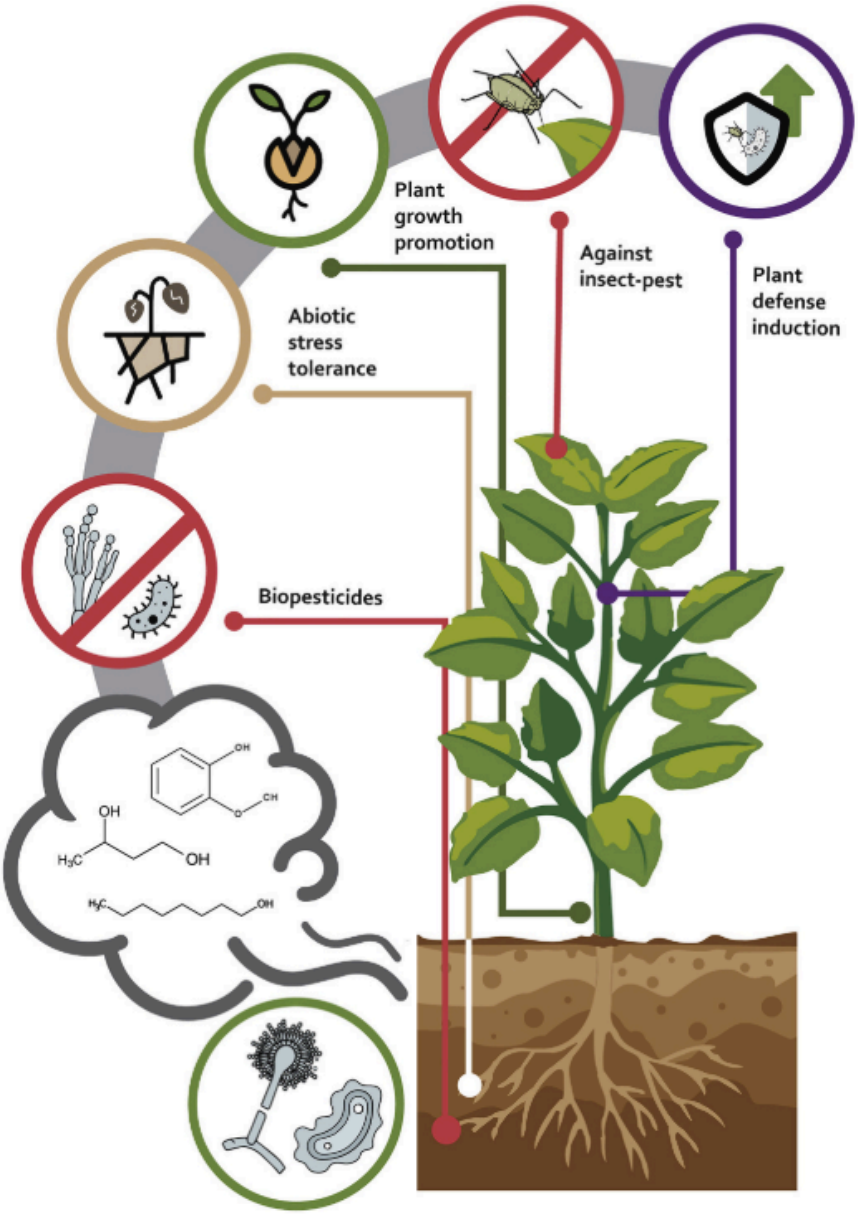

Figure 1 - Graphical representation of the effect of microbial volatiles on plants (from Poveda, 2021).

1.2 The microbiome study: techniques and challenges

In order to achieve a comprehensive picture of the microbiome influence on plant host phenotype and fitness, microbiome studies should aim at capturing as much diversity as possible (Bulgarelli et al., 2013), keeping in mind that 98% of the microbial diversity present in the environment is not culturable (Handelsman, 2004). Therefore, although culture-based methods are proper tools for the characterization and exploitation of microbes, molecular approaches are needed for the assessment of the complexity of

microbial communities (Pineda et al., 2019). **Metagenomics** studies the microbial communities snapshotting the genes present in a certain sample, revealing both their taxonomy and functionalities. In the last years, advancements of high-throughput sequencing (HTS) techniques, together with costs reduction, completely transformed the way microbiotas are analyzed. **Next Generation Sequencing** (NGS), also called second generation sequencing, was developed several years after Sanger DNA sequencing (first generation sequencing). Sanger DNA sequencing is the first sequencing method based on the selective incorporation of chain-terminating dideoxynycleotides by DNA polymerase during *in vitro* DNA replication. During both NGS and Sanger processes, the enzyme adds fluorescent nucleotides onto a growing DNA template strand. The most remarkable difference between the two techniques is the sequencing volume because Sanger sequences one strand at a time whereas NGS is able to sequence millions per run. However, one of the main drawbacks of NGS is its high cost.

NGS platforms generate short-read sequences in a rapid and automatic way. NGS comprises basically four steps: i) library preparation, ii) cluster amplification, iii) sequencing, iv) alignment and data analysis. Library preparation is performed by DNA fragmentation followed by adapter ligation to both fragment ends. Subsequently, DNA is amplified, loaded on a flow cell and sequenced in massive parallel sequencing reactions. Afterwards, sequencing occurs by incorporation of fluorescent-labelled deoxynucleotides in elongating DNA. The sequencing output (reads) consists of strings of bases that have been recognized by the emission wavelength and intensity in the sequencing process. The final step is the alignment of reads to reference sequences. Targeted analysis sequencing is a technique that focuses on amplicons and specific genes and allows researchers to analyze genetic variation in precise genomic regions[1]. The application of HTS techniques to metagenomics is based on the sequencing of highly conserved sequences indicative of definite taxa, such as the region V3 and V4 of the 16S ribosomal subunit gene.

In culture-independent approaches, the study of the functionalities that might be expressed by plant-associated microbes is made possible by the statistical association of genes/pathways of interest with a given microbial species. This is done with the aid of software, such as PICRUSt (Langille et al., 2013) and **Tax4Fun** (Aßhauer et al.,

[1] The description of NGS technique is based on the notes from the e-learning course for Food Microbial Bioinformatician - LEGO project.

2015), allowing the prediction of functional traits by comparing bacterial 16S rRNA genes with information available on the Kyoto Encyclopedia of Genes and Genomes (**KEGG**) database.

Potential errors occurring when applying NGS might include sequencing artefacts and taxonomic misidentifications (Shokralla et al., 2012). Additionally, NGS does not discriminate between live and dead cells, leading to an overestimation of the microbial targets. It is important to note that sampling depth might lead to experimental biases and it is therefore fundamental to **normalize** taxon or OTUs (Xia et al., 2020), in order to reduce data heterogenicity. Normalization means to divide each taxon or OTU by the total sum library size (i.e. total sum of count generated across all taxa in the sample) (Xia et al., 2020).

In Table 1.2, some of the most widely used multivariate statistical tools for correlation and associative methods among OTUs data are reported, together with the advantages and disadvantages linked to their application for microbiome data handling.

Table 1.2- Multivariate statistical tools for microbiome data analysis. This table was drawn based on the information reviewed in Xia et al., 2020.

	Description	**Advantages (PRO) and Disadvantages (CON)**
Principal Component Analysis (PCA)	Data are converted, through an orthogonal transformation, into new variables (defined as Principal Components)	*PRO* Reduction of data dimension. Filter noise. *CON* Inconsistent when the number of variables highly exceeds the sample number. Euclidean distance is used for measuring samples dissimilarity but dataset containing several zeros may produce artifacts
Correspondence Analysis (CA)	Similar to PCA but for counted data	*PRO* CA maximizes the correspondence between measured variables samples of datasets. It assumes a unimodal Model. It uses weighted Euclidean distance.

		CON Mathematical artifact calles "arch effect" might be produced
Principal Coordinate Analysis (PCoA)	Spectral decomposition is used to approximate a matrix of distances/dissimilarities by the distances to reduce dimensions of data points	*PRO* Any distance can be used
Non Metric Multidimension scaling (NMDS)	It explicitly chooses a small number of ordination axes Data are then fitted to those dimensions prior to the analysis.	*PRO* Any distance measure suitable for the data analysis might be used. Linear relationship assumption is not necessary. Point are arranged in order to maximize rank-order correlation between real-world distance and ordination space distance.

Altogether, these tools provide relevant visual insights on the distribution of data among samples whereas Mantel test, ANOSIM and PERMANOVA are among the most used multivariate statistical tools to test whether data are significantly different or not.

One of the main goals of microbiome studies is the understanding of the effect of the microbiome on the physiological properties of the host. Association of microbiome data resulting by NGS with other omics data is not straightforward due to the intrinsic nature of the first ones (Xia et al., 2020). Indeed, NGS data are high dimensional, discrete, sparse and usually with numerous zero values (Xia et al., 2018), therefore several precautions need to be taken into consideration when performing statistical correlations (reviewed in Xia et al., 2020). The most used statistical tools for performing correlation among NGS and other omics data are Redundancy Analysis (RDA) and Canonical Correspondence Analysis (CCA). The main difference among the two is that CCA treats both sets of variables symmetrically and it assumes a unimodal, rather than linear, relationship among variables.

1.3 Practical exploitation of the microbiome in agriculture: applications, limitations and new approaches

To date, several experiments focused on plant microbiome composition and functionalities under natural and agricultural conditions in model and crop plant species. Nonetheless, inadequate coordination among the academic world, industry R&D areas and crop producers did not translate new knowledge into efficient agronomical solutions (Busby et al., 2017). Sustainable plant protection and growth promotion may play a role in ensuring food security to an increasing world population. Application of microbe-based products is in several cases a viable biological alternative to synthetic pesticides and fertilizers that meets the consumers' and regulators' need for zero-residue food and lowers the possibility of pathogen resistance to active principles. However, several challenges limit the effective microbial application in the field. These might be solved by understanding the complexity and ecological behaviour of natural microbiota via omics studies (Sessitsch et al., 2019). In particular, knowledge about the assembly and regulation of microbiomes in different plant genotypes, environments and their response to biotic and abiotic stresses might increase the success of microbial inoculation. Additionally, detailed study of the steps necessary to develop microbe-based products is fundamental for highlighting criticalities that might lead to unsuccessful microbial application in the field.

1.3.1. Toward the development of microbe-based products and related constrains

Developing a microbe-based product consists mainly of two different steps: the selection of the strain and the registration for commercial use (Figure 2).

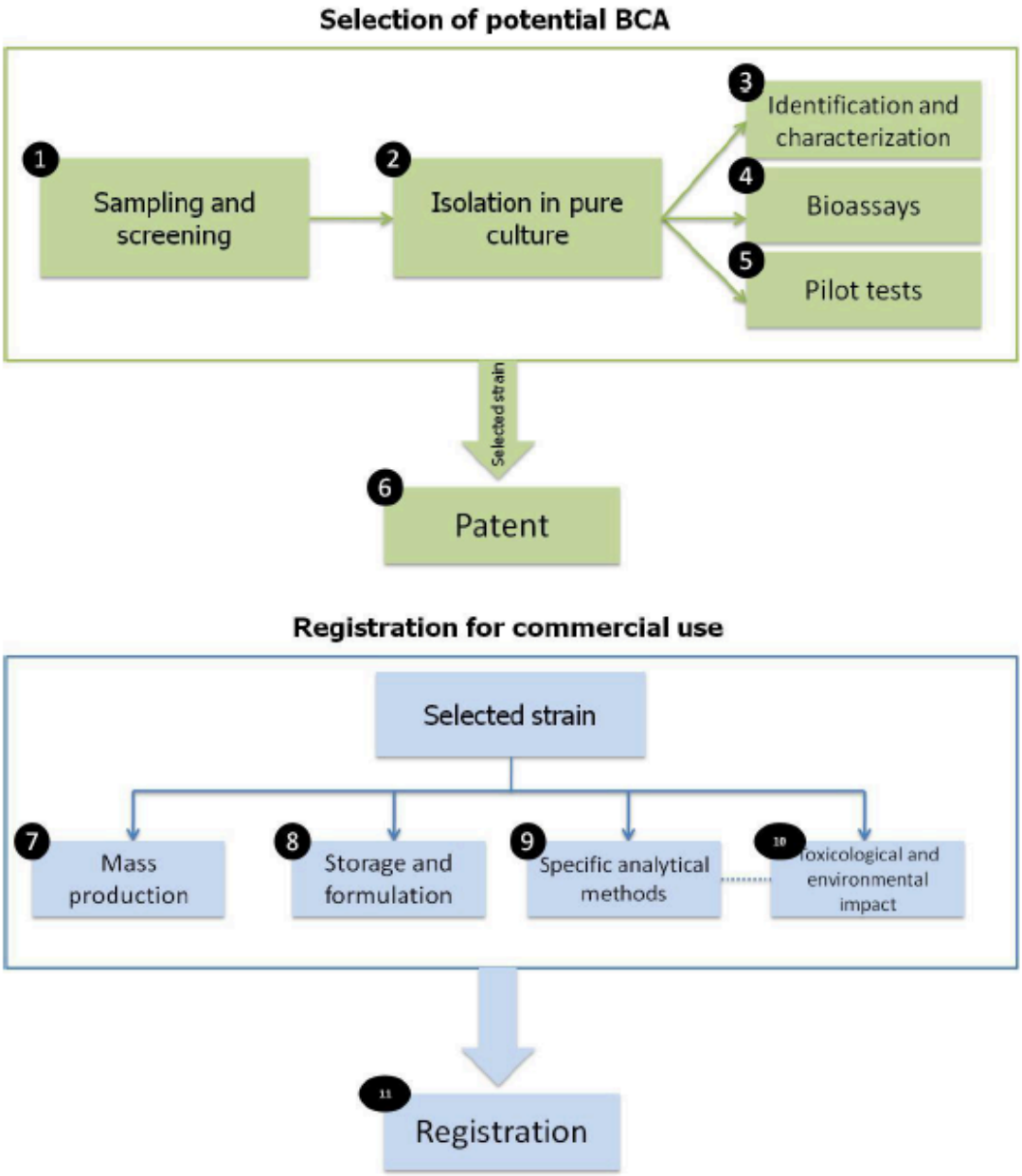

Figure 2 - Schematic representation of microbe-based product development.

Fungal, bacterial and yeast strains are isolated from several environmental sources (i.e. plants, soil, water) and screened *in vitro* for their biological control activity or plant growth-promoting abilities. One of the main challenges encountered in the development of microbe-based products is that when applied in the field, they lack in consistency. Results are often inadequately reproducible and successful application is ensured only within a limited range of host plants. Generally, the introduced microorganism is not able to efficiently compete with the well-established native microbiota. Dosage and delivery method need to guarantee microbes survival,

possibly increasing their competitive ability and survival chances (Compant et al., 2019). Thus, correct formulation is essential to deliver a high number of microbial cells that remain viable for a long time during storage. Cells need to be protected from desiccation and their shelf-life extended by additives application, such as surfactants, adhesives, stabilizers and UV protectants (Preininger et al., 2018). Microorganisms such as endospore-producing *Bacillus* spp. are highly resistant to adverse conditions, whereas Gram-negative bacteria are less likely to be efficient due to their elevated sensibility to environmental conditions (Berninger et al., 2018; Köhl et al., 2011). During mass-production, microbes are generally grown in rich media that might lead to inactivation of mechanisms required to adapt to stresses and to compete in field conditions (Montesinos, 2003). Adaptation treatments aimed at physiologically improving the fitness of microbes under unfavourable conditions might increase their survival chance upon field delivery. *Lactobacillus plantarum* PM411 and TC92 are BCA strains effective against bacterial diseases that affect rosaceous plants. Their field efficacy has been increased by adapting cells to salt and/or acidic conditions during inoculum preparation (Daranas et al., 2018). Additionally, for the successful colonization of plant organs, microbes need to be able to recognize and metabolize exudates and metabolites produced by the host and to efficiently interact with the native microbiota through antagonistic and competitive strategies (Sessitsch et al., 2019). On the other hand, struggling for increasing colonization ability of microbes should not end up in native microflora invasion, environment equilibrium disruption or plant health alteration.

Before approval for commercialization, microbe-based products need to undergo several ecotoxicological studies due to the high safety risk linked to the application of a high microbial load in the environment. Indeed, it is essential to verify that microorganisms do not harm humans, animals or the environment (Sessitsch et al., 2019). Using GRAS microorganisms, i.e. Generally Considered As Safe, speeds up the registration process because these microbes are commonly regarded as not harming by experts.

Nowadays, development of microbial products, such as biofertilizers and biopesticides, mainly relies on culture-dependent approaches, which provide only a partial overview of microbiomes. Thus, deep microbiota understanding through -omics will be essential for their manipulation and it will improve our ability to efficiently apply microbes in the field. Indeed, successful microbial application in the field is a complex goal only achievable when the interactions between plant genotype,

microbiome, the environment and management practices will be untangled (Figure 3). In particular, insights on the influence of biotic and abiotic factors on microbiome assembly, functionalities and core microbiomes will provide practical knowledge essential for microbes exploitation for improving plant health and yield.

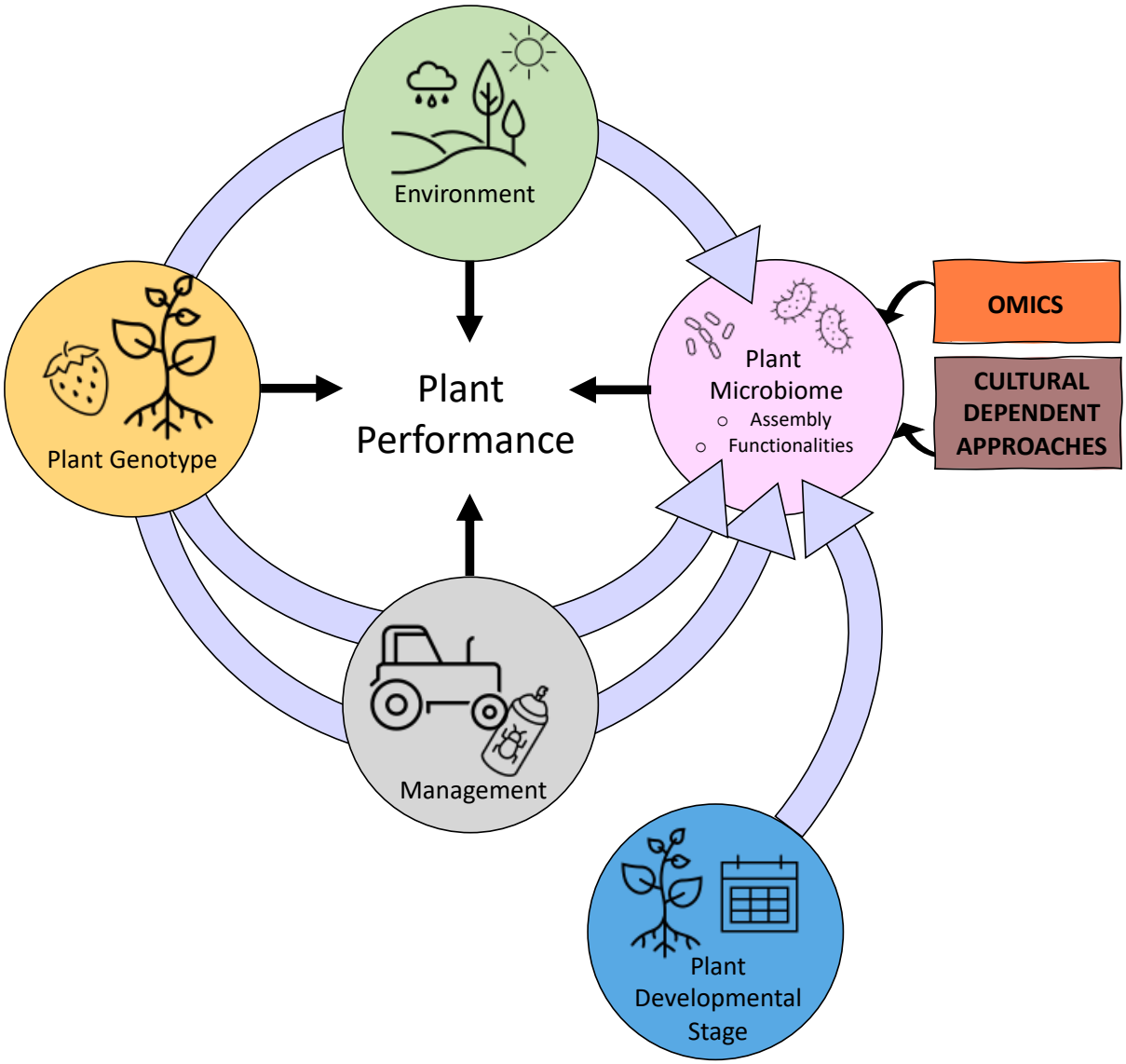

Figure 3 – Factors contributing to plant performance and their interconnections.

1.3.2. Downscaling and modeling of the natural biodiversity: Synthetic Communities

Generating knowledge on networks occurring in plant microbiota, and the influence of environmental changes on the microbial diversity and functionality is pivotal for the successful application of microbes in agriculture. However, microbial

communities are extremely complex and thus their reproduction for research purposes might be challenging. Thus, microbial strains originated in natural microbial communities can be combined in so-called **Synthetic Communities** (SynCom), downscaling and modelling natural biodiversity. SynComs are defined as "artificially created by co-culturing of selected (≥2) species under a (at least initially) well-defined medium" (Grosskopf and Soyer, 2014). The reduced complexity of SynComs, assembled using only culturable microbes, represents the main limitation of this approach. At the same time, SynCom should be designed based on the cultural-independent profiles analyzed by NGS (Vorholt et al., 2017). The SynCom approach allows to accurately investigate its components, unraveling causal links between the taxonomical and functional diversity of microbiome, plant phenotype, performance and response to stresses (Mueller and Sachs, 2015). Additionally, key insights on the best conditions for microbial colonization and survival on plant tissues are possible through SynCom application to plants. Besides being implemented as model systems, SynComs might be exploited for biotechnological purposes. Indeed, application of bioinoculants composed by several strains, with multiple mechanisms of action on plant growth, is one of the proposed methods to overcome inconsistency of microbial inoculation in the field (Tabassum et al., 2017; Etesami and Maheswari, 2018).

1.4 Overview on small berry fruits

The term "berry" refers to small fruits growing in wild bushes, sweet or bitter and characterized by a juicy pulp (Di Vittori et al., 2018). Color, which ranges from red to purple/blue, is the distinctive trait of small berries. However, white fruits such as the white-fleshed raspberry cultivar Anne also exist. Berry consumption and consumer appreciation increased during the last years thanks to their proved benefits provided to human health, which is due to their high content in phytochemicals, dietary fibers, vitamins, mineral and phenolic compounds (Nemzer et al., 2020). One of the main issues of berry cultivation is the high perishability of mature fruits. For example, strawberries and raspberries have high physiological post-harvest activities, which strongly limits fruits shelf-life (usually less than 5 days at 0-4°C) (Han et al., 2004). The most common commercial varieties worldwide belong to several different genera: *Fragaria* (strawberry), *Vaccinium* (blueberry, cranberry, bilberry), *Prunus* (cherries), *Hippophae* (sea buckthorn), *Rubus* (raspberries), *Eiterepe* (acaiberry), *Aristotelia* (Maqui berry), and *Sambucus* (elderberry) (Di Vittori et al., 2018).

1.4.1 Strawberry

The strawberry (*Fragaria* × *ananassa*, Duch.) fruit belongs to the Rosaceae family, subfamily Maloideae, genus *Fragaria*. It is a common fruit, appreciated for its organoleptic properties, such as its characteristic aroma, its bright red color and juicy consistency, and nutraceutical properties. Additionally, due to its short growth cycle and fruiting period, it is recognized by researches as a model plant for studies of fruit trees.

Over time, the selection of traits associated to high yields, large fruits and long shelf-life have negatively influenced strawberry flavor (Yan et al., 2018). Volatile compounds (VOCs) are major determinants of strawberry flavor and aroma, although they account for a tiny percentage of the total fruit weight. More than 350 VOCs have been characterized in strawberry and the VOCs contributing the most to its aroma belong to the esters, terpenes, alcohols, ketones and furans classes (Zabetakis and Holden, 1997). Strawberry health benefits are mainly due to its high content of phenolic compounds, which are characterized by antioxidant and anti-inflammatory properties. Anthocyanidins, in particular pelargonidin and cyanidin derivatives, are the most abundand phenolics in these berries.

Strawberry is characterized by a large consumers market in the world's main economies, having China (6,48 bn US$), Europe (3,49 bn US$) and United States (3,47 bn US$) the biggest market share (FAOSTAT).

Although its first records date back to Pliny's writings (23-79 AD), modern cultivated strawberry originated only 300 years ago from the hybridization between ecotypes of wild octoploid species: *Fragaria chiloensis* subsp. *chiloensis* and *Fragaria virginiana* subsp. *virginiana* (Bertioli, 2019). The first one was discovered in the 1700s in Chile and is characterized by large fruits, whereas *Fragaria virginiana* is native from North America and it is a hardy plant, able to resist to drought and cold. Modern strawberry can be cultivated in both tropical and subtropical areas, however temperate regions are more appropriate for an economically viable cultivation. In Italy, strawberry cultivation can be clearly distinguished in Northern and Southern areas. The production in Northern Italy is characterized by the use of highly productive, although mid-quality cultivars, under protected cultivation systems. On the other hand, in Southern areas the production is mainly on open-field. Basilicata is the first Italian region both in terms of production area and harvested product.

F. × *ananassa* is a perennial accessory aggregate non-climateric fruit which arises from a crown of meristematic tissue or compressed stem tissue. After fertilization, the

receptacle develops into a fleshy fruit, consisting of enlarged flower receptacle, embedded with the numerous true fruits. Strawberries can be diploid, tetraploid, hexaploid, octoploid and even decaploid, which turns traditional breeding into a tedious and time consuming work (Husaini et al., 2016). A large number of different strawberry cultivars exist, differing for their adaptability to various environmental conditions, photoperiod sensitivity, pathogen resistance traits and productivity. Strawberry cultivars can be classified into remontant and non-remontant types, according to the thermophotoperiod they are sensitive to. Indeed, in strawberry, thermophotoperiod influences flower induction. Features discriminating the two types of cultivars are summarized in Table 1.1. However, rigid cultivar classification is not adequate due to the fact that plant responses to day length and environmental conditions are complex. For instance, prolonged warm temperatures totally or partially inhibit flower initiation, disregarding plant photoperiod sensitivity (Husaini et al., 2016 and citations therein).

Table 1.1 - Characteristics of non-remuntant and remuntant strawberry genotypes.

	Non remontant	**Remontant**	
Thermophotoperiodic requirements for flower induction	Short days (<11-16h)	Long days cultivars (>12h)	Day-neutral cultivars (irrespective of photoperiod)
	T 9-21°C, optimal 15-18°C		
	Minimum 7-14 short days		
Harvest	Once in summer/autumn (one year after flower induction)	More times per year	

Strawberry cultivation is affected by several pests and diseases, that might cause considerable economic losses. Major strawberry fungal and bacterial diseases and pests are listed in Table 1.3, together with most important related information.

Table 1.3 - Major biotic stresses affecting strawberry.

Type of biotic stress	Pathogen/ pest	Favourable conditions for spread	Symptoms	Resistant commercial cultivars	Commercial Biological Control Agents
Fungal diseases	*Podosphaera aphanis* (powdery mildew)	Warm and dry climates Greenhouse conditions	Whitish/powdery spots on all plant organs (except roots)	x	*Ampelomyces quisqualis, Trichoderma harzianum, Bacillus subtilis*
	Verticillium dahliae	20-28°C, however it can survive freezing, thawing, heat shocks, dehydration	Wilting of leaves, stunted and flattened plants,	✓	*Trichoderma harzianum, T. viride*
	Botrytis cinerea (botrytis rot fruit)	20°C and prolonged periods of high humidity	Fruit rotting	x	*B. subtilis*
	Colletotrichum acutatum, C. fragariae, C. gloesporioide (anthracnose)	Warm climate	Petiole and crown lesions, irregular and black leaf spots, crown rot, flower blight, fruit rot	x	*Trichoderma* spp.
	Fusarium oxysporum	High temperatures	Wilting, drying, withering foliage, stunting of plants, reduced fruit production	✓	*Trichoderma* spp.
	Phytophtora spp. (red stele, leather	Warm and humid climates	Stunting plants, wilting leaves, deep dark red discoloration of the crown	✓	*Trichoderma* spp.

	rot of the fruit, stem, crown and root rot)				
Bacterial disease	*Xanthomonas fragariae* (angular leaf spot)	2 weeks up to 2 months - leaves are more susceptible	Angular water-soaked spots on leaves	x	x
Pest	Spider mite	Warm and dry climate	Leaf discoloration, white/yellow spots, silky webs	x	*Phytoseiulus persimilis*
	Drosophila suzukii	10-32 °C for oviposition; <30 °C for male fertility; activity and development peak 20- 25 °C	Early fruit mold; holes due to larvae breathing	✓ (reduced emergence from fruits)	Hymenoptera

1.4.2 Raspberry

As strawberry, raspberry (*Rubus idaeus*, L.) belongs to the Rosaceae family, subfamily Rosoideae, genus *Rubus*. It is a soft fruit, highly recognizable for its characteristic flavor and aromatic profile. 300 different VOCs have been reported in raspberry volatilome, being raspberry ketone, α-ionone and β-ionone the most characteristic one (Aprea et al., 2015). Additionally, raspberries are appreciated for their high vitaminic and polyphenolic compounds content. In particular, they are rich in vitamin C, A, B, B1, B2, E, folic acid, flavonoids, phenolic acids, ellagitannins and ellagic acid.

Rubus idaeus is thought to be native of Asia Minor and anciently brought to Europe by Romans. Indeed, first records of domestication can be found in Roman writings that date back to the 4th century. On the other hand, as Europeans settled in North America, raspberry was already consumed and utilized by indigenous populations for medicinal and functional properties. Nowadays, raspberry is worldwide cultivated, being Russian Federation, Mexico, Serbia, United States and Poland the top five

producing countries, whereas Poland is the first country for cultivated area extension (FAOSTAT, 2019 data). In Italy, red raspberry is typically found as spontaneous flora in brushwood and mainly grown in Trentino and Piemonte regions. In fact, the most appropriate cultivation conditions for this berry are areas characterized by mild winters and long, moderate summers, with organic-rich and sub-acids soils, located up to 1200-1500 m a.s.l (Barrel and Diemoz, 2011; Padmanabhan et al., 2016).

Like strawberry, raspberry is an accessory aggregate fruit that develops clustered around a central core, and drupelets are detached from the core when picked.

Raspberry plants can be classified as woody shrubs, characterized both by a perennial root system and by thin canes, having a maximum life span of two year, being either primocanes or floricanes. During a vegetative season, the newly produced shoots (primocanes) are usually mainly vegetative, and become productive (floricanes) during the second year (Kim et al., 2016). Based on this distinction, raspberry cultivars can be primocane- or floricane-fruiting (Table 1.4).

Table 1.4 - Characteristics of floricane- and primocane-fruiting raspberry genotypes.

	Floricane (summer-bearing)	**Primocane (everbearing)**
First year behaviours	No fruit production Only non-fruiting vegetative primocanes	Limited number of flowers and fruits during late summer/fall
Second year behaviour	Large fruit production during summer	Large fruit production during summer
Flowering initiation requirements	Short days and low temperatures followed by bud dormancy	Regardless of day length and temperature Flower development straight to floral initiation
	Floricane (summer-bearing)	Primocane (everbearing)
First year behavior	No fruit production Only non-fruiting vegetative primocanes	Limited number of flowers and fruits during late summer/fall
Second year behavior	Large fruit production during summer	Large fruit production during summer
Flowering initiation requirements	Short days and low temperatures followed by bud dormancy	Regardless of day length and temperature Flower development straight to floral initiation

Several fungi and insects affect raspberry production. The major ones are summarized in Table 1.5 and Table 1.6.

Table 1.5 - Major fungi affecting raspberry.

Pathogen	Favorable conditions for spread	Symptoms	Resistant commercial cultivars	Commercial Biological Control Agents
***Phytophtora fragariae* var. *rubi* (root rot)**	Wet and cool conditions	wilting and leaf redding in young canes; root rots	✓	*Trichoderma* spp.
***Leptosphaeria coniothyrium* (cane blight)**	Wet conditions	Dark lesions in interal tissues; cankers and cane necrosis; failure of bud breaking	✓	x
Botrytis cinerea	20°C and prolonged periods of high humidity	Fruit rotting	x	*B. subtilis*
***Podosphaera macularis* (powdery mildew)**	18-27°C and high relative humidity	Whitish leaves and fruits	✓	*Bacillus* spp.

Several strawberry cultivars resistant to pathogens exist on the market. On the other hand, pest control still relies primarily on the use of chemical pesticides or augemented biological control.

Table 1.6 - Major insects affecting raspberry cultivation.

Pest	Symptoms
***Lasioptera rubi* (raspberry gall midge)**	Wrinkled galls containing larvae
Anthonomus rubi	Flower bud desiccation and death
Byturus tomentosus	Frass-filled tunnels in fruits; larvae feeding on druplets; adults feed on leaves and flower buds creating lesions
Aphis idaei	Bud deformation and interruption of development
Amphorophora idaei	Dangerous because potential virus vector

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2. Thesis overview and general aim

Microbes might play a key role in shaping plant fitness, resistance to biotic and abiotic stresses and ultimately crop productivity and quality. Climate change is acknowledged to produce extreme unfavorable conditions for crop cultivation. At the same time, sustainable approaches that promote plant health and crop quality under adverse circumstances are needed to stop fuelling the climatic crisis. In this view, considering the plant together with its microbiome (i.e. the plant as holobiont) is an innovative approach to address the major bottlenecks impairing the sustainability of horticultural production. Extensive knowledge on interactions occurring between microbes and plants is pivotal for developing new effective agronomical practices and microbe-based products.

The first part of this work (chapter 3) arises from the need to clarify implications of climate change on horticultural crops and to survey biochemical and ecological functions of microbes which might help plants coping with stresses accentuated by the climatic emergency. Thus, we extensively reviewed the current knowledge on microbial biostimulants, highlighting current limitations in their use and defining future perspectives for their successful application.

The subsequent steps of the work involved the characterization of plant-associated microbiomes to exploit their functions for the amelioration of plant conditions and the improvement of fruit quality. For this purpose, strawberry and raspberry were chosen as model species due to their widespread cultivation area around the world and to the high consumption and consumer appreciation. Firstly, our aim was to provide an in-depth and comprehensive view of taxonomic and functional diversity of the strawberry microbiome in relation to plant genotype, health and nutritional status and fruit quality parameters, shedding light on potential practical applications to increase the sustainability of fruit crop production (chapter 4). Additionally, we investigated the *in vivo* growth promoting effect of bacteria isolated from strawberry plants and applied on the same host both individually or in a coordinated inoculation (chapter 5). As we proved that the structure of plant microbiome can be affected by several variables, we next aimed at investigating the role of the cultivation method in determining fruit microbiome. Thus, we studied the effect of organic or integrated management on raspberry fruit microbiome (chapter 6). Besides showing that cultivation method influences fruit quality and aroma, we hypothesized that the resident microflora might contribute to these two fruit features.

To further clarify the contribution of microbes to fruit aroma, we analyzed the volatile organic compounds emitted by naturally colonized, sterile and artificially reinoculated raspberries, highlighting several significant associations between bacterial genera and volatile emissions (chapter 7).

3. Application of microbial biostimulants to mitigate stresses emphasized by climate change in horticultural crops

Abstract: In the current scenario of rapidly evolving climate change, crop plants are more frequently subjected to stresses of both abiotic and biotic origin, including exposure to unpredictable and extreme climatic events, changes in plant physiology, growing season and phytosanitary hazard, and increased losses up to 30% and 50% in global agricultural productions. Plants coevolved with microbial symbionts, which are involved in major functions both at the ecosystem and plant level. The use of microbial biostimulants, by exploiting this symbiotic interaction, represents a sustainable strategy to increase plant performances and productivity, even under stresses due to climate changes. Microbial biostimulants include beneficial fungi, yeasts and eubacteria sharing the ability to improve plant nutrition, growth, productivity and stress tolerance. This work reports the current knowledge on microbial biostimulants and provides a critical review on their possible use to mitigate the biotic and abiotic stresses caused by climate changes. Currently, available products often provide a general amelioration of cultural conditions, but their action mechanisms are largely undetermined and their effects often unreliable. Future research may lead to more specifically targeted products, based on the characterization of plant-microbe and microbial community interactions.

3.1 Introduction

Global climatic records have shown an increase in world temperature since 1970 as well as changes in precipitation regimes leading to several severe consequences for agriculture (Lotze-Campen et al., 2012). In this scenario of climate change, crop plants are more frequently subjected to stresses of both abiotic and biotic origin (Figure 3.1), since, in addition to direct stress on plants, climate change could expand the range of pathogens and pests, and increase the frequency and severity of disease outbreaks (De Wolf and Isard, 2007; Garret et al., 2009). Recent estimations calculated that 50% and 30% losses in global agricultural productions are expected due to abiotic and biotic stress, respectively (Kumar and Verma, 2018). These losses, together with the steady increase of human population, indicate that an increase of 60% of agricultural production is required to meet global needs (Wild, 2003), with a consequent drastic increase in deforestation and reduction of natural habitats (Byerlee et al., 2014). To help ensuring food security with a limited increase in agricultural land, a sustainable

strategy is to increase plant resistance and resilience to counteract climate change-induced stresses. The use of biostimulants could be a valuable option to obtain this objective (Calvo et al., 2014; Yakhin et al., 2017). Compared to xenobiotic agrochemicals, microbial biostimulants do not accumulate in the long term, have a low toxicity, and are less prone to select resistant strains of pests and pathogens, and, therefore, can be considered environment- and human-friendly. Hence, the biostimulant market has steadily increased in the last two decades (Hayat et al., 2010), being Europe the world industry leader, with more than 578 million € of total sales in 2015 (EBIC, 2020).

The compounds grouped within the 'biostimulant' category are heterogenous, including humic substances, protein hydrolysates, aminoacids, seaweed extracts, chitosan and other biopolymers and inorganic molecules. In addition, the subgroup of 'microbial biostimulants' is formed by beneficial microorganisms (i.e. fungi, yeast and eubacteria) sharing the ability to increase plant growth and productivity, promote nutrient uptake and effectiveness, improve abiotic stress tolerance and/or quality of crops (Calvo et al., 2014; Du Jardin, 2015; EBIC, 2020).

Microbial biostimulants are particularly interesting since plants harbour a wide and complex range of microorganisms in their phyllosphere, rhizosphere and endosphere. Indeed, microbial symbiosis is a common and fundamental condition of plants. Plants coevolved with these microbial symbionts, which are involved in major functions such as plant nutrition, plant performance and productivity, and resistance to biotic and abiotic stresses (Vandenkoornhuyse et al., 2015). For example, fossil evidence shows that the association between plants and microorganisms is as ancient, as the emersion from water, thus suggesting that arbuscular mycorrhizal symbiosis has played a key role in the terrestrialization process (Selosse and Le Tacon, 1998).

Microbes exert key functions in ecosystems being involved in nitrogen fixation, carbon and nitrogen cycling, plant nutrient acquisition and soil formation (Wagg et al., 2014). Thus, several microbial symbionts can also act as biofertilizers, providing to the plant complementary limiting nutrients by synergic mechanisms such as nitrogen fixation (e.g. *Azospirillum*, *Azotobacter*, *Rhizobium*), phosphate solubilization (e.g. *Pseudomonas* spp., *Azospirillum*, arbuscular mycorrhiza), cellulolytic activity (e.g. *Trichoderma*, *Penicillium* spp., *Aspergillus*, *Bacillus amyloliquefaciens*), soil acidification (e.g. *Bacillus subtilis*), and siderophore production (e.g. *Pseudomonas* spp. and *Acinetobacter*) (Bhattacharyya and Jha, 2012).

Among fungi, the endosymbiotic genus *Trichoderma* is the most investigated and applied, due to its ability to promote plant growth and defenses, produce antimicrobial substances, parasitize fungal pathogens and prey on nematodes (Szabò et al., 2012; Adnan et al., 2019; Szczalba et al., 2019). In the case of Arbuscule-Forming Mycorrhiza (AFM), the difficulties of *in vitro* cultivation, and the lack of comprehension of host specificity determinants and population dynamics in the agroecosystem may play against their use in commercial products, in spite of the beneficial effects exerted on their host plants, such as the increase in nutritional efficiency and in the protection from biotic and abiotic stresses (Figure 3.1) (Du Jardin, 2015; Szczalba et al., 2019).

Beneficial yeasts are found in the phyllosphere and rhizosphere. Leaf-colonizing yeasts have been reported to control many foliar pathogens through direct antagonism (Preininger et al., 2018) or by elicitation of systemic defenses (Lee et al., 2017). Soil yeasts can promote plant growth by decomposing organic matter, solubilizing phosphate, promoting root growth and soil aggregation, and controlling root pathogens (Sarabia et al., 2018).

Biostimulant bacteria can be distinguished in Plant Growth Promoting Bacteria (PGPB) or Plant Growth Promoting Rhizobacteria (PGPR), the latter specifically colonizing the rhizosphere. The most studied genera are *Burkholderia*, *Bacillus*, *Pseudomonas*, *Serratia* and *Streptomyces* (Bhattacharyya and Jha, 2012; Bonaldi et al., 2015).

Recently, biochemical, physiological and molecular studies of the plant-microbe interactions revealed the existence of microbe-induced plant responses to stress (Farrar et al., 2014), that could activate an Induced Systemic Tolerance against abiotic stresses (Yang et al., 2009) or Induced Systemic Resistance against the biotic ones (Pieterse et al., 2014). It is worth noting that many commercial products based on useful microbes or microbial consortia, such as Subtilex®, Kodiak®, Biota Max®, Trianum-P®, Custom GP®, express multiple functions (competition with pathogens, induction of plant defenses, hormonal stimulation, nutritional exchange) with synergistic and additive effects.

In the current scenario of rapidly evolving climate change, microbial biostimulants represent a sustainable option to support plants coping with biotic and abiotic stresses (Figure 3.1). While laboratory research and technological development of plant-associated microbes have highlighted their beneficial functions, these have often been generically defined, or have not been efficiently reproduced in field conditions. As a

consequence, microbial biostimulants have generally been adopted as accessory treatments, rather than expressing the full potential of microbiome control. The aim of this work is to present the current knowledge on microbial biostimulants, to review their uses in horticulture, and to prospect the development of innovative products to be employed under chronically unfavorable conditions, in particular those exacerbated by the ongoing climate crisis. The information on commercially available microbial species or microbe-based biostimulants has been drawn from on-line archives based in the European Union and in the USA (EU Pesticide DB, 2020; osu.edu, 2020). The understanding of plant-microbe interaction under stressing conditions, together with the identification of limitations and weaknesses in the use of microbes in the current agronomical practices, are pivotal to identify specific scientific questions that need to be addressed, and were therefore investigated.

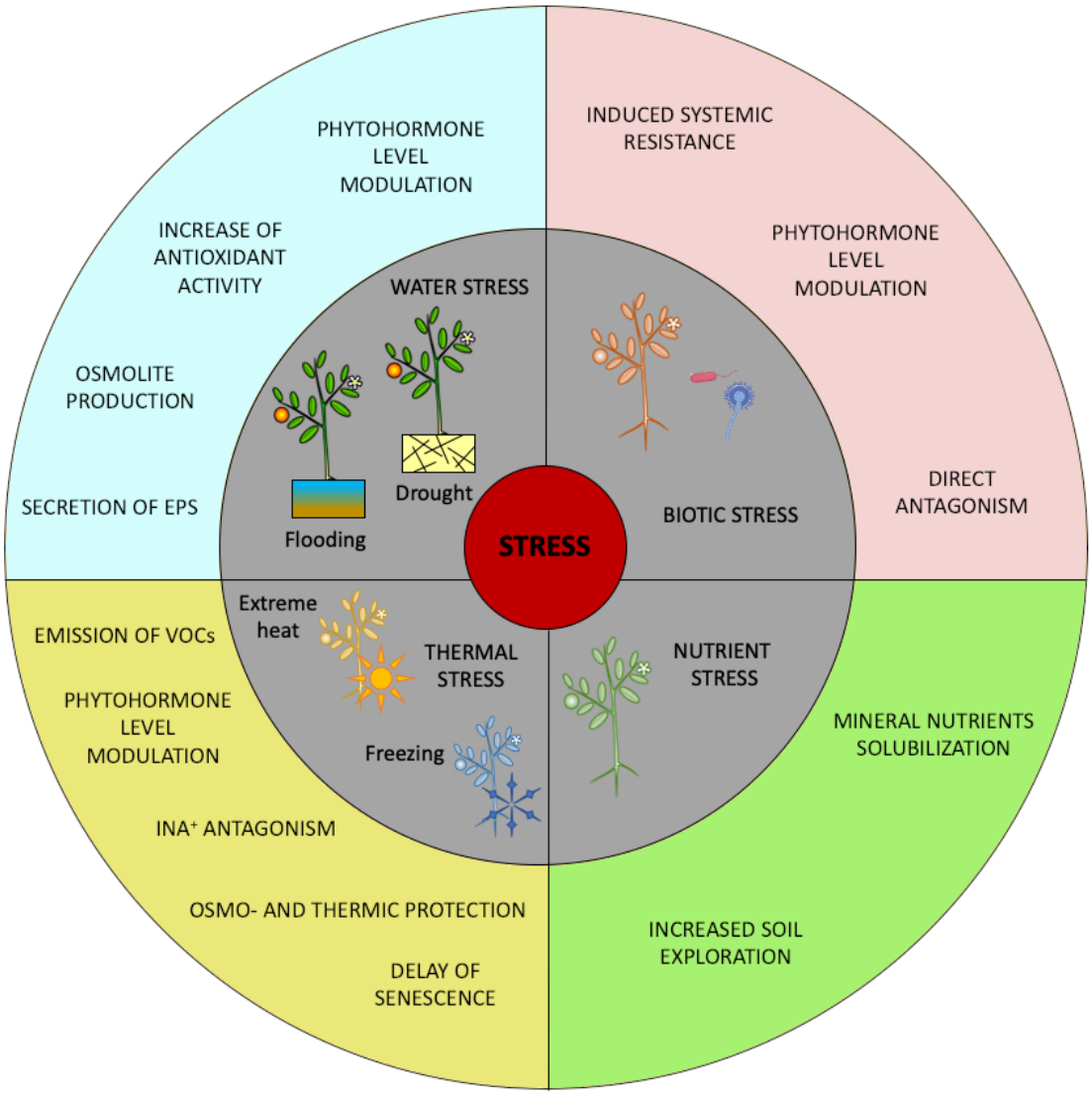

Figure 3.1. Schematic view of the protective mechanisms exerted by microbial biostimulants in relation to the stresses to which plants are subjected.

3.2 Effect of climate change-induced stress on plant and role of microbial biostimulants

3.2.1. Stress induced by extreme thermal events

In comparison to the last 1000 years, the twentieth century has experienced a high climate variability and extreme temperature events, since the frequency of summer heat waves and spring frost has substantially increased (nasa.gov, 2020). Table 3.1 summarizes the results obtained in inducing protection against high or low

temperature by use of microbes, along with the mechanisms underlying the stress protection.

Table 3.1. Microorganisms used against stress induced by extreme thermal events.

Microorganism	Crop Plant	Mode of action	Ref.
PROTECTION FROM HIGH TEMPERATURES			
Paraburkholderia phytofirmans	Potato	ACC deaminase production	(Bensalim et al., 1998)
Mycorrhizae	Tomato	Reduction of lipid peroxidation and H_2O_2, higher ROS scavenging activity in leaves and roots	(Duc et al., 2018)
Bacillus amyloliquefaciens, Azospirillum brasilense	Wheat	ROS reduction, pre-activation of heat shock proteins	(Backer et al., 2018)
Bacillus aryabhatthai SRBO2	Soy	ABA production	(Bashan et al., 2014
Pseudomonas putida AKMP7, *Pseudomonas* sp. AKMP6	Wheat, sorghum	ROS reduction, increase in proline, chlorophyll, sugar, starch, aminoacid and protein content, production of phytohomones	(Tabassum et al., 2017; Nadeem et al., 2014)
PROTECTION FROM COLD AND FROST			
P. fluorescens A506	Apple and pear	Competition with INA^+ bacteria	(Lindow and Brandl, 2003)
Pantoea dispersa 1A, *Serratia marcescens* SRM, *Pseudomonas* spp. PGERs17, NARs9	Wheat	ACC deaminase production	(Mishra et al., 2012; Selvakumar et al., 2008ab; Mishra et al., 2008)
Paraburkholderia phytofirmans	Grapevine	ACC deaminase production	(Barka et al., 2006; Theocharis et al., 2012)

3.2.1.1 High temperature and heat waves

High temperatures impact on plant physiology increasing leaf transpiration and respiration rates, affecting photosynthesis (especially in C3 plants), and modifying allocation of photosynthates (Munns, 2002; Malhotra, 2017). At high temperatures, the affinity of Rubisco to O_2 increases, while it decreases for CO_2 (Jordan and Ogren, 1984). The increase of temperature also reduces CO_2 solubility more than O_2, thus reducing the concentration of CO_2 relative to O_2 in the chloroplast (Ku et al., 1977). Moreover, at high temperature, plants tend to close stomata to reduce water losses by evapotranspiration. When the stomata are closed, CO_2 concentration rapidly drops, becoming the main limiting factor for photosynthesis, while O_2 concentration, in high irradiation conditions, rises due to the high rate of water photolysis by PSII (Lawlor and Fock., 1977; Bhattacharya, 2019). Under these conditions, photosynthetic efficiency is strongly reduced, due both to the limited concentration of CO_2 and to the increased photorespiration activity of Rubisco that can consume up to 25% of the fixed carbon.

When temperature increase occurs during spring, frost risks are lowered and horticultural crops could find a benefit in early flowering (Lawlr and Fock, 1977). This applies mainly to annual crops, such as tomato (Pearson et al., 1997) and lettuce (Bisnbis et al., 2018), in which higher temperatures would allow multiple cycles per year (Maltby, 1995; Wheeler et al., 1993). On the other hand, for some horticultural and fruit species, increasing temperatures could represent a problem for flower differentiation. In cucumber, high temperatures promote masculine flower differentiation instead of productive feminine ones (Wien, 1997). The failure to fulfill cold requirements, needed for flower differentiation in fruit crops as peach, plum (Hazarika, 2013) and apple (Funes et al., 2016) can limit the yield (Luedeling, 2012). In the long term, temperature variations could shift fruit cultivation areas toward northern regions (Luedeling, 2012), in which mild winter temperatures can both induce early flowering, exposing plants to late frost, and extend the vegetative season, delaying dormancy. Furthermore, in this condition, a negative impact on fruit set can be also expected due to an insufficient presence of pollinators (Sunley et al., 2006; Webb et al., 2014). Finally, an increase in temperatures could worsen agriculture in environments, such as tropical areas, characterized by extreme conditions (Gornall et al., 2010), thus causing the total disappearance of particularly sensitive crops.

In plants, heat stress induces complex molecular, biochemical and physiological responses (Kotak et al., 2007), which could lead to the production of heat shock

proteins, enzymes involved in the degradation of Reactive Oxygen Species (ROS), osmoprotecting molecules, amino acids, sulphur compounds and sugars (Shulaev et al., 2008). Heat stress responses are governed by hormonal signaling. Among them, ethylene plays a key role (Kotak et al., 2007; Qu et al., 2013; Byerlee et al., 2014), not only in the physiology, development and senescence of plants but also in response to biotic and abiotic stresses (Dubois et al., 2018). Microbial biostimulants can strengthen plant response to heat stress through different mechanisms (Table 3.1). Production of ROS-degrading enzymes (peroxidases, superoxide dismutase, catalase), reduction of H_2O_2 levels and lipidic peroxidation are mechanisms that promote heat stress tolerance and that have been observed in bacteria of the genera *Pseudomonas* and *Bacillus* and in mycorrhizal fungi in tomato (Bensalim et al., 2018). SoilPro® is a soil improver reinforced with high concentrations of *P. fluorescens* and *P. aeruginosa*, commercialized for its multiple beneficial properties such as phytostimulation, bioremediation and soil fertility enhancement. *Bacillus* spp. have been thoroughly investigated, and several of them have been included in commercial products. Besides registered bio-pesticides (several *B. amyloliquefaciens* strains, *B. pumilus*, *B. firmus*, *B. subtilis*, *B. licheniformis*, *B. thuringensis*, *B. sphaericus*), biostimulant products only containing *Bacillus* species are available, among them Endox® and Activate®. While many commercial products are based on these microbes, either alone or in combination, protection from heat stress is not generally mentioned among their beneficial effects.

The application of microorganisms that reduce ethylene emission has a great potentiality, since the reduction of ethylene in stress conditions could avoid the negative impact of heat stress on plant growth. In particular, the use of bacteria with 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity seems very promising. Indeed, ACC deaminase degrades the ethylene precursor, thus impairing its production in the plant tissues (Figure 3.2). The inoculation of the ACC deaminase-producing bacterium *Paraburkholderia phytofirmans* PsJN in potato allowed to maintain normal plant growth under heat stress conditions (Bensalim et al., 2018). Although its promising beneficial activity, this bacterial species has not found application in commercial products.

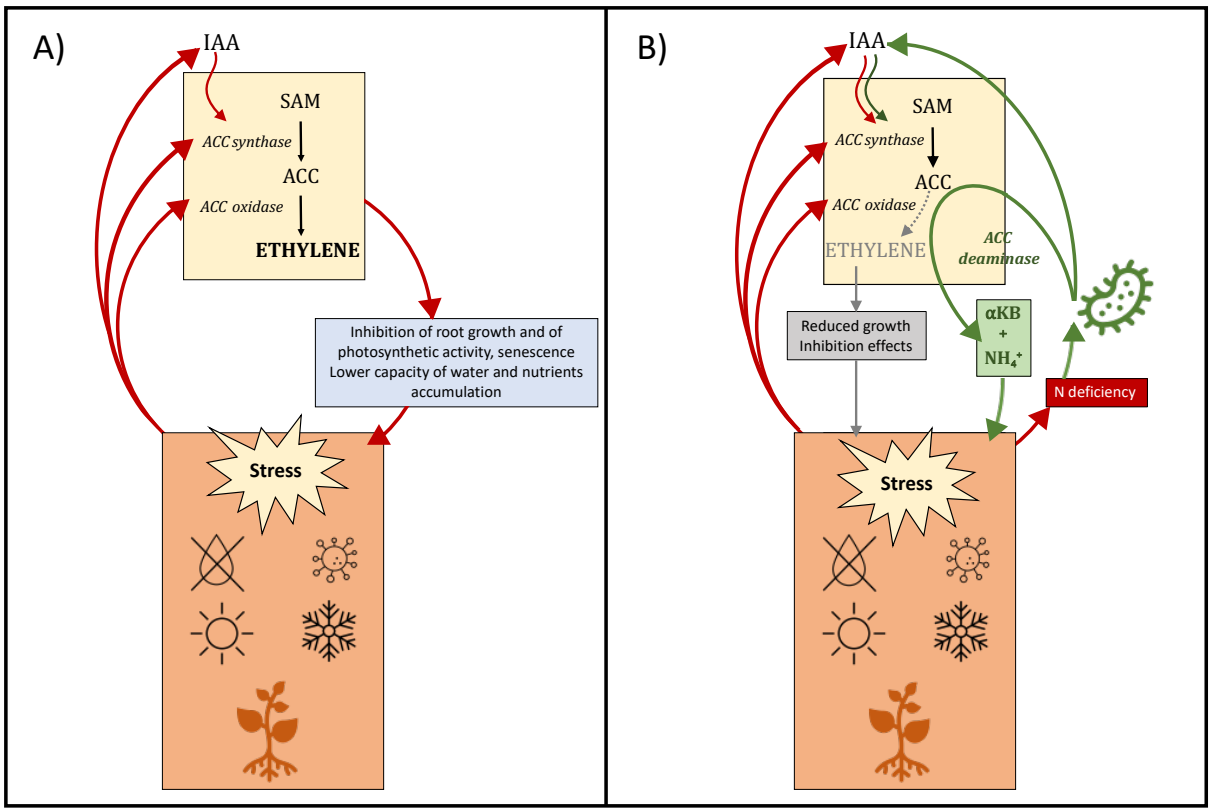

Figure 3.2. Effect of ACC deaminase-producing bacteria on ethylene metabolism of stressed plants. (a) Biotic and abiotic stresses stimulate the production of ethylene and indolacetic acid (IAA) by the plant. Through the enzyme ACC synthase, S-adenosyl methionine (SAM) is converted into 1-aminocyclopropane-1-carboxylate (ACC). ACC synthase converts ACC into ethylene, which causes a general growth inhibition. IAA induces the expression of ACC synthase and contributes to the stimulation of ethylene production. (b) Following the colonization of the plant by ACC deaminase-producing bacteria, ACC is shunted from ethylene to ammonium (NH4+) and α-ketobutyrate (αKB) production. In addition, nutrient stress (including N shortage) stimulates the production of IAA in bacteria. As a result, plant growth promotion is achieved by increased IAA and reduced ethylene contents.

3.2.1.2 Low temperature and frost

Unusually low temperatures are also an important source of stress for cultivated plants. In the last years, frost events causing severe injuries and economical losses to horticulture crops occurred in France, Germany, Italy, Belgium, Switzerland and USA (Vitasse and Rebetez, 2018; De Pascale et al., 2018; Unterberger et al., 2018). Above freezing conditions, low temperatures slow down plant metabolism resulting in diminished photosynthetic levels, foliar growth and early senescence (Huner et al., 1993). Below freezing conditions, bud development can be impaired since low temperatures can destroy re-hydrated and sprouting buds (Mishra et al., 2012).

Finally, frost can cause dehydration of plant tissues, increase in cell cytoplasm osmolite concentrations and consequently plasma membrane disruption (Mishra et al., 2012). Cold damages are promoted by ice core formation, which can occur even at temperatures close to zero. In plants, the formation of ice cores can be caused by the presence of microorganisms with ice nucleating activity (known as INA^+) which can live on leaves, fruits or roots (Lindow, 1983). The cell wall or the extracellular polymeric substance (EPS) of these microorganisms contains proteins, which promote ice crystal formation by acting as ice nucleation centres on their bacterial cell wall (Lee et al., 1995). These microorganisms primarily includes bacteria, but also ice-nucleating fungi have been described (Pouleur et al., 1992) which can colonize the plant both at epiphytic and endophytic level. The first INA^+ bacterial strain identified belonged to *Pseudomonas syringae* species (Arny et al., 1976). Experimental tests showed that the presence of *P. syringae* INA^+ strain increased susceptibility to cold damage in tomato and soy plants (Anderson et al., 1982). In addition to *P. syringae*, other species such as *Erwinia herbicola* (syn: *Pantoea agglomerans*) (Lindow et al., 1978), *Xanthomonas campestris* (Kim et al., 1987) and the Gram-positive bacterium *Lysinibacillus* sp. (Failor et al., 2017) showed some INA^+ activity.

The use of microbial biostimulants able to outcompete INA^+ microorganisms has become an important method to minimize the losses caused by frost damages (Table 3.1). Among them, the use of *Pseudomonas syringae* mutants with inactivated ice nucleating gene reduced frost damages (Xu et al., 1998; Skirvin er al., 2000). Furthermore, several bacterial strains can efficiently compete with INA^+ bacteria and prevent the plant colonization (Wilson and Lindow, 1994; Lindow and Brandl, 2003; Selvakumar et al., 2012). An example of a widely used product to prevent frost damage is Blightban A506® (Nufarm Americas, Inc., Sugar Land, Tex.), which is based on lyophilized *P. fluorescens* A506 (Lindow and Brandl, 2003). This product is also applied for the biological control of fire blight (*Erwinia amylovora*) in apple and pear trees.

Microbial biostimulants can also mitigate the effect of above-freezing temperatures. In fact, microbial symbionts producing growth hormones, such as auxins or gibberellins, can counteract plant growth inhibition due to low temperature. Among auxins, indole-3-acetic acid (IAA) is produced by several microorganisms (Amara et al., 2015). Furthermore, IAA production can be induced by cold temperatures. For example, in *Pantoea dispersa* 1A and *Serratia marcescens* SRM strains from Himalayan northwest, IAA production is induced between 4 and 15 °C. Wheat seeds inoculated

with these strains and grown in cold conditions, showed significantly higher yield and nutrient absorption capacity in comparison to untreated seeds (Selvakumar et al., 2008). Similar results were obtained with *Pseudomonas* sp. PGERs17 and NARs9 strains (Mishra et al., 2008; Mishra et al., 2012). Finally, cold stress increases ethylene production, which further contributes to reduction of plant growth and productivity (Glick, 2014). Despite encouraging results, *Pantoea* and *Serratia* species have not found technological application, possibly because of their relatedness to human pathogens. In grapevine, application of *Paraburkholderia phytofirmans* PsJN, which expresses ACC deaminase activity, increased cold resistance by reducing cell membrane damages (Barka et al., 2006; Theocharus et al., 2012). Bean plants (*Phaseolus vulgaris*) exposed to freezing temperatures and inoculated with psychrophilic, ACC deaminase-producing bacteria such as *Pseudomonas fragi*, *P. chlororaphis*, *P. fluorescens*, *P. proteolytica* and *Brevibacterium frigoritolerans* showed reduced frost damage, lower membrane lipid peroxidation and low ROS production (Tiryaki et al., 2019). Among these bacterial species, *Ps. chlororaphis* and *P. fluorescens* are the only one that found a market outlet, the first being the active ingredient of several registered bio-pesticides (Cedomon®, NematoKill®, BioJect®), whereas the second is found in combination with other PGPR in products such as BFMS®, BioStrain®, HyperGalaxy® and SoilBiotic®.

3.2.2 Stress induced by water scarcity or waterlogging

A list of microorganisms investigated for their ability to protect from stresses linked to water availability is shown in Table 3.2.

Table 3.2. Microorganisms active against water stresses.

Microorganism	Crop Plant	Mode of action	Reference
Phoma glomerata, *Penicillium sp.*, *Exophiala sp.*, *Paecilomyces formosus*, *Glomus intraradices*	Cucumber, bean	Greater soil exploration by roots or by fungal hyphae and better water root conductivity	(Nwodo et al., 2012; Cavagnaro et al., 2015)
P. chlororaphis TSAU13, *Funneliformis mosseae*	Tomato, cucumber, orange	IAA production	(Kaushal and Wani, 2016; Sharifi et al., 2018)

Burkholderia, *Promicromonospora*, *Acinetobacter*, *Pseudomonas* spp.	Cucumber	Gibberellin production	(Ouyang et al., 2017)
Bacillus subtilis	Lettuce	Cytokinin production	(Yuan et al., 2011)
Pseudomonas putida H-2-3	Soybean	ABA production	(Liu et al., 2018)
Achromobacter piechaudii ARV8, *B. licheniformis K11*, *Pseudomonas* spp., *Pseudomonas fluorescens* TDK1	Tomato, pepper, pea, peanut	ACC deaminase	(Khang et al., 2015; Liu et al., 2013; Adesemoye et al., 2009; Finkel et al., 2017)
Glomus intraradices	Carrot, soybean, lettuce	Increased aquaporin activity	(Gong et al., 2013; Jewfa et al., 2010)
Pseudomonas sp.	Basil	Increased antioxidant protection	(Khang et al., 2015)
Pseudomonas putida, *P. aeruginosa* PF23, *Glomus mosseae*, *G. versiforme*, *G. diaphanum*	Sunflower, orange	EPS production	(Barzana et al., 2012; Rouphael et al., 2012; Ali et al., 2014)
Bacillus polymyxa Glomus intraradices Glomus versiforme	Tomato, soybean, tangerine	Osmolite production	(Barzana et al., 2012; Kang et al., 2014; Heidari and Golpayegani, 2012; Ipek et al., 2014

3.2.2.1 Drought and salinity stress

Water stress, caused either by drought or high salinity, occurs when losses by leaf transpiration exceed root absorption, causing a reduction of water content in plant tissues and consequently loss of turgor (El-Daim et al., 2014). Since the 1980s, drought events have become more intense and frequent, particularly in the northern hemisphere and in semi-arid areas (Park et al., 2017; Ali et al., 2009). Furthermore, when rainfalls are of low intensity and sporadic, salt accumulation in the soil can exacerbate the damages due to drought stress (Ali et al., 2011), since the increase of

solutes in saline soils reduces the osmotic potential of soil liquid phase, thus impeding water absorption by roots (Sansavini et al., 2012). Currently, soil salinization has affected about 30% of total arable land and is a very serious problem in the whole Mediterranean area (Wetherald and Manabe, 2002). Additionally, in overexploited areas, drought stress and the consequent soil salinization are the main drivers of desertification. Indeed, drought conditions alter soil composition and edaphic biodiversity, contributing to vegetation degradation and spare soil coverage, which enhance soil erosion (Othman et al., 2006; Trenberth et al., 2014). Drylands currently cover 46% of the global land area, directly affecting 250 million people in developing countries (Rasqol et al., 2013; Sequi, 2006).

Drought stress affects plants both morphologically and physiologically and can cause detrimental ROS accumulation (Vicente-Serrano et al., 2020), ethylene emission (Daliakopoulos et al., 2016) and reduce availability, assimilation and transport of mineral nutrients (Shukla et al., 2019). Microbial biostimulants containing soil microorganisms can improve plant tolerance to drought or salinity through different direct or plant-mediated mechanisms.

Direct mechanisms consist in the alteration of soil composition and structure by the microorganism, improving water uptake. Bacterial exopolysaccharides (EPS) can improve soil structure, through the formation of micro and macro-aggregates (Huang et al., 2020), playing a key role in promoting plant growth under water stress (Bensalim et al., 1998). Additionally, EPS, through the formation of a hydrophilic biofilm, create a microenvironment which increases water retention by protecting microorganisms from drying (Smirnoff, 1993), and binding Na^+ ions, limit their absorption by the plant and favor resistance to saline stress (Ali et al., 2014). Examples of such protective effects have been described in several strains of *Pseudomonas* spp. (Rouphael et al,., 2012; Ali et al., 2014).

Mycorrhizal fungi can strengthen the root capacity of exploring soils (Grover et al., 2011; Nwodo et al., 2012), allowing the increase of root biomass, improving soil structure, increasing water retention and decreasing mineral nutrient leaching (Tewari and Arora, 2014). Arbuscular mycorrhizae of the genus *Glomus* produce a glycoprotein (glomaline) with an aggregating effect on soil structure, inducing better growth and water stress resistance in orange plants (Sandhya et al., 2009; Barzana et al., 2012). Similarly, cucumber plants, colonized by different ascomycetes (*Phoma glomerata* LWL2, *Penicillium* sp. LWL3, *Exophiala* sp. LHL08, *Paecilomyces formosus* LHL10) (Nwodo et al., 2012), showed increased chlorophyll content and leaf growth.

Besides the enhancement of root growth, mycorrhizal fungi are also able to induce a better water uptake via aquaporins, a large family of integral membrane transporters that allow water passage through cell membrane phospholipid double layer. Plant aquaporins mediate roots water absorption and turgor pressure recovery (Khan et al., 2015). Studies on *Phaseolus vulgaris* mycorrhized by *Glomus intraradices*, and subjected to water or saline stress, showed that the fungus regulated aquaporin activity, leading to a better root water conductivity (Cavagnaro et al., 2012). *Glomus intraradices*, grown in symbiosis with carrot plants, showed a high expression of two fungal aquaporins (GintAQPF1 and GintAQPF2) which improved water transfer between the two symbionts, thus conferring to the plant a greater resistance to water scarcity (Gong et al., 2013). Among the above mentioned microbes, *Glomus intraradices*, together with several other mycorrhizae, and *Paecilomyces* spp., are the only ones that have been brought to the market. In commercial products, *Glomus intraradices* is mainly found in combination with other beneficial fungi and bacteria (such as in MycoApply® All Purpose and OroSoil®), but it has been marketed in single formulation (Agtiv® and Groundwork®) as well.

Concerning the plant-mediated effects, microbial biostimulants can influence the associated plants at several levels, including the modulation of phytohormone levels, the antioxidant defenses, the production of protective osmolites such glycine betaine and the emission of volatile organic compounds (VOCs), which in turn influence and coordinate the ecological contour (neighboring plants, rhizosphere microbiome, associated insects) of the plant (Bray et al., 2000; Aroca et al., 2007; Wu et al., 2008; Dimpka et al., 2009; Li et al., 2013; EU Pesticide DB, 2020).

Water limitation also impairs nutrient uptake by plant roots, including nitrogen compounds. This multistress condition promotes ethylene production, that, triggering stress responses, inhibits plant growth and carbon availability for associated microbes (Figure 2a). Thus, mechanisms enacted by plant symbiotic microbes can be based on ACC subtraction, reducing ethylene production and relieving ethylene-mediated inhibition (Glick, 2014), and/or IAA production to stimulate plant growth and root branching (and consequent exchange of resources with rhizospheric microflora). IAA production was found responsible of plant growth promoting effects by several microbes under nitrogen shortage conditions (Paul and Lade, 2014; Kavamura et al., 2013). Notably, some of the identified genera (*Sinorhizobium/Ensifer*, *Serratia*, *Arthrobacter*, *Alcaligenes*, *Bacillus*) are likely N-fixing bacteria. In addition, IAA- and ACC deaminase-based metabolisms are mutually integrated, since IAA may stimulate

ACC synthase, and ACC deaminase recirculates ammonium, making it available for other plant or bacterial metabolic needs (Figure 3.2b).

The bacterium *Pseudomonas chlororaphis* TSAU13, an IAA producer strain, when inoculated on salt stressed tomato and cucumber plants can increase plant water conductance and resistance to salinity and drought (Kaushal and Wani, 2016). Similar results were obtained in orange trees treated with the mycorrhizal fungus *Funneliformis mosseae*, which showed to increase root IAA levels, root hair growth and plant performance under drought stress (Sharifi and Ryu, 2018). *Funneliformis mosseae* is one of the active ingredients of Biologic Systems Wettable Mycorrhizae Blend®, a commercial product improving the plant's ability to absorb water and nutrients. Similarly, gibberellin- and cytokinin-producing bacteria showed their efficacy in controlling water stress damages, stimulating shoot growth and stomatal opening in condition of low water availability (Yuan et al., 2011; Egamberdieva, 2012). *Burkholderia*, *Promicromonospora*, *Acinetobacter* and *Pseudomonas* spp. strains have been described as Plant Growth Promoting Rhizobacteria (PGPR) that can produce active gibberellins. These bacteria, when inoculated on horticultural plants, such as cucumber, can increase plant growth in drought and salinity conditions (Ouyang et al., 2017). Despite their promising beneficial activity, no commercial product has been released based on these species.

The production of abscisic acid (ABA) is physiologically stimulated in plants following water stress to induce stomatal closure. In soybean, the inoculation with *Pseudomonas putida* H-2-3 reduced the production of ABA substantially mitigating the effects of drought stress on plant productivity (Liu et al., 2018a). *Ps. putida* is marketed in combination with *B. subtilis* in the commercial product N-Texx® for its ameliorative effect on soil fertility, although not specifically for drought stress relief. In lettuce, the inoculation with *Glomus intraradices* decreased ABA concentration and reduced salt stress susceptibility (Arkhipova et al., 2007). Both ABA and water scarcity increase ethylene production in plants. High ethylene concentration can reduce plant growth, especially at the root level, further increasing plant sensitivity to water scarcity. Therefore, the application of microorganisms showing ACC deaminase activity may alleviate these negative effects. For example, *Achromobacter piechaudii* ARV8 in tomato and pepper (Liu et al., 2018b), or *Pseudomonas fluorescens* TDK1 in peanut seedlings (Kang et al., 2015), have been successfully used to enhance fresh and dry weight of yielded crops under drought or salinity stresses. *Achromobacter* spp. in combination

with *Pseudomonas* spp. and other beneficial microbes are responsible for plant growth promotion and soil improvement of SOS® and SSB® products.

ROS production, and consequent oxidative damage to proteins, lipids and nucleic acids, is frequently observed under water stress. Several microorganisms can reduce negative effects resulting from ROS increase via the production of antioxidant molecules, or the enhancement of antioxidant enzyme activity, such as catalase or peroxidases (Kang et al., 2014). Basil plants grown in conditions of water deficit showed an increase in catalase activity when inoculated either with *Pseudomonas* sp. alone or by microbial consortia composed by *Pseudomonas* sp., *Bacillus lentus* and *Azospirillum brasilense*. In the latter condition, also glutathione peroxidase and ascorbate peroxidase activity increased (Jahromi et al., 2008). These microbe combination is the base for several successfully commercialized products, such as BFMS®, Environoc®, SoilBiotics® and HyperGalaxy®.

Osmocompatible solute accumulation is a reaction to stress, which involves the accumulation of organic or inorganic solutes respectively in the cytosol or in the vacuole, thus lowering the osmotic potential of the cell and maintaining its turgor potential under water stress (Mayak et l., 2004). Several bacteria can produce osmolites (Saravanakumar and Samiyappan, 2007), which can act in combination with plant osmolites showing also a detoxifying action on ROS (such as proline) and/or stabilizing proteins, enzymes and cell wall components (Vurukonda et al., 2016). When inoculated in tomato plants, proline production by the phosphate-solubilizing bacterium *Bacillus polymyxa* was observed, thus reducing the negative effects induced by water stress (heidari et al., 2012; Kang et al., 2014). In rice, betaine produced by rhizosphere osmotolerant bacteria acted in concert with that produced by the host plant, increasing water stress tolerance (Wu et al., 2008). Despite encouraging results, *B. polymixa* has not landed on the market yet.

Some microbes can interact with plants by means of VOCs, that stimulate adaptation responses to stress conditions. Such responses include root expansion, water saving and activation of mineral uptake systems (Morgan, 1984; Paul et al., 2008; Sanders et al., 2012; Shintu and Jayaram, 2015). The mechanisms underlying plant-microbe interactions under stress are largely obscure, although the implication of hormone signaling cascades has been observed (Zhang et al., 2007; Cho et al., 2008; Bailly et al., 2014). Since the discovery of the effects of the microbial metabolite 2,3-butanediol on plant fitness (Zhang et al., 2007), including regulation of stomata closure and production of osmoprotectants (Sanders et al., 2012; Shintu et al., 2015), other

beneficial VOCs have been identified. For instance, 2-undecanone, 1-heptanol and 3-methyl-butanol from *Parabulkholderia phytofirmans* (Zhang et al., 2010) contribute to salt tolerance, while 1-butanol and butyrolactone promote root development and carbon exchange in the rhizosphere (Ryu et al., 2003). Future exploitation of VOC-based plant promotion will probably depend on the clarification of signaling pathways induced by stress conditions.

Despite the relatively high number of microbial species able to protect plants from water stress, only a few products are specifically commercialized to this purpose. Most of these products (Ryze®, Micosat F®, Suma Grolux®) are based on complex microbial communities, including *Glomus*, *Trichoderma*, *Bacillus* and/or *Pseudomonas* spp., that exert water stress protection, along with the general amelioration of plant growth, nutrition and yield, as a result of interaction of multiple mechanisms, including hormone production or stimulation, enrichment of soil organic matter and nutrients, and production of EPS.

3.2.2.2 Heavy rainfall, flooding and water stagnation

Among the consequences of the ongoing climate change, seasonal variability and interannual rainfall trends are one of the main problems (Bhattacharyya and Garladinne, 2015; Ledger et al., 2016; Tahir et al., 2017). Currently, flood problems involve 13% of earth's surface (Gutierrez-Luna et al., 2010) and, in the future, extreme rainfall frequency and intensity will globally increase (nasa.gov, 2020; Meehl et al., 2007). Heavy rainfall and flood cause water stagnation and roots hypoxia or anoxia. Under flooding conditions, roots produce high levels of the enzyme ACC synthase, which is involved in the biosynthesis of ethylene. In absence of oxygen, the ethylene precursor ACC cannot be converted into ethylene since the enzyme ACC oxidase, which catalyzes the final step in ethylene biosynthesis (Figure 2), is oxygen-dependent. Thus, ACC is translocated through the xylem to the aerial part of the plant (Meehl et al., 2007; Foster and Rahmstorf, 2011), where it can be converted to ethylene, causing wilting, leaf chlorosis or necrosis, flower and fruit drop and reduced yield (Glick, 2014).

The use of PGPB can contribute to minimize problems associated with water stagnation due to their ACC deaminase activity, which reduces endogenous ethylene levels (Bradford et al., 1980; Stevenson et al., 2011; Cramer et al., 2011; Glick, 2014). The pioneering research about microorganism utilization to reduce anoxia stress was

conducted on tomato seeds inoculated with ACC deaminase-producing strains of *Enterobacter* and *Pseudomonas* spp., and submerged for nine consecutive days. The presence of the microorganisms conferred to the germinated seedlings a higher tolerance to this extreme stress condition (Stevenson et al., 2011). Although the application of the commercial product SumaGrow®, containing, among others, *Enterobacter* spp. and *Pseudomonas* spp., provides significant yield increase and better stress tolerance, specific protection from waterlogging stress is not claimed. Using *Pseudomonas* sp. on cucumber seeds (Cramer et al., 2011), and the endophytic *Streptomyces* sp. GMKU 336 strain in association with Indian bean plants (*Vigna radiata*) (Else and Jackson, 1998), plant elongation, biomass, chlorophyll content, leaf area and adventitious roots formation were promoted, together with a reduction of ethylene levels. *Streptomyces* K61 and *S. lydicus* WYEC 108 are the active substances of Mycostop® and Actinovate®, respectively. Although *Streptomyces* spp. are well-known soil beneficial bacteria, commonly used as base of several commercial products, they are mainly applied for targeting biotic stresses such as seed and soil borne fungi. Unfortunately, the use of the above mentioned bacterial species as stimulators of plant tolerance under anoxic conditions is still poorly investigated.

3.3 Role of microbial biostimulants in response to biotic stresses

Plant diseases cause losses estimated for 20-40% of global crop (Grichko and Glick 2001). Climate change has a very complex effect on plant-pathogen interactions since environmental conditions affects the whole disease triangle: they modify plant susceptibility, the biological cycles of parasites and pathogens (Li et al., 2013; Ali et al., 2018), and host-pathogen physiology and interactions (Wild, 2003; Ratchaniwan et al., 2018). Although protection against biotic stresses falls outside the generally accepted definition of biostimulation (Du Jardin, 2015), disease resistance induction is sometimes elicited by *stricto sensu* biostimulants, and will be discussed here as a desirable additional trait for future products to be developed. These considerations exclude however microorganisms directly acting on pests and pathogens (such as entomopathogenic and antibiotic-producing microbes), which are categorized as bio-pesticides rather than biostimulants.

Grapevine and potato downy mildew, gray mold and bacterial canker of kiwifruits represent crop diseases whose incidence has been increased due to climate change (Lim and Kim, 2013). The presence of even more frequent frost events during the

vegetative season lead to an increase of disease incidence of the bacterial canker of kiwifruit (*Pseudomonas syringae* pv. *actinidiae*), as plant tissues were more subjected to frost damages, which can be exploited by the pathogen as entry points (Zahir et al., 2008).

Increase in temperature, CO_2 levels, acid rains and tropospheric O_3 concentration can cause multiple chronical stresses to plants, lowering their ability to respond to pathogen attack (Ali and Kim, 2018). Furthermore, even though the rise of CO_2 can cause a reduction in stomata density, which are important entry points for several epiphytic pathogens, increase in acid rains and O_3 may reduce the protective efficacy of the cuticles and facilitate pathogen penetration. Climate change is also likely to increase the frequency of pesticide application (Porcel and Ruiz-Lonzano, 2004). In fact, higher winter temperature will anticipate bud break and, thus, the length of the growing seasons and the number of pesticide applications. In addition, changes in temperature and precipitation may alter the dynamics of pesticide persistence on the crop foliage. An increase in the frequency of intense rainfall events could result in increased fungicide wash-off and, consequently, reduced control. The physiological and morphological changes in crop plants resulting from growth under elevated CO_2 could also affect uptake, translocation, and metabolism of systemic pesticide. The CO_2-induced increase in crop growth rate may results in bigger and denser canopy, that could negatively affect spray penetration and coverage. The increased use of pesticides (including the variety of compounds applied, their doses and application frequencies) (Porcel and Ruiz-Lonzano, 2004 ; Bltes et al., 2017) and the possible reduction of their efficacy (Ali and Kim, 2018) may result in a rise of pathogen resistance (Logan et al., 2003). In this scenario, the use of microbial biological control agents will become a key option to prevent the environmental, social and economic impact of the increase in pesticide use.

In addition to the classical biological control, based on a direct effect of beneficial microorganisms against the pathogens, an innovative and sustainable approach applied for plant diseases control is the use of microorganisms which enhance plant disease resistance. Systemic acquired resistance (SAR) and induced systemic resistance (ISR) are two different mechanisms for plant resistance (Coakley et al., 1999). In the case of SAR, after a pathogen attack, salicylic acid is accumulated in infected plant tissues. This hormone activates immune responses, such as the expression of pathogenesis-related (PR) genes, encoding for antimicrobial products (Velasquez et al., 2018). On the other hand, beneficial microorganisms more frequently

act via ISR induction, which consists in a plant immune system stimulation (priming) against a broad spectrum of pathogens, leading to a more rapid and intense reaction after pathogen recognition (Tripathi et al., 2016), but generally not affecting plant growth and yield. Bacterial species of the genera *Pseudomonas*, *Serratia* and *Bacillus*, and fungi such as *Trichoderma* spp. and *Piriformospora indica* are among the most studied organisms for the induction of resistance (Ferrante et al., 2014; Delcour et al, 2015). The commercial formulate Trianum-P®, for instance, employs a *Trichoderma harzianum* isolate to induce ISR and protection against soil pathogens. In *Pseudomonas* and *Bacillus* spp.-based products, resistance induction effect may as well exist, although not documented. Several microbial molecules can activate ISR, such as flagellar proteins, Gram-negative bacteria lipopolysaccharides, siderophores (Delocour et al., 2015), some antibiotics, N-alkylated benzylamines, VOCs (Bloomfield et al., 2016) and N-acyl homoserine lactones, a class of signal molecules involved in bacterial quorum sensing (Noyes et al., 2009). The antifungal compound 2,4-diacetylfloroglucinol (Garrett et al., 2011) and cyclic lipopeptides are also recognized as microbial elicitors (Durrant and Dong, 2004; Van Oosten et al., 2008; Tripathi et al., 2016). These elicitors stimulate plant immune response via the activation of the regulatory genes involved in ethylene and jasmonic acid biosynthesis (Kloepper et al., 2004; De Vleesschauwer, 2009). *Enterobacter asburiae* R57 strain, recently isolated from raspberry, showed the ability to control *Botritys cinerea in vitro* via the production of siderophores and acetoin, a volatile precursor of 2,3-butanediol (Bakker et al., 2007). Other microorganisms can be beneficial for plant resistance, increasing plant constitutive barriers, for example by promoting callose deposition in cell wall (Ryu et al., 2004) following ABA stimulation (Schuhegger et al., 2006). Even preventive treatments with ACC deaminase-producing bacteria could help protecting plants from bacteria, fungi and nematodes (Glick, 2014), impeding the development of symptoms and decreasing disease severity. This kind of response was detected following the application of the ACC deaminase producing *Pseudomonas putida* UW4, which limited damages caused by *Pythium ultimum* in cucumber (Iavicoli et al., 2013). A list of resistance-inducing microbes, along with the mechanisms eliciting plant protection and the target pathogens, is presented in Table 3.3.

Several microbes are registered as active principles of pesticides, acting against plant pathogens through direct antagonism mechanisms. In contrast, broad sense biostimulation (i.e., resistance induction) properties against biotic stresses are less considered for commercial formulates. *Bacillus amyloliquefaciens* (formerly *subtilis*)

QST 713 is a EU registered pesticide active substance that, besides directly competing for nutrients on leaf surfaces with fungal pathogens, induces systemic resistance responses in plants, as indicated by peroxidase production (EU Pesticide DB, 2020). Among complex commercial products, Ryze® and Nutribac®, containing both mycorrhizae and PGPR, exhibit not only general beneficial properties for the soil, but also enhance plant resistance to biotic stresses. Direct antagonism seems to have been a more appealing strategy for microbe-based products commercialization. In alternative, resistance-inducing products (such as chitosan, exopolysaccarides and lipopolysaccarides) have been isolated and extracted from their originating organism. While the use of live microbes as resistance inducers may be a less straightforward, thus possibly less reliable protection strategy, it may as well combine the advantages of a long-lasting and stable plant-microbe interaction and of wide-range protection, possibly improving the baseline health status of crops and reducing the synergism within pathogen consortia.

Table 3.3. Resistance-inducing microorganisms with their respective plant protection mechanisms.

Microorganism	Plant / Pathogen(s)	Microbial elicitor	Signaling pathway	Reference
B. pumilus SE34	Tomato / *Phytophtora infestans*		depending on ET/JA; SA independent	(Ruzzi and Aroca, 2015)
B. subtilis S499	Bean / *Botritis cinerea*	Cyclic lipopeptides (surfactin and fengicine)		(Durrant and Dong, 2004)
Burkholderia gladioli	Cucumber / *Colletotrichum orbiculare*	Exopolysaccharides		(Bashan et al., 2016)
P. fluorescens SS101	Tomato / *Phytophtora infestans*	Cyclic lipopeptides	SA-independent	(Strigul and Kravchencko, 2006)
P. fluorescens WCS374	Radicchio / *Fusarium oxysporum* f. sp. *raphani*	Pigment (pseudobactin), lipopolysaccharides		(Vejan et al., 2016)
Rhizobium etli G12	Potato / *Globodera pallida*	lipopolysaccharides		(Mazzola et al., 2017; Etesami et al., 2018)
P. fluorescens WCS417	Eucalyptus / *Ralstonia solanacearum*	Lipopolysaccharides and siderophores		(Purahong et al., 2018)
Serratia liquefaciens MG1	Tomato / *Alternaria alternata*	N-acyl homoserine lactones	Probably depending on SA and ET	(Noyes et al., 2009)
B. amyloliquefaciens IN937a	Arabidopsis / *Erwinia carotovora*	VOCs (2R,3R-butanediol)	depending on ET/JA; NPR1/SA-independent	(Bloomfield et al., 2006)

Paenibacillus polymixa BMP-11	Arabidopsis / *Phytophthora capsici, Alternaria brassicicola, Botrytis cinerea, Colletotrichum capsici, Fusarium oxysoprum*	VOCs (1-octen-3-ol)		(berendsen et al., 2012; Lemanceau et al., 2017)
B. amyloliquefaciens IN937a	Pepper, cucumber / *Xanthomonas axonopodis pv. vesicatoria*	VOCs (3-pentanol)		(Bouffaud et al., 2018)
Trichoderma atroviride TRS25	Cucumber / *Pseudoperonospora cubensis*		Probably depending on SA and JA/ET	(Almario et al., 2013)
Bacillus sp. CHEP5 and *Pseudomonas* sp. *BREN6*	Peanut / *Sclerotium rolfsii*		Increased ACC conversion capacity	(Ahkami et al., 2017)
Paenibacillus P16	Cabbage / *Xanthomonas campestris*			(Ricci et al., 2019)

3.4 Limitations and future perspectives in the application of microbial biostimulants

The development of a new microbial biostimulant displays some specific difficulties. Firstly, commercial registration process is usually complex and a harmonized international legislation is still lacking (Ongena and Jacques, 2008). Secondly, product efficacy is strictly dependent on the horticultural crop on which it is applied and on its phenological state. The development of phytostimulant products needs to evaluate the relationship microorganisms establish with the host plant. A positive and long-lasting plant colonization is an essential prerequisite for biostimulant effectiveness. Finally, the best formulation to guarantee products efficacy and conservation has to be defined, to minimize the influences from environmental and cultural conditions

(Lugtember and Kamilova, 2009). Biostimulants with Gram-positive bacteria allow powder formulations with durable stability and drying tolerance due to spore production by bacteria (Pieterse et al., 2000).

Even if there are many examples of the efficacy of microorganism application in promoting plant growth under unfavorable conditions, very few biostimulant products specifically addressing stresses emphasized by climate change are available. In general, the high costs related to the production of the commercial biostimulant and the variability in the efficacy observed in field conditions (Verhagen et al., 2004) are major hindrances to the development of biostimulant products, resulting in a relatively low number of commercialized products and a limited diffusion in horticultural practice.

Several parameters have in fact to be considered before the application of a microbial biostimulant:

1. Soil and crop characteristics: no microorganisms can be universally applied in any ecosystem (Perpetuini et al., 2019) or on any vegetable host (Van der Ent et al., 2008), thus choosing a particular strain for a biostimulant product needs consideration of soil properties and specific crop requirements, in order to select microbial strains with the best adaptation to each particular conditions (Kumar et al., 2012).
2. Competition for nutrients and ecological niche occupation between selected microbial strains and indigenous microflora, which can reduce biostimulant efficacy (Yan et al., 2002; Cheng et al., 2007; Tran et al., 2007; Park et al., 2008).
3. Mode of application of the microbial biostimulant, that should reduce microorganisms dispersion or death due to abiotic factors (UV, temperature) (Leeman et al., 1995).
4. Specific characteristics of the microbial strain: microbial strains with multiple PGP traits are preferable over microbial strains characterized by only one PGP, because they can reduce different stresses simultaneously (Pieterse et al., 2000; Reitz et al., 2002).
5. Integration of microbial and plant genetic resources: future crop breeding programs should consider the plant's capacity to establish stable symbiotic relationships with useful microorganisms as a highly desirable trait, closely linked to stress resistance, productivity, and resilience. Concurrently, the deeper characterization of microbial

functions and mechanisms of interaction may enable the selection of specific biostimulants for a particular crop/cultivar in a given cultural condition.

In-depth characterization of the plant microbial biocoenosis by next-generation sequencing (NGS), the real-time monitoring of the dynamics of microbial functions and the development and optimization of microbial synthetic communities are pivotal strategies to fully achieve the potential of microbial biostimulants. Several studies suggest that microbes isolated from the microbiome of the host plant have a superior efficacy in comparison to non-indigenous microbial inoculants (Reitz et al., 2000). Thus, the characterization of the native microbiome through the application of high-throughput NGS technologies is a key step for the successful selection of microbial biostimulants. Together with meta-analysis of population association, NGS technologies could lead to the identification of microbes able to persist on the plant under stressful environments (Ran et al., 2005). In fact, their persistence would be a likely result of positive selection, due to their beneficial effect on plant growth and protection (Zhao et al., 2011).

Additionally, investigation of the microbiome based on functional markers, besides taxonomic ones, is fundamental to understand and exploit plant-microbe ecological interactions (Kishimoto et al., 2007). Indeed, the application of real-time monitoring techniques for beneficial microbial functions could address to agricultural practices or conditions to maximize the action of microbial inoculants. Current real-time PCR methods allow broad-range quantification of microbial functional genes, and could be adapted to agroecological functional monitoring in the future (Choi et al., 2014; Szczech et al., 2017).

Finally, the construction of synthetic microbial communities, i.e. integration of several microorganisms with different PGP functions, presents a unique opportunity to increase the efficacy and reliability of microbial biostimulants, although engineering a microbial community represents a significant challenge (Tonelli et al., 2011). Indeed, the complexity of ecological interactions that occur between microbes (e.g. commensalism, competition) have to be deeply investigated in order to assure success of the beneficial community.

3.5 Conclusions

Microbial biostimulants potentially represent a sustainable and effective strategy to reduce abiotic and biotic stresses accentuated by climate change. Moreover, the use of microbial biostimulants could contribute to the maintenance of agro-ecosystem ecological balance, minimizing the use of pesticides and/or heavy metals in agriculture. Nonetheless, in pursuing a better product efficacy and a more widespread employment, some issues should be considered both at the regulation level, and at the research and development stage.

The definition of plant biostimulant is claims-based, meaning that the function itself defines the product (Ghazalibiglar et al., 2016). Multiple active ingredients can be present in one product, with different functions and targets. Therefore, the intrinsic heterogeneous nature of biostimulants eludes legislative categories (e.g. amendant, fertilizer, fungicide). According to the country of registration, products may undergo long and expensive trial procedures before approval. The lack of a coherent international regulation (Schuhegger et al., 2006) forms an impediment to product marketing, and may discourage the development of new products.

With regard to biological and agroecological research, the use of microbial biostimulants still presents several limitations mainly linked with their lower efficacy and higher sensitivity to environment in comparison with chemical growth regulators, fertilizers and pesticides. Furthermore, microbial biostimulants often showed inconsistent results from crop to crop or from region to region. Thus, to maximize the efficacy of microbial biostimulants fostering the constancy of the results, future research should be aimed at obtaining better targeted products, for instance, by in-depth exploring plant-associated microbiomes, by characterizing and controlling plant-microbe interactions, by functionally integrating the community of species included in one biostimulant product, by isolating microbes specifically adapted to the agricultural stress or local conditions of interest, or even by allowing on-field selection of useful microbes rather than introducing new ones. Biostimulants should be coupled with agricultural practices able to increase agroecosystem biodiversity, and ensuring a long-lasting and stable symbiotic relationship with crop plants. In this scenario, the use of microbial biostimulants represents a sustainable and effective solution against plant productivity losses due to changing climatic conditions, and could help optimize human inputs in agricultural ecosystem.

Finally, the experimental results obtained by the research on microbial biostimulants should be used to promote pilot or demonstration trials for all the relevant

stakeholder, from growers, to extension services and policy makers, to ensure the straightforward application of this methodology on different crops, regions and environmental conditions. A close cooperation and a constant exchange of information between the scientific community and the stakeholders is the key to the successful validation of research results in real conditions and their adaptation to practical applications.

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4. Genotype-dependent taxonomical and functional assembly of strawberry microbiome

Abstract: Specific microbial communities are associated to host plants, influencing their phenotype and fitness. Despite the rising interest in plant microbiome, the role of microbial communities associated to perennial fruit plants remains overlooked. Here, we provide the first comprehensive description of the taxonomical and functional bacterial and fungal microbiota of below- and above-ground organs of three commercially important strawberry genotypes ('Monterey', 'Elsanta', 'Darselect') under cultural conditions. We characterized strawberry-associated fungal and bacterial microbiomes by Next-Generation Sequencing and we analysed the potential functions expressed by the bacterial microbiome by both *in silico* and *in vitro* characterization of plant growth promoting abilities of native bacteria. Additionally, we investigated the association between strawberry microbiome, plant disease tolerance, plant mineral nutrient content and fruit quality. Our results showed that the strawberry core microbiome included 24 bacteria and 15 fungal operational taxonomic units (OTUs). However, plant organs and genotype had a significant role in determining the taxonomical and functional composition of microbial communities. Interestingly, the cultivar with the highest tolerance against powdery mildew (*Podosphaera aphanis*) and leaf spot and the highest fruit productivity was the only able to ubiquitously recruit the beneficial bacterium, *Pseudomonas fluorescens*, and to establish a mutualistic symbiosis with the arbuscular mycorrhiza *Rhizophagus irregularis*. Altogether, our work sheds light on the interaction of cultivated strawberry genotypes with a variety of microbes and highlights the importance of their applications to increase the sustainability of fruit crop production.

4.1 Introduction

Crop plants are associated with a wide diversity of microorganisms, which differently colonize plant compartments (Purahong et al., 2018; Compant et al., 2019). Such microbial biocoenosis influences plant phenotype fitness, growth, fruit production and quality, by contributing to plant nutrition, tolerance to abiotic stresses, and control of pathogenic or opportunistic species (Busby et al., 2014; Compant et al., 2019). In this view, individual plants can be considered as an holobiont, i.e. the superorganism encompassing individual host and its associated microbial community (Lemanceau et

al., 2017; Cregger et al., 2018). The association between terrestrial plants and microbes developed at least 460 million years ago, as suggested by the fossil evidence of arbuscular mycorrhizae on some of the earliest land plants (Cregger et al., 2018; Hassani et al., 2018). To date, many important questions regarding these associations remain unanswered, especially concerning the factors determining the communities assemblage and diversity of the plant microbiome (Busby et al., 2014). Increasing evidence suggests that plants can actively recruit a beneficial microflora to facilitate their adaptation to environmental conditions and changes (Busby et al., 2014; Xu et al., 2018; Kong et al., 2019). However, further studies are needed to generalize this hypothesis, and enable practical applications, especially for horticultural perennial crops grown in cultural conditions (Sangiorgio et al., 2020). To date, most experiments on plant microbiome have focused either on specific model plants (i.e. *Arabidopsis thaliana*) or economically important, annual herbaceous monocotyledons, such as corn and rice (Busby et al., 2017). Perennial plants, on the other hand, are exposed to radically changing environmental conditions (including freezing winter temperatures, dry seasons, periodic flooding) (Gutschick and Bassiri, 2008). Therefore, in perennial plants, the microbial community has evolved to last for more than a growing season, thus suggesting an assembly with a more intimate connection with host allowing its endurance to changing environmental conditions. Furthermore, perennial crops may promote plant–microbial linkages, increasing richness of bacterial and fungal beneficial communities, due to their extensive root networks and allocation of belowground carbon (Hargraves and Hofmockel, 2014; Thomson et al., 2016; McGowan et al., 2019). In addition, microbiome research has so far primarily taken into consideration the rhizosphere, while other plant compartments have been relatively neglected (Grady et al., 2019). Finally, bacterial community analysis dominates the microbiome studies (Bergelson et al., 2019). The study of bacterial and fungal microbiota colonizing different plant compartments under agronomic conditions provides key information to unfold agricultural constraints and achieve a successful microbial manipulation in farmlands (Qiu et al., 2019).

The cultivated strawberry (*Fragaria* × *ananassa* Duch., fam. *Rosaceae*) is an important fruit crop, originated approximately 300 years ago from the hybridization between ecotypes of wild octoploid species: *Fragaria chiloensis* subsp. *chiloensis* from South America and *Fragaria virginiana* subsp. *virginiana* from North America (Bertioli, 2019). In the last decade (2008-2018), the global strawberry cultivation area has increased by 14% (FAOSTAT). In 2016, the global strawberry gross production valued 17 billions

US$ with China having the biggest market-share (6.48 bn US$), followed by Europe (3.49 bn US$) and United States (3.47 bn US$) (FAOSTAT). The high adaptability of strawberry to different conditions allows the cultivation under a wide range of environments and agronomical managements (from Mediterranean to the Nordic climates) making the fruit available on the market, almost independently of the season (Mezzetti et al., 2018). For this reason, strawberry fruit represents an important and valuable portion of the daily fresh food consumption (Battino et al., 2018). Strawberry is greatly appreciated for aroma and nutraceutical properties. Among others, strawberry fruit contains phytochemicals, such as anthocyanins and ellagitannins which may prevent human health diseases induced by reactive oxygen species (Giampieri et al., 2015). While strawberry productivity and quality can be positively improved by beneficial microorganisms (Todeschini et al., 2018), the cultivation is challenged by a large variety of pathogens, which cause substantial economic losses and require the frequent application of pesticides. Among these diseases, red stele (*Phytophthora fragariae*), powdery mildew (*Podosphaera aphanis*) and bacterial angular leaf spot (*Xanthomonas fragariae*) are the ones most severely affecting strawberry production worldwide (Husaini and Neri, 2016). Powdery mildew mainly affects photosynthetic ability of strawberries cultivated in humid environments (Amsalem et al., 2006), which leads to strong reduction of growth and productivity with major yield losses (Sargent et al., 2019). Leaf spot diseases, which in severe conditions may led to plant death, are caused by different pathogens, including bacteria (*Xanthomonas fragariae*) and fungi (*Colletotrichum gloeosporiodes*, *Mycosphaerella fragariae*, *Cercospora fragariae, Mycosphaerella louisianae*, *Septoria fragariae, S. aciculosa, S. fragariaecola*, etc.). Multiple resistance to a broad spectrum of diseases such as powdery mildew and leaf spot is still not available among commercial strawberry cultivars (i.e. human-selected clonal genotypes) (Whitaker et al., 2020). Disease control is particularly challenging in strawberry production, since several cultivars present at the same time, flowers, fruits and leaves, and are therefore subjected to a high risk of pesticide residue accumulation on berries (Mezzetti et al., 2018). Biological control is a promising and safer alternative to the use of xenobiotic pesticides. Some commercially available, beneficial microorganisms (i.e. *Ampelomyces quisqualis*, *Bacillus subtilis*, *Trichoderma harzianum*, *Glomus* spp.) have been tested for disease control in strawberry, yet none of them has demonstrated characteristics of reliability, persistence and/or cost-effectiveness justifying their use as an alternative to chemical pesticides (Husaini and Neri, 2016). The unsatisfactory degree of disease control and the high variability of results

obtained in different locations and seasons with commercial beneficial microorganisms can be explained by the fact that those microbes are in most cases non-native to the strawberry plant microbiome. Several studies suggest that biological control agents isolated from the host plant microbiome have a superior efficacy in comparison to non-indigenous microbial inoculants (Haney et al., 2015; Santhanam et al., 2015; Mazzola and Freilich, 2017). Thus, the characterization of the native microbiome is a key step for the successful selection of beneficial microorganisms against plant diseases (Purahong et al., 2018). Unfortunately, the complete microbiome of cultivated strawberry has not yet been described, hindering the identification and selection of the most effective indigenous microorganisms to improve plant fitness and fruit quality and/or provide resistance to biotic and abiotic stresses.

The plant-associated microbial community is pivotal in delivering multiple functions to its host. Traditionally, microbiome studies focused on the taxonomy of plant-associated microbial communities. However, functional diversity should receive reasonable consideration in the context of sustainable agriculture as it has been recognized as the best predictor of ecosystem processes and properties (Johnson and Pomati, 2020). Indeed, plants are sessile organisms hence functionalities provided by the microbiota are essential for supporting plants in coping with adverse environmental conditions (Lemanceau et al., 2017; Sangiorgio et al., 2020). As for taxonomy, microbial functional composition differs between plant niches (Bai et al., 2015). Potential functions expressed by the microbiota might be examined both by a culture-independent and dependent approach, by applying computational techniques and by *in vitro* screening of microbial traits, respectively.

The aim of this study was to provide a complete description of the strawberry holobiome, including both fungal and bacterial populations, and to identify the core microbiome, from soil, plant-soil interface (rhizosphere) and plant compartments (roots and above-ground organs) using Next Generation Sequencing (NGS). For this purpose, three commercially important strawberry genotypes ('Elsanta', 'Darselect' and 'Monterey') were used. Additionally, functions potentially expressed by the bacterial microbiota were analysed both *in silico*, analysing Tax4Fun data of the microbial communities of the three cultivars, and *in vitro,* selecting native bacteria on artificial media and characterizing them for their Plant Growth Promoting (PGP) abilities. Furthermore, the effects of strawberry genotypes, soil and plant compartments on the richness and microbial community composition were studied, with a focus on pathogenic and beneficial microbes. Finally, the links between

strawberry microbiomes, plant mineral nutrient content and fruit quality traits were investigated. To our knowledge, this study provides the first in-depth and comprehensive view of horticultural crop microbiome in relation to plant genotype, health and nutritional status and fruit quality parameters, shedding light on potential practical applications to increase the sustainability of fruit crop production.

4.2 Materials and Methods

4.2.1 Strawberry cultivation, phenotypic characterization and disease severity ranking

Three *Fragaria* × *ananassa* cultivars (genotypes) were used: the everbearing genotypes 'Elsanta' (E) and 'Darselect' (D) (widely cultivated in Northern Italy), and the day-neutral variety 'Monterey' (M). Bareroot strawberries were bought from CREA Forlì, COVIRO Ravenna, SANTORSOLA Trento, for D, M and E genotype, respectively. Plants were transplanted in early June-July 2017 into 48.5 × 22 × 11 cm white plastic pots, filled with a commercial blond sphagnum peat moss soil (pH 5.2-5.8; EC 0.15-0.025 dS/m; 220 kg/mc; total porosity 92%) composed by 20-30 mm blonde peat, 10-20 mm blond peat, mixture of Irish peat, fibre and dusted coconut fibre and granules (Vigorplant s.r.l, Lodi). Each pot contained 6 plants with a distance of 16.7 cm between each other. These pots were maintained at 1.2 m above ground under rainproof tunnel (18 m × 3.50 m × 5.60 m) located in field at the experimental station of Pergine Valsugana (frazione Vigalzano, TN, Italy; 46°07'N, 11°22'E, 450 a.s.l.). Plants were fertigated using a drip system (Table S4.1) with a daily volume of 2 l of fertigation solution for each pot. Over the growing season (25, 35 and 50 days after transplant) root and leaf apparatus, as well as fruits, of 50 plants were weighted. Moreover, 100 additional plants of each genotype grown in same conditions were weekly monitored for powdery mildew and leaf spot symptoms. Disease index on leaves was visually ranked using a 0-5 scale (0 = no symptoms; 5 = plant death) (Table S4.2).

4.2.2 Sampling

At the end of the production cycle, for each genotype, four asymptomatic plant replicates from different pots distributed in the field area were collected. The plants were immediately brought to the laboratory and processed. Definition of the plant-soil compartments were defined as follows: 'bulk soil' is the soil domain explored by the roots, but not attached to them (i.e. approx. 1 cm radius from a feeder root); 'rhizosphere' includes only soil particles firmly adhering to root and extracted by

washing; 'roots' are washed roots (without visible soil particles); 'above-ground organs of strawberry plant' are constituted by crown (short stem), petiole, leaves and runners. More in detail, bulk soil was collected from the growing pots, approx. 10 cm apart from any plant and at 5 cm depth and suspended in sterile 10 mM $MgSO_4$ solution. Plants were divided in above-ground tissues (leaves, stems, crown) and roots. Roots were shaken to release loosely-associated soil, then washed in sterile 10 mM $MgSO_4$ solution under vigorous shaking to collect the rhizospheric soil. Above-ground tissues and root samples (further cleaned with a brush) were ground with mortar and pestle, and suspended in sterile 10 mM $MgSO_4$ solution. No bleaching agent was used for roots and aerial parts samples as it may enter inside the plant tissues and degrade the microbial DNA. One aliquot from each sample was serially diluted and plated on Lysogenic Broth (LB) agar medium (Sigma Aldrich) amended with cycloheximide (100 μg ml^{-1}) to prevent fungal growth. The remaining washing volume was stored at -20 °C until DNA extraction.

4.2.3 Bacteria isolation and functional characterization

LB agar plates, prepared as described above, were incubated at 27°C for 24h. Colonies were phenotypically characterized and for each phenotype in a repetition, a single colony was randomly collected from the plates at the highest dilution. After purification, isolates were stored at -80°C in LB broth supplemented with 20% v/v glycerol. DNA was extracted from each bacterial isolate using GenElute Bacterial Genomic DNA kit, following manufacturer's instructions. REP-PCR was performed using BOXA1R (De Urraza et al., 2000), as described in Gevers et al., 2001. Genomic DNA patterns obtained were visualized on a polyacrylamide gel (Filippetti et al., 2005), in order to screen for bacterial isolates showing equal patterns and later perform identification and functional characterization only on one of them.

Bacterial isolates were identified by 16S rRNA sequencing as follows: 16S rRNA gene extraction was amplified using Lac16S-for (5'-AATGAGAGTTTGATCCTGGCT-3') and Lac16Srev (5'-GAGGTGATCCAGCCGCAGGTT-3') primers, as described in (Bringel et al., 2005). The amplification product was delivered to Biofab Research Srl for sequencing. Sequences were compared with those available on BLAST (NCBI) at date January 2019. 19, 14 and 10 isolates for 'Monterey', 'Elsanta' and 'Darselect', respectively, were functionally characterized for acetoin, IAA, ammonia and

siderophores production, ACC deaminase activity, *X. fragariae* and *B. cinerea* inhibition as described in (Perpetuini et al., 2019).

4.2.4 Analysis of plant mineral composition and fruit quality traits

Ultrapure 65% HNO_3 was obtained from analytical grade HNO_3 (Carlo Erba, Milan, Italy) by means of a SAVILLEX DST 1000 sub-boiling system (Savillex Corp., Eden Prairie, MN, USA). Standard solution and sample preparation were carried out by weight with a Mettler AE200 analytical balance (Mettler Toledo S.p.A, Milan, Italy) with ±0.0001 g sensitivity. Elemental analysis on root and above-ground organs of strawberry plants (percentage of C, H and N) was performed on Thermo Scientific™ FLASH 2000 organic elemental analyser, each sample was analysed in duplicate. Strawberry samples' digestion was performed by a microwave assisted procedure performed with a FKV autoclave, Ultrawave model, on a maximum sample aliquot of 0.4 g, accurately weighted in the microwave quartz vessels, before adding 1.5 mL HNO_3 and 3.5 mL H_2O. At the end of digestion process, an almost colourless, pale yellow sample was obtained. The resulting solutions were diluted up to a total mass of 15 g with Milli-Q water in polypropylene tubes, microfiltered (Ø 0.22 mm) and analysed. Measurements of the Mg, P, K, Ca, Fe, Mn, Co, Ni, Zn. Sr, Ba and Pb content in vegetal samples were performed by using an inductively coupled plasma interfaced to a quadrupolar mass analyzer, ICP/qMS, (XSeries II model, ThermoFisher Scientific, Bremen, Germany) equipped with Peltier cooled (3°C) spray chamber. The collected samples were randomly acquired after being introduced by the autosampler CETAC ASX 520 into the nebulizer, and the positively charged ions were then produced by high temperature, inductively coupled plasma. The ions passed through a sampling cone interface into a high-performance quadruple mass spectrometer, which is computer controlled to carry out multi-element analysis. Data were analysed by PlasmaLab software. The instrument was tuned daily with an ICP-MS tuning solution. In in HNO_3 4% (100 ppb) was used as internal standard. ICP-multi-element solution, IV-ICP-MS-71A (Inorganic Ventures, Christiansburg, VA, USA) was used for the determination of Mg, P, K, Ca, Fe, Mn, Co, Ni, Zn, Sr, Ba and Pb concentrations. Each sample was analysed at least in 3 independent measurements and each experiment comprised three repetitions. Results are given as mean value ± standard deviation (Table S4.6).

Strawberry fruits for each cultivar were weighted. Fruit firmness was measured by a texture analyser (Zwick Roell, Italy) using the penetration test methodology that was

previously developed for raspberry (Giongo et al., 2019). This penetration test outlined a mechanical force displacement using a 5 kg loading cell and a cylindrical flat head probe with a diameter of 4mm entering the berry flesh that was placed on the plate with the receptacle upright to the compression probe. Mechanical profiles were acquired with a resolution of 100 points per second with the following instrumental settings: test speed of 300mm min^{-1}, post-test speed of 1000mm min^{-1}, auto force trigger of 2 g and stop plot at target position. Each berry was penetrated until a 99% penetration strain. In this study only the maximum force value (N) was considered, since this parameter is usually highly related with berry firmness (Giongo et al., 2018). Soluble sugar content was measured on strawberry fruit juice with a hand-held Atago digital refractometer (Optolab, Modena, Italy). Titratable acidity was determined on strawberry juice diluted (1:2) in distilled water by titration with NaOH to pH 8.1 and expressed as citric acid equivalents.

4.2.5 DNA extraction and Illumina sequencing

DNA was extracted from 250 mg of each homogenized bulk soil, rhizosphere, root, and above-ground organs using the MoBio PowerSoil kit (MO BIO Laboratories, Carlsbad, CA, USA), following the manufacturer's instructions. DNA quality and quantity were measured by spectrophotometric quantification with a NanoDrop ND-8000 V1.1.1 spectrophotometer (Thermo Fisher Scientific, Dreieich, Germany). DNA extracts were then stored at –20 °C before further analysis. The extracted DNA samples were sent to RTL Genomics, Lubbock, TX, USA for Paired-end Illumina MiSeq sequencing. The V5, V6 and V7 regions of the 16S rRNA gene and ITS2 regions of the nuclear ribosomal internal transcribed spacer (ITS) rRNA gene were targeted for bacteria and fungi respectively. DNA extracts were amplified for sequencing in a two-step process. The forward primer was constructed with the Illumina i5 sequencing primer (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3') and the 799F (5'-AACMGGATTAGATACCCKG-3') for bacteria (Chelius and Triplett, 2011) and the fITS7 primer (5'- GTGARTCATCGAATCTTTG-3', [1]) for fungi. The reverse primer was constructed with the Illumina i7 sequencing primer (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3') and the 1193r (5'-ACGTCATCCCCACCTTCC-3') for bacteria (Bodenhausen et al., 2013) or the ITS4 primer (5'-TCCTCCGCTTATTGATATGC-3', [2]) for fungi. The selected primer set for bacteria (799F and 1193r) can avoid contamination from plastid DNA. Amplification

reactions in 25 μl reactions with Qiagen HotStar Taq master mix (Qiagen Inc, Valencia, California), including 1μl of each 5μM primer, and 1μl of template were performed. Reactions were performed on ABI Veriti thermocyclers (Applied Biosytems, Carlsbad, CA, USA) under the following thermal profiles: 95°C for 15 min, then 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, followed by one cycle of 72°C for 10 min and 4°C hold (bacteria) and 95°C for 15 min, then 35 cycles of 94°C for 30 sec, 54°C for 40 sec, 72°C for 1 min, followed by one cycle of 72°C for 10 min and 4°C hold (fungi). Products from the first stage amplification were added to a second PCR based on qualitatively determine concentrations. Primers for the second PCR were designed based on the Illumina Nextera PCR primers as follows: Forward - AATGATACGGCGACCACCGAGATCTACAC[i5index]TCGTCGGCAGCGTC and Reverse - CAAGCAGAAGACGGCATACGAGAT[i7index]GTCTCGTGGGCTCGG. The second stage amplification was run the same as the first stage except for 10 cycles. Amplified products were visualized with eGels (Life Technologies, Grand Island, New York). The products were then pooled equimolar, and each pool was size selected in two rounds using Agencourt AMPure XP (BeckmanCoulter, Indianapolis, Indiana) in a 0.75 ratio for both rounds. The size selected pools were then quantified using the Quibit 2.0 fluorometer (Life Technologies) and loaded on an Illumina MiSeq (Illumina, Inc. San Diego, California) 2x300 flow cell at 10pM.

4.2.6 nifH gene and Pseudomonas fluorescens detection

To verify the nitrogen-fixing activity and the presence of putative Plant Growth Promoting species that were found to be discriminant genotypes and/or compartments, specific detection protocols were applied. *nifH* gene were used as target to detect N-fixing activity, being a highly conserved gene (Boyd and Peters, 2013). The presence of *nifH* gene in samples was verified by PCR using *nifH* gene-specific primers (PolF (5'- TGC GAY CCS AAR GCB GAC TC -3') /PolR (5'- ATS GCC ATC ATY TCR CCG GA -3')), as previously described (Hoppe et al., 2014). *Pseudomonas fluorescens* detection in 'Monterey' genotype was performed as follows: DNA from bulk soil and rhizosphere samples was extracted as above; roots and above-ground parts of strawberry plants were surface sterilized two times with deionized water and 70% ethanol and washed 3 times sterile water, organs were let 3h in sterile water. Then, strawberry roots and above-ground part DNA was extracted as above and amplified using *Ps. fluorescens* specific primers (16SPSEfluF (5'-TGC ATT

CAA AAC TGA CTG-3') /16SPSER (5'-AAT CAC ACC GTG GTA ACC G-3')) as described elsewhere (Scarpellini et al., 2004). For both *nifH* and *Ps. fluorescens* detection, amplification products were visualized through agarose gel 1.5% electrophoresis.

4.2.7 Bioinformatics

High quality reads from the paired-end sequences generated by Illumina MiSeq sequencing platform were extracted using Mothur (Schloss et al., 2009) and OBI Tools (Boyer et al., 2016) software suits. PANDAseq was used to merge forward and reverse raw reads from the same sample by using the simple-bayesian algorithm with a minimum overlap of 80 and 20 nucleotides for bacteria and fungi, respectively. All the merged reads were then trimmed with the following parameters: (i) minimum length of 350 (bacteria) and 120 (fungi), (ii) minimum average Phred score of 25 on the trimmed length, (iii) no ambiguities in the sequence length, and (iv) maximum length of 20 homoploymers in the sequence. The reads were then pre-clustered using CD-HIT-EST, allowing a maximum of 1% of dissimilarity and with only one base allowed per indel (Niu et al., 2010), in order to merge those reads arising likely from sequencing errors (Huse et al., 2010). Chimeric sequences were detected using the Uchime algorithm (Edgar et al., 2011) as implemented in Mothur and removed. Reads from each sample were pooled together and were dereplicated into unique sequences and sorted by decreasing abundance. The resulting reads were then clustered into operational taxonomic units (OTUs) using the CD-HIT-EST algorithm (Fu et al., 2012) at a threshold of 97% sequence similarity. The OTU representative sequences (defined as the most abundant sequence in each OTU) were taxonomically assigned against the reference sequences from the SILVA database v132 for prokaryote 16S (Yilmaz et al., 2010) and from the Unite database (version unite.v7) (Kõljalg et al., 2005) for fungal ITS using the naive Bayesian classifier (Bai et al., 2013) as implemented in Mothur using the default parameters. All the sequences identified as non-target organisms were removed from bacterial and fungal datasets. Rare OTUs (singletons), which potentially might represent artificial sequences were removed. The read counts were rarefied to the smallest read number per sample (10,930 and 8,077 reads for bacteria and fungi, respectively). Ecological functions were determined for each OTU using FAPROTAX for bacteria (Louca et al., 2016), and FUNGuild (Nguyen et al., 2016) for fungi. Ecological functions of bacteria obtained by FAPROTAX were also manually checked against other references for their presence in terrestrial system (Sansupa et

al., 2021). We grouped arbuscular mycorrhizae, ectomycorrhizae, ericoid mycorrhizae, endophytes, dark septate endophytes and mycoparasites as potential beneficial fungi. All fungal plant pathogens were checked again for their taxonomic identifications and their DNA-based Species Hypotheses (SH) are presented in Supplementary Table S4.3. Potential beneficial bacteria (N fixing, plant growth promoting and biological control agents) were manually assigned using all available references (Table S4.4). Prediction of the bacterial functions according to the KEGG database was also performed. Tax4fun package (Aßhauer et al., 2015) to perform the functional prediction was used, and the results were analyzed following MicrobiomeAnalyst online pipeline ((https://www.microbiomeanalyst. ca/) (Dhariwal et al., 2017; Chong et al., 2020). KEGG orthologs and modules involved in PGP mechanisms were chosen using the KEGG database and several reviews. The Illumina sequencing of all bacterial and fungal datasets are deposited in The National Center for Biotechnology Information (NCBI) database under BioProject: PRJNA556362
(https://dataview.ncbi.nlm.nih.gov/object/PRJNA556362?reviewer=4d8dskbpvkqqcci8o1inim36b).

4.2.8 Statistical analysis

To assess the coverage of the sequencing depth, individual rarefaction analysis for each sample using the function 'diversity' in Past was performed. At the analyzed sequencing depth, all individual rarefactions shown to be sufficient to infer bacterial and fungal community composition and richness in our samples (Figure S4.1). We defined core microbiome as the bacterial and fungal communities that are comprised of OTUs that were detected in all strawberry genotype and present in more than 75% of the samples (Xu et al., 2018). The effects of strawberry genotype, soil and plant compartments (bulk soil, rhizosphere, root and above-ground organs) on bacterial and fungal OTUs richness were analyzed using two-way analysis of variance (ANOVA), incorporating the Jarque-Bera JB test for normality. The effects of strawberry genotype, soil and plant compartment on bacterial and fungal community compositions were visualized using Non-metric MultiDimensional Scaling (NMDS) based on the presence–absence data and Jaccard distance measure. Colored ellipses in NMDS ordinations are 95% confidence intervals of species centroids for each

treatment level. The significant effect of the strawberry genotype, soil and plant compartment on bacterial and fungal community compositions were determined using two-way Analysis of Similarity (ANOSIM) and two-way Permutational multivariate analysis of variance (PERMANOVA) based on the presence–absence data and Jaccard distance measure over 999 permutations. Since relative abundance data from Next Generation Sequencing may not be fully used quantitatively (Amend et al., 2010), we analyzed the microbial community composition using both presence/absence and relative abundance data sets. The results from presence/absence data are presented in the main text and the corresponding results using relative abundance data (with Bray–Curtis distance measure) are presented in Supplementary Information (Table S4.5). NMDS ordination based on presence/absence data and the Jaccard dissimilarity measure coupled with the envfit function of the vegan package in R version 3.2.2 (Oksanen et al., 2013) were used to investigate the links between each of bacterial and fungal community composition (bulk soil, rhizosphere, root and above-ground organs) and soil nutrient parameters, strawberry genotypes, fruit quality parameters (soluble sugar content and titratable acidity). NMDS stress values were between 0.06 – 0.13. Differences in phenological characteristics, Plant Growth Promoting KEGG modules and KOs and chemical composition of roots and above-ground strawberry of the three genotypes were tested by One-way ANOVA, followed by multiple comparisons by Tukey's test, performed using Past version 2.17 (Hammer et al., 2001).

4.3 Results

4.3.1 Phenotypical differences of strawberry cultivars

Flower differentiation, fruit production, plant architecture and growth rate were observed during the growing season. We found that both leaf and root apparatus of 'Elsanta' were significantly heavier in respect to the other genotypes, whereas 'Monterey' had the highest productivity, besides producing the biggest fruits (Table 4.1).

The susceptibility of the different genotypes to the main strawberry diseases was also evaluated (Table S4.2). Plants of 'Monterey' showed the highest tolerance to leaf spot and powdery mildew in comparison to the other genotypes. On the other hand, 'Elsanta' showed to be less sensitive than 'Darselect' to leaf spot, but highly susceptible to powdery mildew.

Table 4.1. Phenological characteristics of different strawberry genotypes are shown. Data are expressed as mean ± SE. Different letters indicate significant differences between genotypes according to One-way ANOVA followed by multiple comparisons by Tukey's test.

Cv	Leaves g/plant at 50 days after transplant	Root g/ plant at 50 days after transplant	Harvested red fruit /week/plant (g)	Single fruit weight (g)
Monterey	7.76 ± 0.90 **b**	30.04 ± 3.29 **ab**	53.87 ± 5.48 **a**	16.24 ± 0.76 **a**
Elsanta	15.32 ± 1.54 **a**	39.54 ± 2.74 **a**	34.24 ± 5.81 **ab**	10.24 ± 0.55 **b**
Darselect	9.44 ± 1.39 **b**	20.25 ± 3.64 **b**	30.87 ± 6.73 **b**	9.43 ± 1.07 **b**

4.3.2 Composition of strawberry microbiomes

Bacterial 16S rRNA and ITS gene communities were profiled in bulk soil, rhizosphere, root and above-ground organ samples in the three strawberry genotypes (Figure 4.1a). In roots and above-ground organs, we targeted epiphytic and endophytic microorganisms jointly. In total, we generated 1,531,637 (average of 31,909 reads per sample) and 739,458 (average of 15,405 reads per sample) high quality reads excluding chimeric sequences for bacteria and fungi, respectively. We removed singletons which may come from sequencing errors and normalized all bacterial and fungal datasets to 10,930 sequences for bacteria and 8,077 for fungi. Rarefaction curves showed the sufficient sequencing effort for most of the samples (Figure S4.1a,b). In addition, OTU richness estimates, predicted with Chao1 are shown (Figure S4.2). We used observed richness directly as diversity measure for both bacteria and fungi (Figure 4.1b,c). In total, we detected 26,434 bacterial and 1,716 fungal OTUs. Among the three strawberry genotypes, 'Darselect' displayed the lowest bacterial richness in all compartments (Figure 4.1d). In general, above-ground organs had a lower bacterial richness compared to the other compartments. Differently, fungal microbiome diversity was rather homogenous among different compartments (Figure 4.1e).

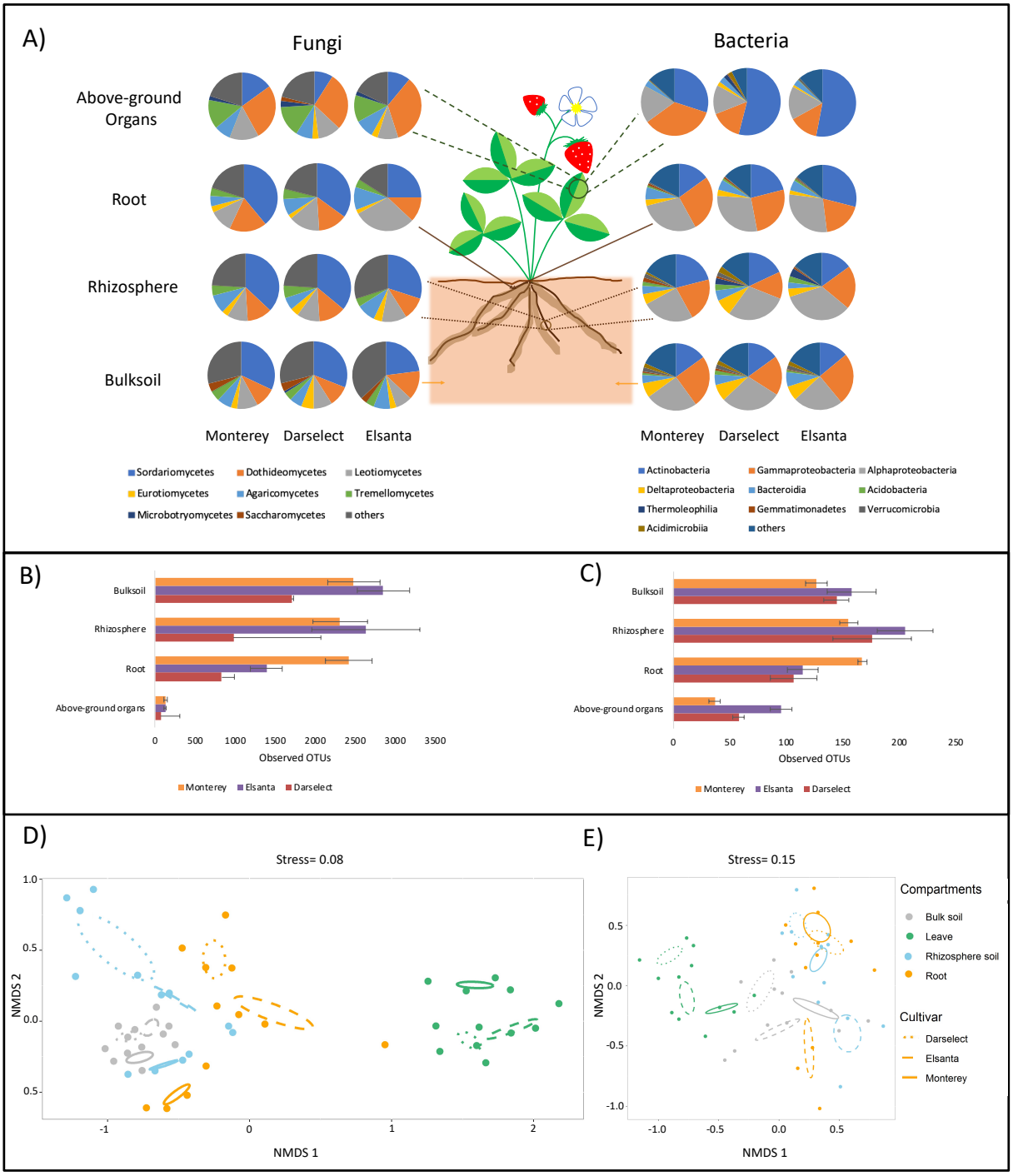


Figure 4.1. Composition of strawberry microbiomes. (A) Overall community composition of bacteria and fungi in different soil and plant compartments and genotypes, estimated using presence-absence data. (B) Bacterial and (C) fungal OTU richness in different soil and plant compartments and genotypes. Bacterial (D) and (E) fungal composition similarity among different soil and plant compartments and genotypes, shown by nonmetric multidimensional scaling (NMDS) plot.

The total bacterial and fungal community assemblages were compared using two-way PERMANOVA to identify the main drivers of the microbiome composition (Table 4.2).

Microbial compositions are strongly dependent on the analysed genotype (bacteria $F = 1.87$, $P = 0.002$; fungi $F = 2.93$, $P = 0.001$), on the compartment (bacteria $F = 4.27$, $P = 0.001$; fungi $F = 3.56$, $P = 0.001$), and on genotype × compartment interaction (bacteria $F = 1.44$, $P = 0.001$; fungi $F = 1.51$, $P = 0.001$; Table 4.2). In particular, NMDS highlighted that both bacterial and fungal above-ground microbiome compositions strongly differ from below-ground ones (Figure 4.1 d,e). Similar results were obtained by means of two-way ANOSIM analysis (Table 4.2) and by using relative-abundance data, instead of presence-absence one (Table S4.6).

Table 4.2. Effect of genotype, soil and plant compartment on richness and community composition of strawberry microbiome. Nd = not determined; Significant *P* values are highlighted in bold.

Microorganisms/ Factors	Richness (Two way ANOVA)		Community composition (Two-way ANOSIM)		Community composition (Two-way PERMANOVA)	
	F	***P***	***R***	***P***	**Pseudo*F***	***P***
Total bacteria						
Genotype	12.15	**0.000**	0.65	**0.001**	1.87	**0.002**
Compartment	32.47	**0.000**	0.83	**0.001**	4.27	**0.001**
Genotype x compartment	2.55	**0.037**	nd	nd	1.44	**0.001**
Potential beneficial bacteria						
Genotype	4.92	**0.013**	0.34	**0.001**	1.61	**0.001**
Compartment	20.86	**0.000**	0.48	**0.001**	2.87	**0.001**
Genotype x compartment	1.81	0.125			1.35	**0.001**
Fungi						
Genotype	1.74	0.191	0.78	**0.001**	2.93	**0.001**
Compartment	19.00	**0.000**	0.76	**0.001**	3.56	**0.001**
Genotype x compartment	2.00	*0.092*	nd	nd	1.51	**0.001**
Potential beneficial fungi						
Genotype	9.23	**0.001**	0.22	**0.001**	2.05	**0.004**
Compartment	13.13	**0.000**	0.47	**0.001**	4.05	**0.001**
Genotype x compartment	3.46	**0.008**	nd	nd	1.33	**0.033**
Plant pathogenic fungi						
Genotype	3.65	**0.036**	0.43	**0.001**	3.34	**0.001**
Compartment	4.30	**0.011**	0.51	**0.001**	3.90	**0.001**
Genotype x compartment	2.92	**0.020**	nd	nd	1.58	**0.002**

Community composition based on presence-absence data indicates that Actinobacteria, Alphaproteobacteria, Gammaproteobacteria, Deltaproteobacteria and Bacteroidia are the bacterial groups representing the backbone of strawberry bacterial microbiome in all plant and soil compartments, all together accounting on average for 80% of total detected OTUs (Figure 4.1a). Above-ground organs of 'Darselect' and 'Elsanta' were dominated by Actinobacteria (54 and 53% respectively), whereas 'Monterey' was mostly colonized by Gammaproteobacteria (Figure 4.1a). Below-ground compartments of the three cvs were dominated by Alphaproteobacteria (Figure 4.1a).

Regarding the strawberry mycobiome, Sordariomycetes, Dothideomycetes, Leotiomycetes and Agaricomycetes were the most represented fungal classes in all plant and soil compartments accounting for 64% of total OTUs based on presence-absence data (Figure 4.1a), but their percentages varied depending on cv and compartment. Dothideomycetes were predominant in leaves of the three *F.* × *ananassa* genotypes (34% 'Elsanta', 28% 'Darselect', 27% 'Monterey'), whereas below-ground compartments of all genotypes were mostly dominated by Sordariomycetes (Figure 4.1a).

Overall bacterial and fungal microbial community composition was analysed based on relative-abundance data, too (Figure S4.2). In this view, Actinobacteria was the predominant bacterial group in all genotypes and compartments analysed. The lowest percentages were found in the rhizosphere and bulk soil of 'Elsanta' (31 and 33%, respectively), whereas 'Elsanta' and 'Darselect' above-ground compartments showed a high group homogeneity, being dominated by Actinobacteria for 98 and 99%, respectively (Figure S4.2). Alphaproteobacteria were homogenously represented in plant and soil compartments of the three genotypes. Gammaproteobacteria were almost absent in above-ground compartments of 'Elsanta' and 'Darselect', while they were the second most represented group in 'Monterey' (26%) (Figure S4.2). The fungal community composition, based on relative-abundance data, showed a good level of homogeneity between different genotypes. Dothideomycetes was the predominant taxa in above-ground organs of the three genotypes (Figure S4.2) followed by Tremellomycetes, Leotiomycetes and Sordariomycetes. whereas below-ground organs of the three cultivars were dominated by Sordariomycetes (Figure S4.2).

Commonalities in the microbiomes of the three cultivars in the different plant compartments are shown (Figure 4.2). Alphaproteobacteria, Gammaproteobacteria, Actinobacteria, Deltaproteobacteria and Bacteroidia are common to the three

genotypes in all plant compartments, except for above-ground organs where Deltaproteobacteria and Bacteroidia do not form part of the overlap (Figure 4.2). Regarding the fungal microbiome, Sordariomycetes, Dothideomycetes, Leotiomycetes, Eurotiomycetes and Agaricomycetes constituted the commonalities of below-ground organs of the three genotypes. The above-ground core was similar except for the absence of Eurotiomycetes and Agaricomycetes (Figure 4.2).

We identified ubiquitous microbes in all the studied environments from the soil to the above-ground plant organs of all three strawberry genotypes. Among these core microbes, 24 OTUs were bacterial (mainly Micrococcales) and 15 fungal (mainly Ascomycota) (Table S4.7).

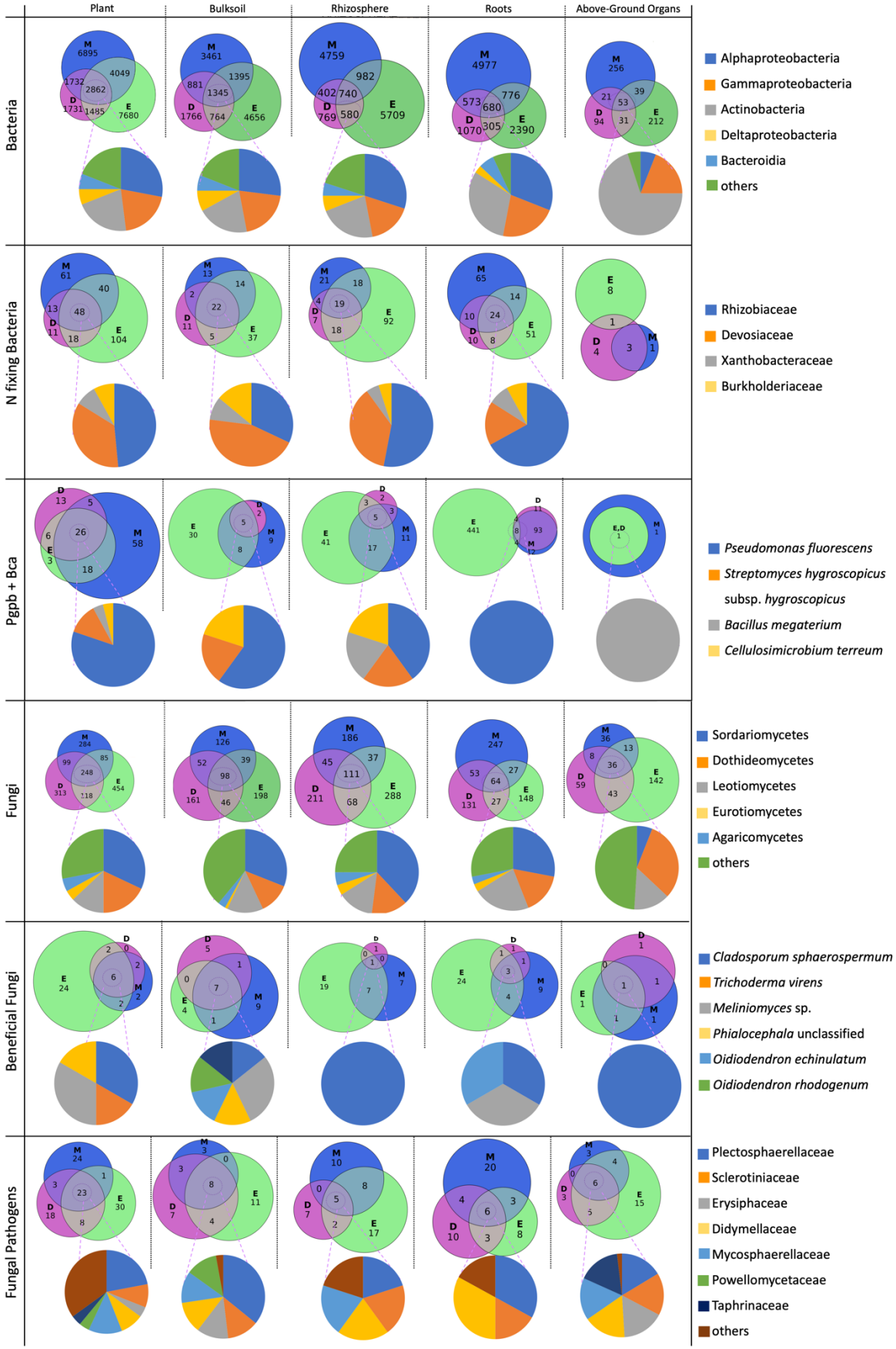

Plant
Bulksoil
Rhizosphere
Roots
Above-Ground Organs
Bacteria
N fixing Bacteria
Pgpb + Bca
Fungi
Beneficial Fungi
Fungal Pathogens
Alphaproteobacteria
Gammaproteobacteria
Actinobacteria
Deltaproteobacteria
Bacteroidia
others
Rhizobiaceae
Devosiaceae
Xanthobacteraceae
Burkholderiaceae
Pseudomonas fluorescens
Streptomyces hygroscopicus subsp. hygroscopicus
Bacillus megaterium
Cellulosimicrobium terreum
Sordariomycetes
Dothideomycetes
Leotiomycetes
Eurotiomycetes
Agaricomycetes
others
Cladosporum sphaerospermum
Trichoderma virens
Meliniomyces sp.
Phialocephala unclassified
Oidiodendron echinulatum
Oidiodendron rhodogenum
Plectosphaerellaceae
Sclerotiniaceae
Erysiphaceae
Didymellaceae
Mycosphaerellaceae
Powellomycetaceae
Taphrinaceae
others

Figure 4.2. Identification of bacterial and fungal core microbiomes. Core microbiome taxonomic compositions, both concerning the whole plant and for each organ, are described. Quantity of bacterial and fungal OTU unique for each strawberry genotype, as well as overlaps, are reported.

4.3.3 Functions potentially expressed by plant-associated bacteria and fungi

Plant-associated microbial community is pivotal in delivering multiple functions, to the plant, such as plant growth promotion and increase of abiotic/biotic stress resilience. In this work, functional diversity of bacterial and fungal microbiomes was studied following several approaches.

In silico prediction of the microbial functions was first performed using FAPROTAX and FUNGuild, functional prediction tools providing guild characteristics of bacterial and fungal taxa, respectively. 3,845 bacterial (15% of all detected bacteria) and 706 fungal (41% of all detected fungi) OTUs were assigned to a putative functional group. Twenty bacterial and sixteen fungal functional groups colonized different soil and plant compartments of strawberry plants (Table S8). Chemoheterotrophy, methanol oxidation, intracellular parasitism, predation/exoparasitism were the dominant bacterial functions, while saprophytism, plant pathogenic and endophytic colonization were dominant among the fungal functions.

Within the bacterial OTUs we further explored specific functions relevant to plant health, fitness and growth (Table S4.4). We assigned 285 OTUs to 16 potential N-fixing genera, and 129 OTUs as species known for their activity as biological control agents (BCA) and/or plant growth promoter (PGPB). Both compartment and genotype had a significant role in defining plant-associated beneficial bacterial community, according to ANOSIM and PERMANOVA ($P < 0.001$) (Figure S4.3a; Table 4.2; Table S4.5). In below-ground organs, commonalities of N-fixing bacteria between the three strawberry genotypes consisted of Rhizobiaceae, Devosiaceae, Xanthobacteraceae and Burkholderiaceae, whereas in above-ground organs no overlap was identified (Figure 4.2). Besides the identification of commonalities of the three genotypes in different organs, we further highlighted the distribution of OTUs among organs of the same genotype (Figure 4.3). N-fixing bacteria have been found to be widely distributed both in below- and above-ground compartments. However, above-ground organs are colonized by fewer N-fixing bacteria in respect to below-ground compartments. Above-ground organs of 'Monterey' and 'Darselect' were characterized by the presence of *Methylobacterium* spp., which was found only in this compartment.

Aminobacter spp. was uniquely found in the root and rhizosphere of 'Darselect', whereas *Phyllobacterium* spp. was characteristic of 'Monterey' (Figure 4.3a).
Regarding the bacterial beneficial microbiome, in 'Monterey', 19% of beneficial OTUs were able to simultaneously colonize below- and above-ground organs, whereas in 'Elsanta' and 'Darselect' only one OTU (identified as *B. megaterium*) was found to colonize both underground and above-ground organs. *Ps. fluorescens* is known for its several Plant Growth Promoting traits and the ability to control several plant diseases (Bakker et al., 2007). NGS analysis revealed that 'Monterey' was the only genotype colonized by *Ps. fluorescens* in the below as well as in the above-ground organs (Figure 4.3b). Therefore, its colonization ability of different plant compartments was further confirmed by PCR analysis. Indeed, we proved the ability of *Ps. fluorescens* to establish detectable populations in the soil and tissues (both internal and external) of 'Monterey' (Figure S4.4).

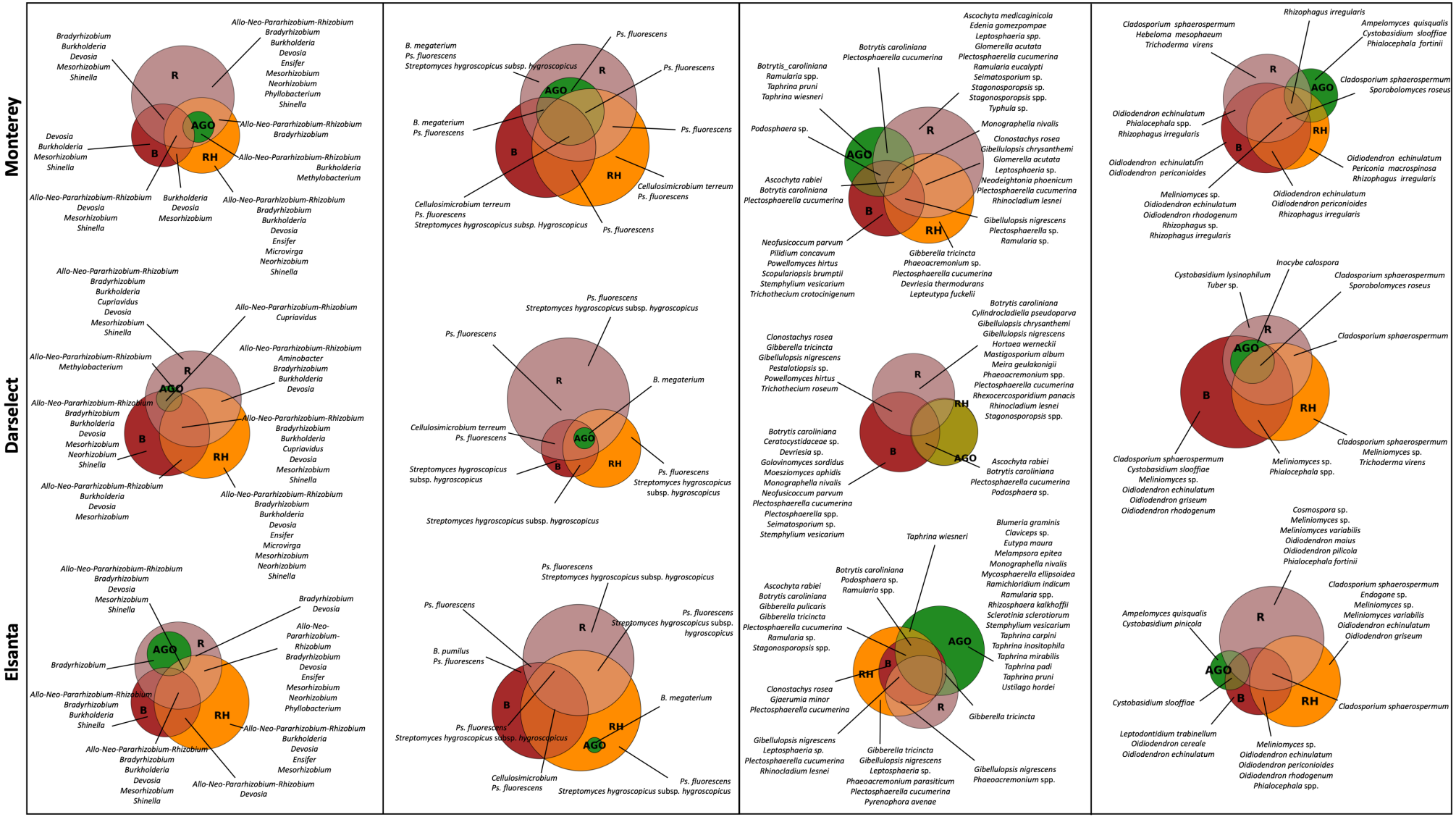

A) Potential N Fixing Bacteria
B) Potential Pgpb and Bca
C) Potential Fungal Pathogens
D) Potential Beneficial Fungi
Monterey
Darselect
Elsanta
R
AGO
B
RH
Allo-Neo-Pararhizobium-Rhizobium
Bradyrhizobium
Burkholderia
Devosia
Ensifer
Mesorhizobium
Neorhizobium
Phyllobacterium
Shinella
Methylobacterium
Microvirga
Cupriavidus
Aminobacter
Ps. fluorescens
B. megaterium
Streptomyces hygroscopicus subsp. hygroscopicus
Cellulosimicrobium terreum
B. pumilus
Botrytis caroliniana
Plectosphaerella cucumerina
Botrytis_caroliniana
Ramularia spp.
Taphrina pruni
Taphrina wiesneri
Podosphaera sp.
Ascochyta rabiei
Botrytis caroliniana
Plectosphaerella cucumerina
Neofusicoccum parvum
Pilidium concavum
Powellomyces hirtus
Scopulariopsis brumptii
Stemphylium vesicarium
Trichothecium crotocinigenum
Ascochyta medicaginicola
Edenia gomezpompae
Leptosphaeria spp.
Glomerella acutata
Plectosphaerella cucumerina
Ramularia eucalypti
Seimatosporium sp.
Stagonosporopsis sp.
Stagonosporopsis spp.
Typhula sp.
Monographella nivalis
Clonostachys rosea
Gibellulopsis chrysanthemi
Glomerella acutata
Leptosphaeria sp.
Neodeightonia phoenicum
Plectosphaerella cucumerina
Rhinocladium lesnei
Gibellulopsis nigrescens
Plectosphaerella sp.
Ramularia sp.
Gibberella tricincta
Phaeoacremonium sp.
Plectosphaerella cucumerina
Devriesia thermodurans
Lepteutypa fuckelii
Clonostachys rosea
Gibberella tricincta
Gibellulopsis nigrescens
Pestalotiopsis sp.
Powellomyces hirtus
Trichothecium roseum
Botrytis caroliniana
Cylindrocladiella pseudoparva
Gibellulopsis chrysanthemi
Gibellulopsis nigrescens
Hortaea werneckii
Mastigosporium album
Meira geulakonigii
Phaeoacremonium spp.
Plectosphaerella cucumerina
Rhexocercosporidium panacis
Rhinocladium lesnei
Stagonosporopsis spp.
Botrytis caroliniana
Ceratocystidaceae sp.
Devriesia sp.
Golovinomyces sordidus
Moesziomyces aphidis
Monographella nivalis
Neofusicoccum parvum
Plectosphaerella cucumerina
Plectosphaerella spp.
Seimatosporium sp.
Stemphylium vesicarium
Ascochyta rabiei
Botrytis caroliniana
Plectosphaerella cucumerina
Podosphaera sp.
Taphrina wiesneri
Botrytis caroliniana
Podosphaera sp.
Ramularia spp.
Ascochyta rabiei
Botrytis caroliniana
Gibberella pulicaris
Gibberella tricincta
Plectosphaerella cucumerina
Ramularia sp.
Stagonosporopsis spp.
Clonostachys rosea
Gjaerumia minor
Plectosphaerella cucumerina
Gibellulopsis nigrescens
Leptosphaeria sp.
Plectosphaerella cucumerina
Rhinocladium lesnei
Gibberella tricincta
Gibellulopsis nigrescens
Leptosphaeria sp.
Phaeoacremonium parasiticum
Plectosphaerella cucumerina
Pyrenophora avenae
Gibellulopsis nigrescens
Phaeoacremonium spp.
Blumeria graminis
Claviceps sp.
Eutypa maura
Melampsora epitea
Monographella nivalis
Mycosphaerella ellipsoidea
Ramichloridium indicum
Ramularia spp.
Rhizosphaera kalkhoffii
Sclerotinia sclerotiorum
Stemphylium vesicarium
Taphrina carpini
Taphrina inositophila
Taphrina mirabilis
Taphrina padi
Taphrina pruni
Ustilago hordei
Gibberella tricincta
Rhizophagus irregularis
Cladosporium sphaerospermum
Hebeloma mesophaeum
Trichoderma virens
Ampelomyces quisqualis
Cystobasidium slooffiae
Phialocephala fortinii
Oidiodendron echinulatum
Phialocephala spp.
Rhizophagus irregularis
Oidiodendron echinulatum
Oidiodendron periconioides
Meliniomyces sp.
Oidiodendron echinulatum
Oidiodendron rhodogenum
Rhizophagus sp.
Rhizophagus irregularis
Oidiodendron echinulatum
Periconia macrospinosa
Rhizophagus irregularis
Oidiodendron echinulatum
Oidiodendron periconioides
Rhizophagus irregularis
Inocybe calospora
Cystobasidium lysinophilum
Tuber sp.
Cladosporium sphaerospermum
Sporobolomyces roseus
Cladosporium sphaerospermum
Cladosporium sphaerospermum
Cystobasidium slooffiae
Meliniomyces sp.
Oidiodendron echinulatum
Oidiodendron griseum
Oidiodendron rhodogenum
Meliniomyces sp.
Phialocephala spp.
Cladosporium sphaerospermum
Meliniomyces sp.
Trichoderma virens
Cosmospora sp.
Meliniomyces sp.
Meliniomyces variabilis
Oidiodendron maius
Oidiodendron pilicola
Phialocephala fortinii
Cladosporium sphaerospermum
Endogone sp.
Meliniomyces sp.
Meliniomyces variabilis
Oidiodendron echinulatum
Oidiodendron griseum
Ampelomyces quisqualis
Cystobasidium pinicola
Cystobasidium slooffiae
Cladosporium sphaerospermum
Leptodontidium trabinellum
Oidiodendron cereale
Oidiodendron echinulatum
Meliniomyces sp.
Oidiodendron echinulatum
Oidiodendron periconioides
Oidiodendron rhodogenum
Phialocephala spp.

Figure 4.3. Genotype specific functional microorganisms. Venn diagram showing (A) Potential Nitrogen fixing genera, (B) Potential Plant Growth Promoting Bacteria and Biological Control Agents species, (C) Potential Fungal pathogens species and (D) Potential Fungal Beneficial species present in each compartment (B = bulk soil; RH = rhizosphere; R = Roots, AGO = Above-ground organs) of the three strawberry cultivars. Intersections indicate microbes simultaneously present in more than one organ are also presented (genera abbreviations *Ps.* = *Pseudomonas*; *B.* = *Bacillus*).

Nitrogen (N) is one of the essential nutrients for ensuring plant growth and productivity. In agriculture it might become a limiting factor and therefore external application is generally needed. N-fixing microorganisms able to convert the unavailable atmospheric N_2 into an accessible form belong to Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Firmicutes and Cyanobacteria phyla (Pathania et al., 2020). *nifH* is a highly conserved gene, generally used as marker for nitrogen fixation processes in natural habitats. In our work, PCR analysis assessed the presence of *nifH* gene in bulk soil, rhizosphere and root samples of the three strawberry genotypes.

Besides investigating bacterial microbiome potential functionalities using FAPROTAX and databases research, biochemical functions were analyzed using Tax4fun (Aßhauer et al., 2015). Tax4Fun allows the prediction of functional traits comparing bacterial 16S rRNA genes with information available on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. According to Two-way PERMANOVA, the compartments had a significant role in shaping the functionalities expressed by the microbiome (Table 4.3). Modules and KOs related to PGP traits were strongly linked both to the organ and the genotype. Altogether, 26 modules and KOs were found to be significantly different (Table 4.4). Interestingly, we found that in above-ground organs of 'Monterey', genes linked to jasmonic acid (JA) biosynthesis, isoprenoid biosynthesis and H_2S production were significantly more abundant. On the other hand, potential beneficial traits associated to below-ground organs were homogeneously found across the three genotypes.

Table 4.3. Effect of genotype and soil/plant compartment on overall KEGG modules and KO and Plant Growth Promoting KEGG modules and KO composition throughout strawberry microbiome, according to Two-way PERMANOVA analysis, is shown. Significant *P* values are highlighted in bold.

Feature/Factors	Two-way PERMANOVA	
	Pseudo F	*P*
KEGG Modules		
Organ	22.46	**0.0001**
Cultivar	1.76	0.12
Organ x Cultivar	1.43	0.15
KEGG KO		
Organ	39.007	**0.0001**
Cultivar	**1.95**	**0.1111**
Organ x Cultivar	1.6	0.1088
PGPB Kegg Modules		
Organ	18.74	**0.0001**
Cultivar	2.09	**0.0564**
Organ x Cultivar	1.42	0.1182
PGPB Kegg KO		
Organ	24.04	**0.0001**
Cultivar	2.72	**0.0361**
Organ x Cultivar	1.39	0.1904

Table 4.4. Significantly different Plant Growth Promoting KEGG modules and KOs in soil/plant compartments are shown. Data are expressed as mean ± SE. Different letters indicate significant differences between genotypes according to One-way ANOVA followed by multiple comparisons by Tukey's test.

Organ	KEGG PGP MODULE or GENE	Monterey	Elsanta	Darselect
BULK SOIL	Pectin degradation	42.25 ± 0.94 b	67 ± 5.93 a	34 ± 2.20 c
	Polyamine biosynthesis, arginine => agmatine => putrescine => spermidine	1315.75 ± 27.67 ab	1389.25 ± 36.81 a	1199.75 ± 33.61 b
	C10-C20 isoprenoid biosynthesis, bacteria	650.5 ±11.13 b	704 ±7.22 a	643.75 ± 7.92 b
	C10-C20 isoprenoid biosynthesis, archaea	215.5 ± 5.5 b	277.5 ± 15.22a	211 ± 10.98 b
	Dissimilatory sulfate reduction, sulfate => H2S	434 ± 10.36 b	506 ± 18.41 a	442.75 ± 12.26 b
	1-aminocyclopropane-1-carboxylate deaminase	42 ± 2.97 b	56 ± 2.55 a	42.75 ± 1.31 b

	Pyocyanine biosynthesis, chorismate => pyocyanine	5.75 ± 0.63 b	19.25 ± 2.59 a	8,75 ± 2.02 b
	Abscisic acid biosynthesis, beta-carotene => abscisic acid	3.5 ± 0.29 b	5.5 ± 0.29 a	2.75 ± 0.25 b
RHIZOSPHERE	Assimilatory sulfate reduction, sulfate => H2S	2578.5 ± 26.43 c	2847.75 ± 54.76 a	2774.5 ± 45.10 b
	Betaine biosynthesis, choline => betaine	1265.5 ± 15.78 b	1486 ± 77.35 a	1429.5 ± 40.70 ab
	Butanoate metabolism	4654.5 ± 26.36 b	5321 ± 217.89 a	5044.25 ± 181.02 ab
ROOT	GABA (gamma-Aminobutyrate) shunt	1911.5 ± 45.42 ab	2184.25 ± 92.87 a	1833.75 ± 65.80 b
	C5 isoprenoid biosynthesis, non-mevalonate pathway	3498.75 ± 31.54 ab	3597.25 ± 83.17 a	3277.25 ± 66.64 b
	Ascorbate biosynthesis, plants, fructose-6P => ascorbate	2918.75 ± 20.30 b	2970.25 ± 41.62 a	2717 ± 72.87 c
	Ascorbate biosynthesis, animals, glucose-1P => ascorbate	1523.75 ± 5.65 ab	1580.25 ± 5.12 a	1477.25 ± 40.07 b
	Polyamine biosynthesis, arginine => agmatine => putrescine => spermidine	1231.25 ± 39.21 b	1044.5 ± 23.36 c	1287.75 ± 57.05 a
	GABA biosynthesis, eukaryotes, putrescine => GABA	2914 ± 36.25 ab	3086.75 ± 129.51 a	2541 ± 178.62 b
	Methanogenesis, methanol => methane	150.5 ± 18.19 a	72.75 ± 20.00 b	130.25 ± 15.43 ab
	Betaine biosynthesis, choline => betaine	1458 ± 40.38 ab	1599.25 ± 49.18 a	1285.25 ± 48.17 b
	methionine-gamma-lyase	277.25 ± 13.47 a	252.75 ± 10.94 a	190 ± 13.23 b
	IAA	1006.75 ± 15.13 ab	1059.25 ± 60.20 a	827.25 ± 56.85 b
	salycilic acid	391.5 ± 10.32 b	458.5 ± 13.11 a	319.5 ± 15.60 c
ABOVE-GROUND ORGANS	Jasmonic acid biosynthesis	710.25 ± 45.82 a	552 ± 44.68 b	624.5 ± 23.90 ab
	C10-C20 isoprenoid biosynthesis, non-plant eukaryotes	130.75 ± 8.08 a	99.75 ± 9.09 b	116.5 ± 5.63 ab
	Purine degradation, xanthine => urea	1378.5 ± 104.68 b	1775 ± 81.18 a	1595 ± 98.93 ab
	Dissimilatory sulfate reduction, sulfate => H2S	764.5 ± 22.26 a	669.75 ± 15.14 b	710.75 ± 30.13 ab
Organ	KEGG PGP MODULE or GENE	Monterey	Elsanta	Darselect
BULK SOIL	Pectin degradation	42.25 ± 0.94 b	67 ± 5.93 a	34 ± 2.20 c
	Polyamine biosynthesis, arginine => agmatine => putrescine => spermidine	1315.75 ± 27.67 ab	1389.25 ± 36.81 a	1199.75 ± 33.61 b
	C10-C20 isoprenoid biosynthesis, bacteria	650.5 ±11.13 b	704 ±7.22 a	643.75 ± 7.92 b
	C10-C20 isoprenoid biosynthesis, archaea	215.5 ± 5.5 b	277.5 ± 15.22a	211 ± 10.98 b
	Dissimilatory sulfate reduction, sulfate => H2S	434 ± 10.36 b	506 ± 18.41 a	442.75 ± 12.26 b
	1-aminocyclopropane-1-carboxylate deaminase	42 ± 2.97 b	56 ± 2.55 a	42.75 ± 1.31 b

	Pyocyanine biosynthesis, chorismate => pyocyanine	5.75 ± 0.63 b	19.25 ± 2.59 a	8,75 ± 2.02 b
	Abscisic acid biosynthesis, beta-carotene => abscisic acid	3.5 ± 0.29 b	5.5 ± 0.29 a	2.75 ± 0.25 b
RHIZOSPHERE	Assimilatory sulfate reduction, sulfate => H2S	2578.5 ± 26.43 c	2847.75 ± 54.76 a	2774.5 ± 45.10 b
	Betaine biosynthesis, choline => betaine	1265.5 ± 15.78 b	1486 ± 77.35 a	1429.5 ± 40.70 ab
	Butanoate metabolism	4654.5 ± 26.36 b	5321 ± 217.89 a	5044.25 ± 181.02 ab
ROOT	GABA (gamma-Aminobutyrate) shunt	1911.5 ± 45.42 ab	2184.25 ± 92.87 a	1833.75 ± 65.80 b
	C5 isoprenoid biosynthesis, non-mevalonate pathway	3498.75 ± 31.54 ab	3597.25 ± 83.17 a	3277.25 ± 66.64 b
	Ascorbate biosynthesis, plants, fructose-6P => ascorbate	2918.75 ± 20.30 b	2970.25 ± 41.62 a	2717 ± 72.87 c
	Ascorbate biosynthesis, animals, glucose-1P => ascorbate	1523.75 ± 5.65 ab	1580.25 ± 5.12 a	1477.25 ± 40.07 b
	Polyamine biosynthesis, arginine => agmatine => putrescine => spermidine	1231.25 ± 39.21 b	1044.5 ± 23.36 c	1287.75 ± 57.05 a
	GABA biosynthesis, eukaryotes, putrescine => GABA	2914 ± 36.25 ab	3086.75 ± 129.51 a	2541 ± 178.62 b
	Methanogenesis, methanol => methane	150.5 ± 18.19 a	72.75 ± 20.00 b	130.25 ± 15.43 ab
	Betaine biosynthesis, choline => betaine	1458 ± 40.38 ab	1599.25 ± 49.18 a	1285.25 ± 48.17 b
	methionine-gamma-lyase	277.25 ± 13.47 a	252.75 ± 10.94 a	190 ± 13.23 b
	IAA	1006.75 ± 15.13 ab	1059.25 ± 60.20 a	827.25 ± 56.85 b
	salycilic acid	391.5 ± 10.32 b	458.5 ± 13.11 a	319.5 ± 15.60 c
ABOVE-GROUND ORGANS	Jasmonic acid biosynthesis	710.25 ± 45.82 a	552 ± 44.68 b	624.5 ± 23.90 ab
	C10-C20 isoprenoid biosynthesis, non-plant eukaryotes	130.75 ± 8.08 a	99.75 ± 9.09 b	116.5 ± 5.63 ab
	Purine degradation, xanthine => urea	1378.5 ± 104.68 b	1775 ± 81.18 a	1595 ± 98.93 ab
	Dissimilatory sulfate reduction, sulfate => H2S	764.5 ± 22.26 a	669.75 ± 15.14 b	710.75 ± 30.13 ab

Using the predictive tool FUNGuild, fungi were divided into beneficial or pathogenic (Table S4.8). As for bacteria, beneficial fungal community showed to be strongly correlated to both genotype ($F = 2.05$, $P = 0.004$) and compartment ($F = 4.05$, $P = 0.001$) (PERMANOVA values genotype × compartment $F = 1.33$, $P = 0.033$; Figure S4.3b; Table 4.2; Table S4.5). *Cladosporum sphaerospermum* was the unique taxa common to above-ground and rhizosphere of the three genotypes (Figure 4.2). While most of the potentially beneficial fungal groups are similarly represented in the three strawberry genotypes, the arbuscular mycorrhizae *Rhizophagus irregularis* showed a high

frequency only in 'Monterey', while being completely absent in 'Elsanta' and 'Darselect' (Figure 4.3d). In addition to the beneficial fungi recognized with FUNGuild, we highlighted some species previously documented as beneficial to plants (Table S4.9).

Both genotype ($F = 3.34$, $P = 0.001$) and plant compartment ($F = 3.90$, $P = 0.001$), as well as their interaction (PERMANOVA values genotype × compartment $F = 1.58$; $P = 0.002$; Fig 4.3c; Figure 4.4b; Table 4.2; Table S4.5) play a key role in the abundance of pathogens in the fungal community associated to strawberry. Regarding commonalities of fungal pathogens in the three strawberry cultivars in different organs, Powellomycetaceae and Taphrinaceae were unique of bulk soil and above-ground organs, respectively (Figure 4.2) whereas *Podosphaera* spp. was present in both below- and above-ground compartments of the three genotypes (Figure 4.3c).

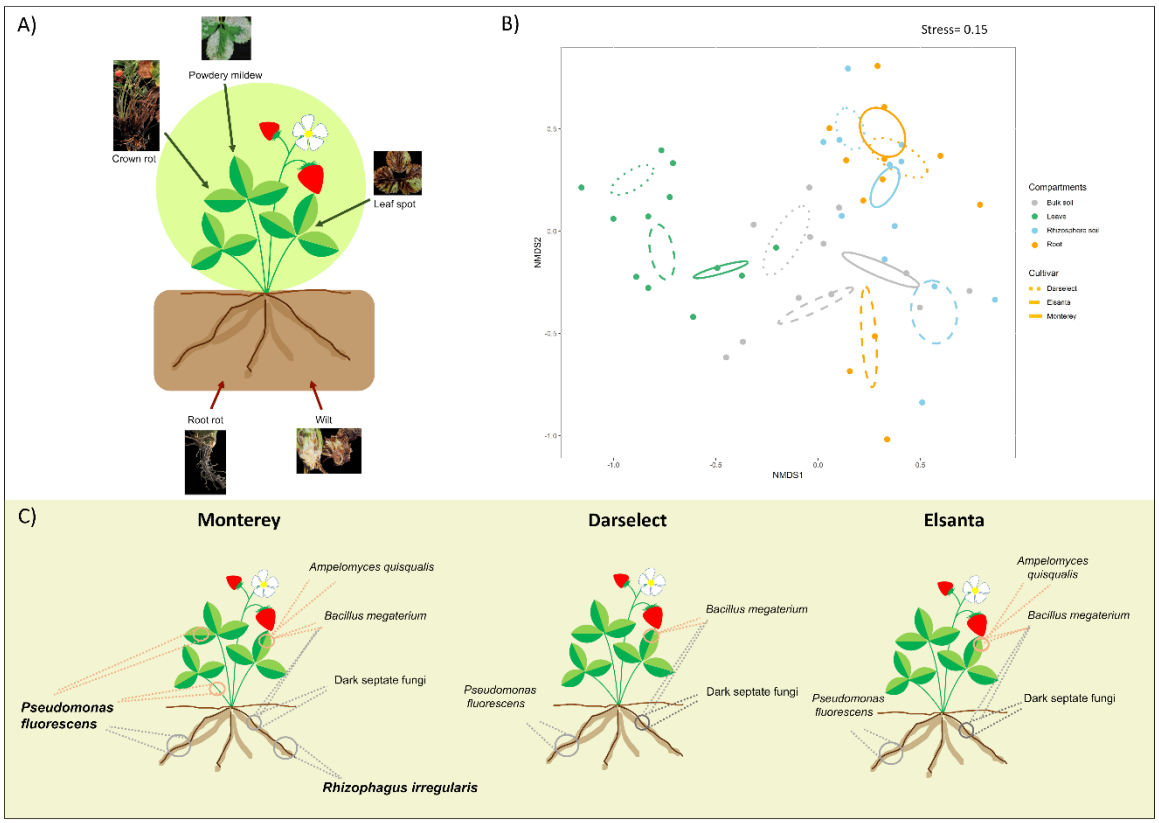


Figure 4.4. Different strawberry cultivars show unique microbial populations to cope with plant diseases. (A) Fungal and bacterial pathogens affecting strawberry plant cultivation. (B) Nonmetric multidimensional scaling (NMDS) plot showing fungal pathogens composition similarity among different soil and plant compartments and genotypes. (C) Bacterial and fungal beneficial species colonizing the different strawberry genotypes.

4.3.4 Isolation and functional characterization of bacteria isolates

Viable bacterial counts were on average 5.02×10^7, 4.65×10^8, 4.29×10^7, 1.12×10^8 CFU/ml for leaves, rhizosphere, root and soil, respectively (Figure S4.5). No significant differences were found among CFU/ml detected on the same organ of different genotypes. A total of 70 colonies were selected from the highest countable dilution.

'Monterey' is the genotype showing the highest diversity, and ten genera were uniquely isolated from this variety. One and three genera were unique for 'Elsanta' and 'Darselect', respectively. *Pseudomonas* and *Vagococcus* genera were common to the three genotypes (Table S4.10), being the first one isolated only from below-ground compartments. 43 isolates were tested *in vitro* for their plant growth promoting activities. PGP traits were qualitatively screened (0 = no activity, 3 = highest activity). Bacteria isolated from 'Monterey' showed the highest plant-growth promoting potentiality both in the above- and below-ground organs (Table 4.5). Interestingly, in above-ground organs, 'Monterey' showed the highest number of indoleacetic acid (IAA)- and NH_4^+-producing bacteria, as well as more *Xanthomonas fragariae* antagonists when compared to 'Elsanta' and 'Darselect'. ACC deaminase producing bacteria were not found in above-ground organs. Compared to above-ground organs, plant-growth traits are far more homogenously spread in below-ground organs across the three cultivars.

Table 4.5. Plant Growth Promoting *in vitro* activities of bacteria isolated either in above-ground or below-ground organs of the three strawberry genotypes were characterized and are shown here. Plant Growth Promoting traits were qualitatively screened (0 = no activity, 3 = highest activity) for each isolate. For each trait, the indicated values represent the sum of activity levels of single isolates from each genotype.

		Monterey	*Elsanta*	*Darselect*
Above-Ground Organs	Acetoin Production	2	0	1
	IAA production	6	0	0
	Siderophores	0	0	0
	Botrytis cinerea antagonism	0	0	1
	Xanthomonas fragariae antagonism	5	0	2
	NH4+ production	3	1	0
	ACC deaminase activity	0	0	0
Below-Ground Organs	Acetoin Production	6	2	6
	IAA production	0	3	3

	Siderophores	6	3	4
	Botrytis cinerea antagonism	2	0	0
	Xanthomonas fragariae antagonism	6	1	2
	NH4+ production	5	7	2
	ACC deaminase activity	0	1	4

4.3.5 Effects of strawberry microbiome on plant mineral composition and fruit quality

In this work, the contribution of bacterial and fungal microbiomes to the plant mineral composition (Figure 4.5a) and fruit quality (Figure 4.5b) was investigated. Significant correlations were observed between the mineral composition of the plant organs and the microbial community assemblage across different soil and plant compartments (Figure 4.5a). In particular, the fungal microbiome colonizing the root of strawberry has been found to be strongly determined by the genotype and to be associated to the availability of B, Sr and N. On the other hand, fungal community associated to above-ground organs was not correlated to plant mineral composition. In general, compared to the fungal microbiome, the bacterial community was associated to the mineral elements to a smaller extend. However, below-ground bacterial microbiomes strongly correlate with Sr and Ca. In addition, microbes, and particularly those associated with soil and roots, contributed substantially, although indirectly, to sensorial fruit quality (Table S4.11). In details, the fungal microbiome associated to the bulk soil and rhizosphere contributed the most to the development of SSC and TA of fruits (Figure 4.5b). Contrarily, rhizopheric and above-ground bacterial microbiome played a similar role in SSC of fruits whereas TA was related only to bacterial bulk soil microbiome.

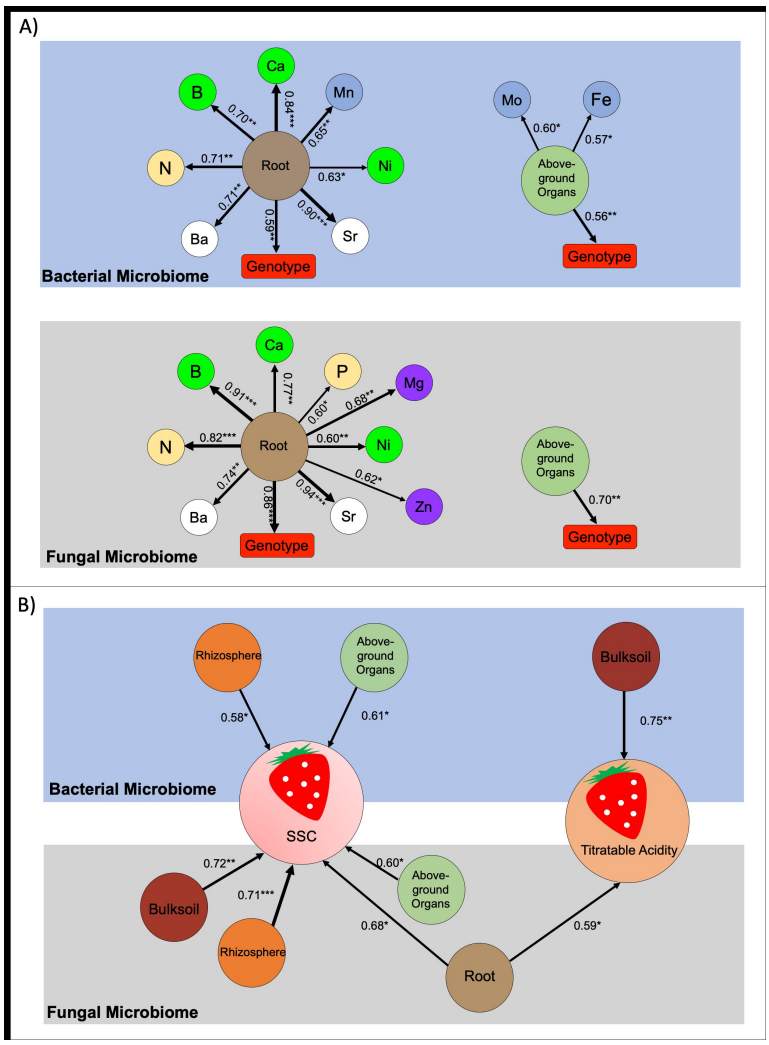


Figure 4.5. Interactions between strawberry microbiome, plant mineral composition and effect of microbiome on fruit quality. (A) Correlations between bacterial and fungal microbiomes colonizing roots and above-ground compartments with genotype and mineral nutrient content of the respective organ. Nutrients are indicated as essential macro-nutrients (yellow), universally essential cations (purple), elements playing an important ecological role both for plants and microbes (light blue), elements with specialized purposes in microbes (green), micro-nutrients (white); (B) Correlation between bacterial and fungal microbiomes colonizing different plant compartments and total soluble solid content (SSC) and titratable acidity of fruits. Arrows thickness indicate strength of the correlation, R^2 and p-values (* = 0.05; ** = 0.01; * = 0.001) are shown.**

4.4 Discussion

4.4.1 Microbiome composition

In agreement with previous studies (De Souza et al., 2016; Cregger et al., 2018), above-ground organs displayed a lower bacterial and fungal alpha-diversity than below-ground ones. Such diversity in microbial community composition (Figure 4.1a; Figure S4.1a) and richness could be explained by the differences in the physical and chemical properties of the two environments. In fact, above-ground organs are subjected to oligotrophic and unstable conditions, with daily and seasonal fluctuations in temperature, humidity and UV light (Lindow and Brandl, 2003; Redford and Fierer, 2009), whereas the soil compartment is relatively more protected, stable and nutrient-rich. Despite the influence of plant genotype in the assembly of the cultivar-specific microbiota, we report the existence of a core microbiome, common to the three strawberry plant genotypes (Figure 4.2). The identification of ubiquitous microbes suggests that they are either able to colonize all soil and plant compartments, or they can move across soil and the plant organs with a passive or active translocation from roots to the above-ground organs (e.g. leaves, runners).

Interestingly, several strawberry pathogens colonized the plants (Figure 4.3c), and some of them were found among the core fungal microbiome, namely *Plectosphaerella cucumerina* (fruit, root and collar rot), *Botrytis caroliniana* (gray mold) and *Alternaria alternata* (black leaf spot) (Table S4.7), although no evident disease symptoms were observed. The pathobiome concept refers to the fact that pathogens are integrated in a complex biotic environment where they interact with the plant microbial community (Vayssier-Taussat et al., 2014). It may be speculated that the overall composition of the microbiome, as well as antagonistic and mutualistic interactions occurring within microbes, contribute to the control of such pathogens population, making the mere organ colonization not sufficient, *per se*, for a successful infection causing disease symptoms (Donati et al., 2020). Altogether, our observations suggest that abiotic (e.g. temperature, humidity, nutrient availability) (Pandey and Kumar, 2019) and biotic factors (e.g. plant-associated microbial consortia, plant resistance) (Amsalem et al., 2006; Zhang et al., 2013; Ab Rahman et al., 2018) may have contributed in determining the fate of plant-pathogen interactions.

4.4.2 Plant growth promoting functions of the microbiota and their contribution to plant phenotype

Domestication of crop plants has been suggested to have determined a reduction in the biodiversity of the associated microflora, in particular for functions regarding nutrition and stress tolerance (Pérez-Jaramillo et al., 2016). On the other hand, it is also possible that cultivated plants recruit microbes specifically exerting beneficial functions under cultural conditions. In this view, the ability to interact with such microbes may be regarded as a trait selected by domestication (Pérez-Jaramillo et al., 2018). In this work, we found that, even after centuries of domestication and complex hybridization (Edger et al., 2019; Bertioli, 2019), cultivated strawberry plants are associated with 16 nitrogen fixing bacterial genera (Figure 3a), which is more than what reported in wild strawberry plants relatives (*F. chiloensis*, *F. virginiana* ssp. *platypetala*, *F.* × *ananassa* ssp. *cuneifolia*) (7 genera) (Wei and Ashman, 2018) and comparable to the number of nitrogen-fixing genera (18) able to establish a nitrogen fixing symbiosis with legumes (Table S4.12). Although bacterial taxa known to have N-fixing potential were surprisingly found in the above-ground habitat (Figure 4.3a), we did not detect any *nifH* gene in this compartment. Considering that nitrogenase is inactivated by oxygen (Gallon, 1981), the ability of these bacteria to interact with plant hosts is not necessarily related to their ability to fix nitrogen.

Tax4Fun predictive functional analysis of the bacterial microbiome showed that both the plant genotype and the organ significantly contributed to the overall assembly of bacteriome metabolic functionalities (Table 4.3), which is in accordance to previous findings (Chamam et al., 2013; Zipfel, 2017).

Modules and KOs specifically related to PGP traits were also investigated and the organ was found to significantly contribute to the assembly of metabolic functionalities of the microbiota. Metabolites or signals differently emitted by plant organs may explain organ colonization by microorganisms potentially delivering different functions (Zipfel, 2017).

Additionally, both Tax4fun predictive analysis and *in vitro* screening of PGP traits showed that several PGP functions were observed to be different among cultivars (Table 4.4).

However, in the case of below-ground organs, differences in PGP traits of the three genotypes were less marked. Similarly, studies about functional microbiomes of grapevine rootstocks of different genotypes, showed that the potential ecological

services were maintained across below-ground organs of different genotypes (Marasco et al., 2018).

PGP functions might result in the production of specific metabolites, signals or activation of pathogen defense mechanisms that may explain differences observed in the phenotype of different strawberry cultivars. ACC deaminase producing bacteria have not been isolated from 'Monterey'. On the other hand, 'Elsanta' and 'Darselect' hosted ACC deaminase producing bacteria. Thus, it might be the case that, being these two genotypes more susceptible than 'Monterey', they played out an active recruitment of these interesting plant growth promoting bacteria. Indeed, this enzyme cleaves the ethylene precursor (ACC) into ammonia and alpha-ketobutyrate, lowering plant ethylene levels, which when present at high concentration can have severe negative effects on plant growth (Glick, 2014).

'Elsanta' plants showed a significantly heavier root and leaf apparatuses, which might relate to functions linked to IAA and betaine production expressed by below-ground microflora. Indeed, root growth can be simulated through secretion of IAA by a variety of microbial species, including plant growth-promoting, stress tolerance-inducing, as well as pathogenic ones (Spaepen et al., 2007). Betaine is synthesized by plants in response to several environmental stresses, such as drought, salinity and low temperatures (Ashraf et al., 2007; Rajashekar et al., 1999), but betaine-producing bacteria (including *B. subtilis* and *Arthtobacter globiformis* (Zou et al., 2016) may also contribute to plant stress tolerance.

Our work also revealed the vast diversity of fungal partners of strawberry, which have not been thoroughly investigated so far, and include ectomycorrhizae, arbuscular mycorrhizae, ericoid mycorrhizae, endophytes, dark septate endophytes and mycoparasites (Figures 4.2 and 4.3c,d).

4.4.3 Effect of the microbiota on disease resistance

The environmental factors, soil conditions and pool of natural microbial inoculum are assumed to be comparable for all three strawberry genotypes, as plants were grown in the same cultural and environmental conditions. Therefore, the observed differences in associated bacterial and fungal communities (Figures 4.2, 4.3) can be explained with the ability of the plant to adjust the composition of the associated microflora (Morella, 2019). In this view, the lower susceptibility to powdery mildew and leaf spot observed in 'Monterey' over the season (Table S4.2), which is in

agreement with existing literature (Masny et al., 2016), may be at least partly due to its ability to establish exclusive beneficial microbial relationships (Figure 4.4a,c). At the same time, 'Elsanta' showed to be less sensitive than 'Darselect' to leaf spot, but highly susceptible to powdery mildew (Bestfleisch et al., 2015 ab). Finding unique beneficial microbial patterns for a genotype that showed to be more tolerant than others to biotic stresses suggests an important contribution of the microbiota in the defense strategy of strawberry plants.

Indeed, 'Monterey' was characterized by the presence of *Rhizophagus irregularis* and above-ground *Ps. fluorescens* populations (Figure 4.4). In several crop plants, the colonization of the root systems by *R. irregularis* has been demonstrated to confer plant resistance to broad-spectrum of pathogens by induced systemic resistance (ISR) and mycorrhizal-induced resistance (MIR) (Cameron et al., 2013; Velivelli et al., 2015). Many *Ps. fluorescens* strains promote plant growth or protection, by mechanisms such as phosphorus solubilization, phytohormone production, competition against phytopathogens, elicitation of ISR, or production of antimicrobial compounds, such as cyanide or phenolics (Bakker et al., 2007; David Baliah et al., 2018). *Ps. fluorescens* strains isolated from raspberry fruit produce high levels of siderophores, which provide a competitive advantage against plant pathogens (Perpetuini et al., 2019). Furthermore, the same strains showed a strong ACC-deaminase activity that may regulate plant ethylene and, in turn, influence induced systemic resistance. Non-indigenous *Ps. fluorescens* strains have been already applied to strawberry plants, allowing to anticipate flowering and fruiting, increase fruit yield and vitamin content (Bona et al., 2015), and to control crown rot (*Phytophtora cactorum*) (Agusti et al., 2011). Notably, the inoculation of rice seed with a *Ps. fluorescens* strain for riceblast control resulted in the colonization of roots, stems and leaves (Vidhyasekaran et al., 1997), supporting that this species does not have strict organ preferences. In this work, three different *Ps. fluorescens* strains were isolated from the three strawberry genotypes. Remarkably, only the strain isolated from 'Monterey' showed to have *in vitro* antagonistic activity against the pathogen *Xanthomonas fragariae* (Table S4.10).

Interestingly, the combined action of *Pseudomonas* spp. and *Rhizophagus* spp. has been explored in several crop species (Velivelli et al., 2015; Bahmani et al., 2018; Ma et al., 2019). In particular, a mixture of arbuscular mycorrhizae, which included *Rhizophagus* spp., and *Pseudomonas fluorescens* was successfully applied to strawberry, resulting in increased fruit production and quality (Bona et al., 2015). The combination of *Rhizophagus* spp. and *Ps. fluorescens* has been proven to elicit plant systemic defense

system in tomato via the activation of ethylene response to pathogen attack (Velivelli et al., 2015).

B. megaterium was the only OTU able to ubiquitously colonize 'Elsanta' and 'Darselect'. This bacterium has attracted considerable attention as a functional microbe in several crop species, including strawberry, since it is able to solubilize phosphate and produce phytohormones (Dias et al., 2009). Furthermore, it has been proven to be effective for the control of *B. cinerea* (Donmez et al., 2011).

In above-ground organs, the KEGG modules responsible for jasmonic acid, isoprenoid biosynthesis and H_2S formation were higher in 'Monterey' than in the other genotypes. Jasmonic acid and its derivatives are responsible for many essential processes involved in plant growth and development, such as the immune response against necrotrophic pathogens and herbivorous insects (Ali and Baek, 2020) and regulation of ISR (Pieterse et al., 2014). The production of JA from plant-associated bacteria is scarcely documented (Piccoli et al., 2011), although oxylipins have been reported to act as quorum sensing messengers in bacterial communication (Martínez et al., 2019). Volatile isoprenoids and H_2S may exert a direct antimicrobial action, and/or enhance plant defenses against pests and pathogens (Abbas et al., 2017).

A further indication of the role of the microbiota in disease protection of 'Monterey' is offered by the high number of native *X. fragariae* antagonists isolated from this cultivar, in agreement with its low susceptibility to leaf spot disease.

For these reasons, disease tolerance in 'Monterey' may be partially explained by a contribution of phyllospheric microflora in plant signalling and biochemical functions, and by the plant's ability to recruit microbiota components with a protective action.

4.4.4 Interactions between strawberry microbiome, plant mineral composition and fruit production and quality

Bacterial and fungal microbiomes were correlated to plant mineral composition (Figure 4.5a). Indeed, plant associated microbiomes play a key role in improving plant nutrition by promoting both nutrient acquisition and nutrient use efficiency (Trivedi et al., 2020). On the other hand, the host plant and its nutrient preferences impact its microbiome recruitment (Mendes et al., 2017). Lambert et al., 1980 observed a strong relationship between adequate fertility of soils and a proper mycorrhizal activity in red clover, pointing to an interconnection similar to that found in this work between the fungal microbiome colonizing the below-ground compartments of strawberry and

B content in the roots. Additionally, root Sr significantly correlated with fungi colonizing the below-ground compartment which might be related to the presence of specific taxa. Indeed, Ramirez-Flores et al., 2019 observed an increase in root mineral content, including Sr, after root inoculation with *R. irregularis*. In order to access nutrients, plants are dependent on soil microbes able to depolymerize and mineralize organic nutrients which might explain the relationship between below-ground fungi and mineral nutrient content of roots (including N) observed in this work. Besides fungi, bacteria strongly correlate with root mineral content, in particular with Sr and Ca. Recent studies observe an increase in Ca content following bacterial inoculation (Karlidag et al., 2007; Devarajan et al., 2021), confirming an involvement of bacteria in plant nutrition.

Significant correlations between fruit total soluble solid content and titratable acidity and bacterial and fungal microbiomes emerged from this work (Figure 4.5b), suggesting a role of plant-associated microflora in fruit development. Such a role has been previously observed in strawberry (Todeschini et al., 2018) as well as in other species, e.g. for *Bacillus* spp. on flowers and leaves of sour cherry (Arikan and Prilak, 2016), and is not limited to interactions with above-ground organs. In fact, fruit development and ripening are finely regulated by phytohormones, in particular by ethylene, auxin and gibberellins that can be produced by both fungi and bacteria. Ethylene is produced by a wide range of microbes starting from two alternative precursors, 2-keto-4-methyl-thiobutyric acid (KMBA) or 2-oxoglutarate (Nagahama et al., 1992; Hausinger, 2004). Furthermore, several bacterial species present the 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity. ACC deaminase degrades the ethylene precursor, thus, impairing its production in the plant tissues (Glick, 2014). Several of the bacterial genera found in our study (i.e. *Methylobacterium* sp., *Pantoea* sp., *Erwinia* sp., *Pseudomonas* sp.) have been found to exert ACC deaminase activity on strawberry (this work) and other berry fruits (Perpetuini et al., 2019).

A high plant productivity and fruit weight, as observed in 'Monterey', may be partially explained by hormonal and signalling effects of the associated microflora (Sangiorgio et al., 2021). Indeed, this cultivar was rich in OTUs predicted to produce JA and isoprenoids (Table 4), as well as in IAA-producing isolates (Table 4.5). In strawberry, JA and derived compounds stimulate development and ripening of fruits (Han et al., 2019). In addition to volatile compounds, responsible for fruit aroma, the isoprenoid family includes abscisic acid, brassinosteroids, cytokinins, gibberellins, phytoecdysteroids and strigolactones, all with key roles in plant growth regulation

(Tarkowská and Strnad, 2018). IAA regulates fruit size and ripening at early stages of fruit development (Symons et al., 2012). The relative importance of these signals, and of the microbes that release them, on fruit production and quality remains to be assessed.

Arbuscular mycorrhizae have been proven to affect plant hormonal balance and metabolism. Indeed, their beneficial effect has been observed both in below- and above-ground organs (Bona et al., 2015). Besides arbuscular mycorrhizae, PGPB are also able to affect fruit quality, mainly by modulating the interplay between ethylene and auxin metabolisms and providing essential nutrients (Perpetuini et al., 2019; Sangiorgio et al., 2020). Altogether, these correlations suggest that bacteria and fungi can contribute to the host's adaptation to growing conditions and, consequently, to fruit development.

4.5 Conclusions

Together with the characterization of the taxonomical composition, the prediction of plant microbiota functional properties facilitate the study and exploitation of metabolic potentialities of the microbiome (Aßhauer et al., 2015; Lemanceau et al., 2017) . Thus, several aspects, such as the plant performance, resistance and resilience to stresses might be influenced by functional, rather than taxonomic diversity (Johnson and Pomati, 2020).

Although it may be difficult to dissect the plant properties uniquely deriving from its genotype, the contribution of its microflora, and the interactive effects of plant genotype with the specifically associated microbiota, it is important to note that the culture-dependent, biochemical characterization of the bacterial isolates led to results similar to those obtained by *in silico* prediction of metabolic functionalities (viz. a culture-independent approach). These data offer two independent lines of confirmation of phenotypic observations, thus strengthening our results.

In conclusion, this work highlighted the interaction of cultivated strawberry genotypes with a variety of microbial species. Such interactions are specific to genotypes and compartments. These microbiomes play a key role in the plant ability to cope with biotic stress and in modulating fruit quality. Our findings suggest that a comprehensive picture of plant holobiome is needed in order to shed light on the influence of microbial communities and key microbes on plant phenotype and performances. Further studies on microbiomes of crop plants can contribute to the

advancement of plant production science, by providing a deeper insight in the interactions between crops and the microflora and evidencing applicative tools and strategies for an efficient and environmentally sustainable horticultural practice. However, the complexity and specificity of the patterns described in this work suggests that the idea to replace agro-chemicals by a few universal beneficial microorganisms is not realistic. Therefore, breeding programs should aim at the selection of high quality, climate-change resilient horticultural varieties with remarkable capacity to establish symbiotic relationships with useful microorganisms (Sangiorgio et al., 2019). The inclusion of microbial markers in marker-assisted selection will represent a paradigm shift in plant breeding.

Availability of data and materials: The datasets Illumina sequencing of all bacterial and fungal datasets generated during the current study are available in The National Center for Biotechnology Information (NCBI) database under BioProject: PRJNA556362 (https://dataview.ncbi.nlm.nih.gov/object/PRJNA556362?reviewer=4d8dskbpvkqqcci8o1inim36b).

4.6 Supplementary Material

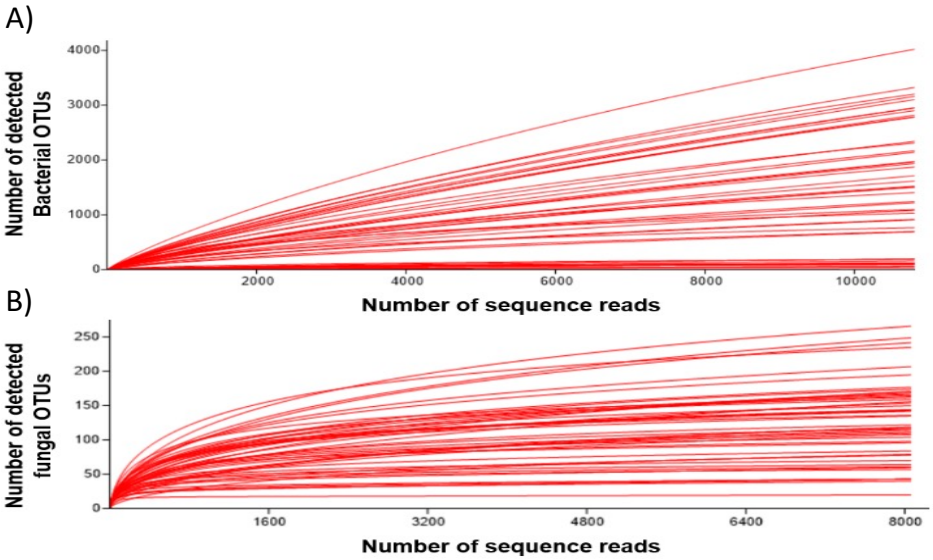


Fig. S4.1 Rarefaction curves for (A) bacteria and (B) fungi are shown.

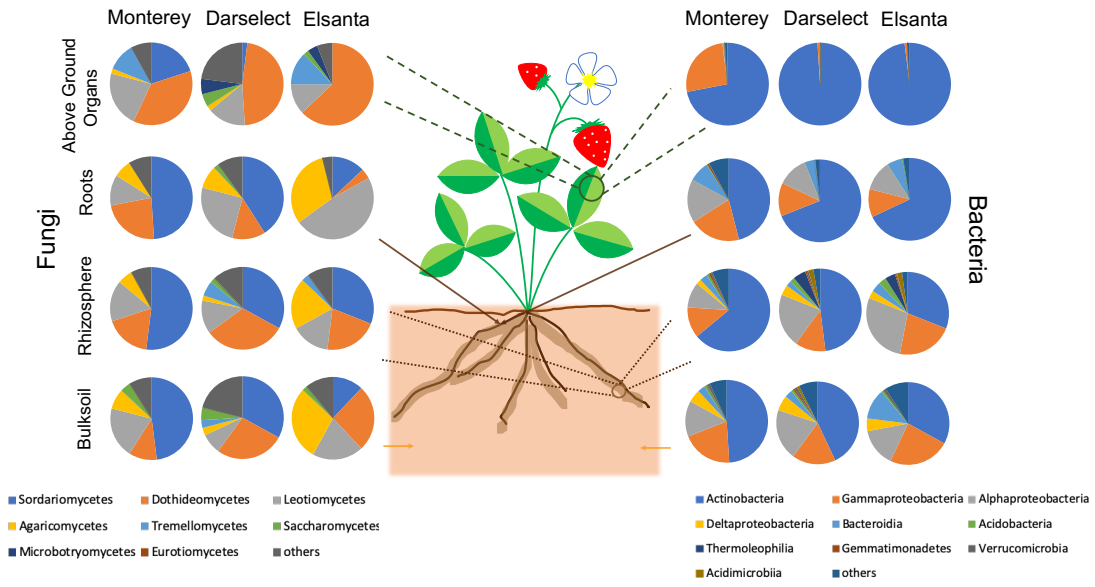


Fig. S4.2 Overall community composition based on relative-abundance data

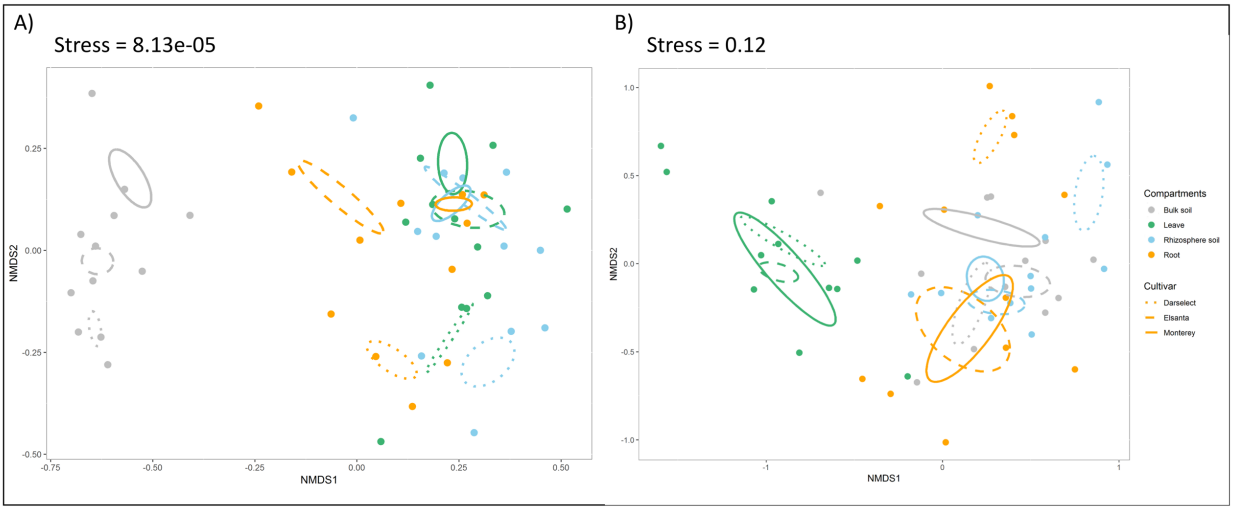


Fig. S4.3 Nonmetric multidimensional scaling (NMDS) plot showing beneficial community composition similarity of beneficial bacteria (A) and fungi (B) among different soil and plant compartments and genotypes.

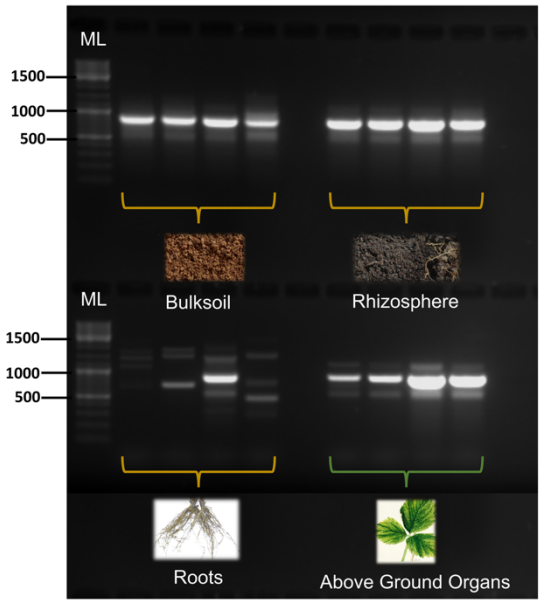


Fig. S4.4 Gel electrophoresis of PCR amplified products using species-specific primers for *Pseudomonas fluorescens*. Colonization of Monterey genotype has been investigated in bulk soil, rhizosphere, internal tissue of roots and above ground plant compartments; 4 replicates for each organ.

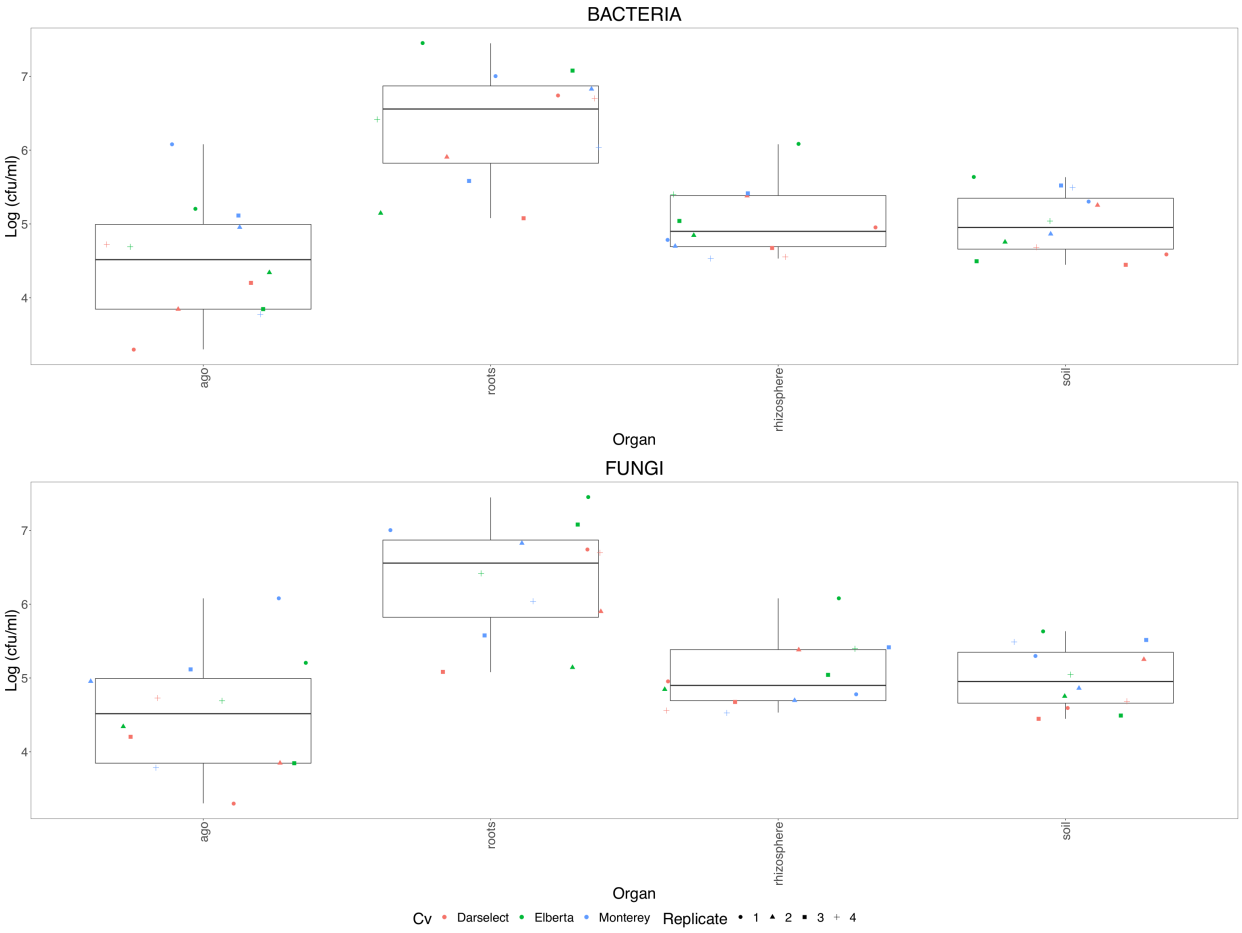


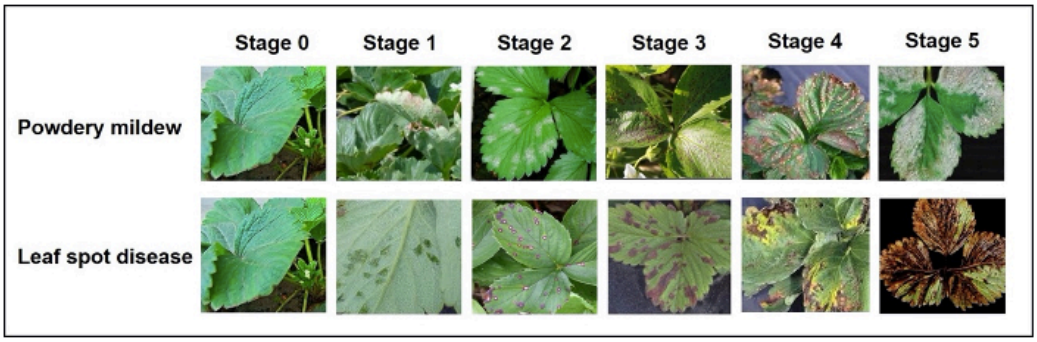
Fig. S4.5 Boxplots visualizing results for viable bacterial and fungal cells (colony-forming unit, CFU) present in plant and soil compartments of strawberry cvs Darselect, Elsanta, Monterey.

Table S4.1 Mineral composition of the fertigating solution of strawberry plants in this work.

Macro elements	**mmol**
NO_3	11
NH_4	0.5
H_2PO_4	1
K	6.5
Ca	4
Mg	1.5
SO_4	1.5
Meso elements	**µmol**
Fe	20
$MnSO_4$	15
Micro elements	**µmol**
$ZnSO_4$	5
$Na_2B_4O_7$	4
$CuSO_4$	0.75
Na_2MnO_4	0.5

Table S4.2 Disease Index (a) of cvs Monterey, Elsanta and Darselect under cultural conditions, based on symptoms incidence and severity determined according to visual scale (b) (0=no symptoms, 5=severe symptoms).

a)



b)

	Monterey	Elsanta	Darselect
Leaf spot	0	1	3
Powdery mildew	2	5	4

Table S4.3 Potential strawberry fungal pathogens (Unite Species Hypotheses).

OTU	Phylum	UNITE species identification	UNITE species hypotheses (%)	Short ID
Otu0003	Ascomycota	*Botrytis caroliniana*	*Botrytis caroliniana (100)*	SH1189120.08FU
Otu0007	Ascomycota	*Plectosphaerella cucumerina*	*Acremonium nepalense (100)*	SH1234026.08FU
Otu0016	Ascomycota	*Plectosphaerella cucumerina*	*Acremonium nepalense (100)*	SH1234026.08FU
Otu0020	Ascomycota	*Ascochyta rabiei*	*Didymella exigua (100)*	SH1174007.08FU
Otu0036	Ascomycota	*Podosphaera sp*	*Podosphaera (100)*	SH1144824.08FU
Otu0040	Ascomycota	*Leptosphaeria sp*	*Paraphoma radicina (100)*	SH1193181.08FU
Otu0053	Ascomycota	*Clonostachys rosea*	*Clonostachys rosea (100)*	SH1155535.08FU
Otu0073	Ascomycota	*Gibellulopsis nigrescens*	*Gibellulopsis piscis (100)*	SH1234027.08FU
Otu0103	Ascomycota	*Stagonosporopsis unclassified*	*Didymella exigua (100)*	SH1174007.08FU
Otu0109	Ascomycota	*Gibberella tricincta*	*Gibberella tricincta (100)*	SH1173588.08FU
Otu0140	Ascomycota	*Mastigosporium album*	*Pezizomycotina (98.64)*	SH1173291.08FU
Otu0145	Ascomycota	*Claviceps sp*	*Hypocreales (100)*	SH1234312.08FU
Otu0160	Ascomycota	*Ramularia sp*	*Ramularia cynarae (100)*	SH1194043.08FU
Otu0169	Ascomycota	*Rhinocladium lesnei*	*Parascedosporium putredinis (100)*	SH1192748.08FU
Otu0177	Ascomycota	*Phaeoacremonium unclassified*	*Sordariomycetes (98.80)*	SH1196099.08FU
Otu0183	Ascomycota	*Ramularia unclassified*	*Ramularia cynarae (100)*	SH1194043.08FU
Otu0230	Ascomycota	*Taphrina wiesneri*	*Taphrina wiesneri (100)*	SH1153415.08FU
Otu0261	Ascomycota	*Taphrina padi*	*Taphrina padi (100)*	SH1153419.08FU
Otu0279	Ascomycota	*Ramularia unclassified*	*Ramularia cynarae (100)*	SH1194043.08FU
Otu0292	Ascomycota	*Seimatosporium sp*	*Xylariales (99.34)*	SH1237195.08FU
Otu0303	Ascomycota	*Trichothecium roseum*	*Trichothecium roseum (100)*	SH1181148.08FU
Otu0325	Ascomycota	*Gibberella pulicaris*	*Fusarium venenatum (100)*	SH1212037.08FU
Otu0355	Ascomycota	*Pestalotiopsis sp*	*Neopestalotiopsis steyaertii (100)*	SH1177548.08FU
Otu0364	Ascomycota	*Cylindrocladiella pseudoparva*	*Cylindrocladiella variabilis (100)*	SH1212036.08FU
Otu0380	Chytridiomycota	*Powellomyces hirtus*	*Powellomyces hirtus (100)*	SH1187133.08FU
Otu0397	Ascomycota	*Neodeightonia phoenicum*	*Neodeightonia palmicola (100)*	SH1143975.08FU
Otu0408	Ascomycota	*Scopulariopsis brumptii*	*Microascus paisii (100)*	SH1146112.08FU
Otu0421	Ascomycota	*Monographella nivalis*	*Monographella nivalis (100)*	SH1179475.08FU
Otu0425	Ascomycota	*Gibellulopsis chrysanthemi*	*Gibellulopsis chrysanthemi (94.67)*	SH1234048.08FU
Otu0427	Ascomycota	*Rhizosphaera kalkhoffii*	*Scleroconidioma sphagnicola (100)*	SH1149663.08FU
Otu0451	Ascomycota	*Taphrina inositophila*	*Taphrina inositophila (98.50)*	SH1153418.08FU
Otu0459	Ascomycota	*Plectosphaerella unclassified*	*Acremonium nepalense (100)*	SH1234026.08FU

OTU	Phylum	UNITE species identification	UNITE species hypotheses (%)	Short ID
Otu0467	Ascomycota	*Gibberella tricincta*	*Gibberella tricincta (100)*	SH1173588.08FU
Otu0479	Ascomycota	*Taphrina pruni*	*Taphrina pruni (99.53)*	SH1153426.08FU
Otu0499	Basidiomycota	*Gjaerumia minor*	*Gjaerumia minor (99.55)*	SH1186349.08FU
Otu0507	Ascomycota	*Blumeria graminis*	*Blumeria graminis (100)*	SH1159375.08FU
Otu0516	Ascomycota	*Bipolaris cynodontis*	*Bipolaris peregianensis (100)*	SH1157992.08FU
Otu0588	Ascomycota	*Devriesia sp*	*Devriesia (100)*	SH1231801.08FU
Otu0601	Ascomycota	*Plectosphaerella cucumerina*	*Acremonium nepalense (95.86)*	SH1234026.08FU
Otu0612	Ascomycota	*Dendryphion europaeum*	*Dendryphion europaeum (100)*	SH1191756.08FU
Otu0615	Ascomycota	*Stemphylium vesicarium*	*Stemphylium vesicarium (100)*	SH1157993.08FU
Otu0645	Ascomycota	*Mycosphaerella ellipsoidea*	*Dothistroma pini (100)*	SH1194045.08FU
Otu0657	Ascomycota	*Botrytis caroliniana*	*Botrytis caroliniana (97.95)*	SH1189120.08FU
Otu0661	Ascomycota	*Eutypa maura*	*Diatrypaceae (100)*	SH1190467.08FU
Otu0664	Ascomycota	*Trichothecium crotocinigenum*	*Trichothecium crotocinigenum (98.31)*	SH1181149.08FU
Otu0688	Basidiomycota	*Typhula sp*	*Typhula (98.67)*	SH1238811.08FU
Otu0700	Ascomycota	*Golovinomyces sordidus*	*Golovinomyces leucheriae (100)*	SH1210305.08FU
Otu0734	Ascomycota	*Ceratocystidaceae sp*	*Thielaviopsis cerberus (100)*	SH1227619.08FU
Otu0736	Ascomycota	*Pilidium concavum*	*Pilidium concavum (100)*	SH1213824.08FU
Otu0757	Ascomycota	*Taphrina carpini*	*Taphrina carpini (100)*	SH1153417.08FU
Otu0760	Ascomycota	*Pyrenophora teres*	*Pleosporaceae (100)*	SH1192922.08FU
Otu0826	Ascomycota	*Taphrina unclassified*	*Taphrina inositophila (95.50)*	SH1153418.08FU
Otu0842	Basidiomycota	*Melampsora epitea*	*Melampsora epitea (100)*	SH1190669.08FU
Otu0877	Ascomycota	*Stagonosporopsis sp*	*Didymella exigua (98.72)*	SH1174007.08FU
Otu0907	Ascomycota	*Plectosphaerella cucumerina*	*Acremonium nepalense (100)*	SH1234026.08FU
Otu0942	Ascomycota	*Devriesia thermodurans*		
Otu0983	Basidiomycota	*Thecaphora melandrii*	*Thecaphora saponariae (100)*	SH1142764.08FU
Otu0989	Ascomycota	*Venturia hystrioides*	*Fusicladium fagi (98.62)*	SH1222281.08FU
Otu1117	Ascomycota	*Ascochyta medicaginicola*	*Pleosporales (100)*	SH1213830.08FU
Otu1125	Ascomycota	*Edenia gomezpompae*	*Pezizomycotina (100)*	SH1146626.08FU
Otu1141	Basidiomycota	*Ustilago hordei*	*Ustilago hordei (97.26)*	SH1145429.08FU
Otu1204	Ascomycota	*Gibellulopsis nigrescens*	*Gibellulopsis piscis (98.24)*	SH1234027.08FU
Otu1206	Ascomycota	*Neofusicoccum parvum*	*Neofusicoccum mangiferae (99.35)*	SH1143963.08FU
Otu1257	Ascomycota	*Phaeoacremonium parasiticum*	*Phaeoacremonium rubrigenum (100)*	SH1196092.08FU

OTU	Phylum	UNITE species identification	UNITE species hypotheses (%)	Short ID
Otu1281	Ascomycota	*Lectera colletotrichoides*	*Lectera longa (100)*	SH1234028.08FU
Otu1284	Basidiomycota	*Moesziomyces aphidis*	*Moesziomyces parantarcticus (100)*	SH1145427.08FU
Otu1321	Ascomycota	*Rhexocercosporidium panacis*	*Rhynchosporium commune (98.64)*	SH1173280.08FU
Otu1330	Ascomycota	*Plectosphaerella cucumerina*	*Acremonium nepalense (100)*	SH1234026.08FU
Otu1338	Ascomycota	*Glomerella acutata*	*Colletotrichum acerbum (98.69)*	SH1171875.08FU
Otu1400	Ascomycota	*Hortaea werneckii*	*Hortaea werneckii (100)*	SH1154533.08FU
Otu1431	Ascomycota	*Sclerotinia sclerotiorum*	*Botrytis caroliniana (100)*	SH1189120.08FU
Otu1437	Basidiomycota	*Meira geulakonigii*	*Meira geulakonigii (99.56)*	SH1237725.08FU
Otu1524	Ascomycota	*Clonostachys rosea*	*Clonostachys rosea (90.91)*	SH1155535.08FU
Otu1531	Ascomycota	*Bipolaris sorokiniana*	*Bipolaris peregianensis (100)*	SH1157992.08FU
Otu1577	Ascomycota	*Clonostachys rosea*	*Clonostachys rosea (100)*	SH1155535.08FU
Otu1694	Ascomycota	*Gibberella tricincta*	*Gibberella tricincta (100)*	SH1173588.08FU
Otu1709	Ascomycota	*Botrytis caroliniana*	*Botrytis caroliniana (100)*	SH1189120.08FU
Otu1713	Ascomycota	*Curvularia americana*	*Curvularia lunata (100)*	SH1157991.08FU
Otu1731	Ascomycota	*Rhexocercosporidium panacis*	*Rhynchosporium commune (97.26)*	SH1173280.08FU
Otu1756	Ascomycota	*Clonostachys rosea*	*Clonostachys rosea (97.58)*	SH1155535.08FU
Otu1761	Ascomycota	*Plectosphaerella cucumerina*	*Acremonium nepalense (100)*	SH1234026.08FU
Otu1781	Ascomycota	*Erysiphe arcuata*	*Erysiphe arcuata (99.45)*	SH1184259.08FU
Otu1821	Ascomycota	*Leptosphaeria sp*	*Paraphoma radicina (100)*	SH1193181.08FU
Otu1841	Ascomycota	*Plectosphaerella cucumerina*	*Acremonium nepalense (99.31)*	SH1234026.08FU
Otu1866	Ascomycota	*Plectosphaerella cucumerina*	*Acremonium nepalense (97.63)*	SH1234026.08FU
Otu1875	Ascomycota	*Clonostachys rosea*	*Clonostachys rosea (100)*	SH1155535.08FU
Otu1881	Ascomycota	*Plectosphaerella cucumerina*	*Acremonium nepalense (98.19)*	SH1234026.08FU
Otu1896	Ascomycota	*Erysiphaceae unclassified*	*Erysiphe (100)*	SH1184255.08FU
Otu1906	Ascomycota	*Plectosphaerella cucumerina*	*Acremonium nepalense (99.31)*	SH1234026.08FU
Otu1909	Ascomycota	*Leptosphaeria sp*	*Paraphoma radicina (98)*	SH1193181.08FU
Otu1927	Ascomycota	*Pyrenophora avenae*	*Pleosporaceae (100)*	SH1192921.08FU
Otu1930	Ascomycota	*Plectosphaerella cucumerina*	*Acremonium furcatum (99.42)*	SH1234029.08FU
Otu2003	Ascomycota	*Botrytis caroliniana*	*Botrytis caroliniana (96.25)*	SH1189120.08FU
Otu2023	Ascomycota	*Plectosphaerella cucumerina*	*Acremonium nepalense (100)*	SH1234026.08FU
Otu2033	Ascomycota	*Botrytis caroliniana*	*Botrytis caroliniana (100)*	SH1189120.08FU
Otu2059	Ascomycota	*Plectosphaerella cucumerina*	*Acremonium nepalense (97.63)*	SH1234026.08FU
Otu2086	Ascomycota	*Plectosphaerella unclassified*	*Acremonium nepalense (99.41)*	SH1234026.08FU

OTU	Phylum	UNITE species identification	UNITE species hypotheses (%)	Short ID
Otu2119	Ascomycota	*Leptosphaeria unclassified*	*Paraphoma radicina (100)*	SH1193181.08FU
Otu2135	Ascomycota	*Glomerella acutata*	*Colletotrichum acerbum (98.66)*	SH1171875.08FU
Otu2146	Ascomycota	*Plectosphaerella cucumerina*	*Acremonium nepalense (99.32)*	SH1234026.08FU
Otu2150	Ascomycota	*Glomerella acutata*	*Colletotrichum acerbum (98..08)*	SH1171875.08FU
Otu2197	Ascomycota	*Plectosphaerella cucumerina*	*Acremonium nepalense (98.22)*	SH1234026.08FU
Otu2226	Ascomycota	*Ramularia eucalypti*	*Ramularia cynarae (97.87)*	SH1194043.08FU
Otu2233	Ascomycota	*Plectosphaerella cucumerina*	*Acremonium nepalense*	SH1234026.08FU
Otu2240	Ascomycota	*Gibberella unclassified*	*Gibberella fujikuroi (98.65)*	SH1212030.08FU
Otu2314	Ascomycota	*Gibellulopsis nigrescens*	*Gibellulopsis piscis (95.88)*	SH1234027.08FU
Otu2360	Ascomycota	*Plectosphaerella cucumerina*	*Acremonium nepalense (99.35)*	SH1234026.08FU
Otu2365	Ascomycota	*Taphrina mirabilis*	*Taphrina pruni-subcordatae (99.53)*	SH1153416.08FU
Otu2369	Ascomycota	*Plectosphaerella cucumerina*	*Acremonium nepalense (97.63)*	SH1234026.08FU
Otu2382	Ascomycota	*Plectosphaerella cucumerina*	*Acremonium nepalense (98.22)*	SH1234026.08FU
Otu2384	Ascomycota	*Gibellulopsis nigrescens*	*Gibellulopsis piscis (100)*	SH1234027.08FU
Otu2407	Ascomycota	*Ramichloridium indicum*	*Ramichloridium indicum (100)*	SH1177266.08FU
Otu2423	Ascomycota	*Lepteutypa fuckelii*	*Lepteutypa fuckelii (92.40)*	SH1184890.08FU
Otu2454	Ascomycota	*Plectosphaerella cucumerina*	*Acremonium nepalense (97.63)*	SH1234026.08FU
Otu2490	Basidiomycota	*Urocystis agropyri*	*Urocystis agropyri (98.85)*	SH1177340.08FU
Otu2527	Ascomycota	*Leptosphaeria sp*		

Table S4.4 Identification of potential bacterial beneficial functions based on literature research.

Function	Taxa
Nitrogen fixation	Allorhizobium, Bradyrhizobium, Ensifer, Mesorhizobium, Rhizobium, Neorhizobium, Pararhizobium, Aminobacter, Devosia, Methylobacterium, Microvirga, Phyllobacterium, Shinella, Burkholderia, Paraburkholderia, Cupriavidus (Khan et al., 2010)

Plant growth promotion and Biological control activity	*Bacillus megaterium* and *B. pumilus* (Choudhary and Johri, 2009)
	Cellulosimicrobium sp. (Nabti et al., 2014)
	Pseudomonas fluorescens (Purahong et al., 2018)
	Streptomyces hygroscopicus (Prapagdee et al., 2008)

Table S4.5 Effect of genotype, soil and plant compartment on community composition of strawberry microbiome based on relative abundance data and Bray-Curtis distance measure. Nd = not determined; Significant *P* values are highlighted in bold.

	Community composition (Two-way ANOSIM)		**Community composition (Two-way PERMANOVA)**	
Microorganisms/Factors	***R***	***P***	**Pseudo*F***	***P***
Total bacteria				
Genotype	0.3	**0.001**	3.19	**0.003**
Compartment	0.54	**0.001**	10.43	**0.001**
Genotype x compartment	nd	nd	1.97	**0.005**
Potential beneficial bacteria				
Genotype	0.30	**0.001**	2.05	**0.001**
Compartment	0.48	**0.001**	5.69	**0.001**
Genotype x compartment	nd	nd	1.47	**0.002**
Fungi				
Genotype	0.45	**0.001**	4.14	**0.001**
Compartment	0.65	**0.001**	7.77	**0.001**
Genotype x compartment	nd	nd	1.92	**0.002**
Potential beneficial fungi				
Genotype	0.2	**0.001**	2.4926	**0.001**
Compartment	0.53	**0.001**	9.8272	**0.001**
Genotype x compartment	nd	nd	1.8785	**0.005**
Plant pathogenic fungi				
Genotype	0.36	**0.001**	3.6854	**0.001**
Compartment	0.45	**0.001**	5.2552	**0.001**
Genotype x compartment	nd	nd	1.9834	**0.001**

Table S4.6 Chemical composition of roots and above-ground strawberry organs.

	Above-ground Organs			Roots		
	Darselect	**Elsanta**	**Monterey**	**Darselect**	**Elsanta**	**Monterey**
Macro-elements, % dry weight						
C	43.50 ± 0.82	44.40 ± 0.98	43.11 ± 0.83	46.20 ± 3.13	48.25 ± 0.54	45.77 ± 0.58
N	3.25 ± 0.43	3.02 ± 0.34	3.36 ± 0.49	1.36 ± 0.10	1.09 ± 0.15	1.60 ± 0.11
H	5.75 ± 0.12	5.73 ± 0.14	5.52 ± 0.13	5.10 ± 0.23	5.23 ± 0.04	5.24 ± 0.21
Macro-elements, µg/g						
P	4425.60 ± 1021.05	3099.75 ± 905.37	5598.78 ± 528.59	1285.95 ± 198.23	752.55 ± 124.10	1544.53 ± 431.20
K	31527 ± 5251.49	17834.63 ± 4314.25	30119.10 ± 1284.10	1158.75 ± 249.05	822.30 ± 424.05	1657.15 ± 1271.44
Ca	14685.33 ± 1708.42	11495.10 ± 1281.37	10188.78 ± 978.89	11636.68 ± 2139.82	12512.58 ± 1943.92	17586.35 ± 1616.47
Mg	4117.93 ± 615.78	4675 ± 731.02	5103.70 ± 669.97	2600.35 ± 346.89	2174.43 ± 246.41	3638.43 ± 579.04
Micro-elements, µg/g						
B	59.90 ± 6.93	65.18 ± 19.91	67.65 ± 9.26	24.53 ± 3.66	19.33 ± 0.56	33.18 ± 2.55
Mn	32.88 ± 5.39	86.88 ± 55.97	41.20 ± 15.08	45.43 ± 31.63	35.83 ± 24.36	74.75 ± 23.16
Fe	71.08 ± 10.45	89.85 ± 18.70	106.68 ± 17.44	517.95 ± 281.97	218.08 ± 73.42	533.83 ± 387.58
Co	0.00 ± 0.00	0.03 ± 0.05	0.00 ± 0.00	0.25 ± 0.13	0.20 ± 0.00	0.48 ± 0.42
Ni	0.30 ± 0.14	0.45 ± 0.24	0.30 ± 0.00	0.90 ± 0.37	0.65 ± 0.13	1.83 ± 0.77
Zn	23.43 ± 3.29	21.70 ± 9.35	29.60 ± 7.66	81.73 ± 28.97	39.68 ± 18.35	26.58 ± 3.01
Sr	93.28 ± 14.46	77.08 ± 10.84	63.88 ± 7.85	82.70 ± 15.55	81.25 ± 10.09	132.40 ± 5.35
Mo	0.95 ± 0.37	1.08 ± 0.36	2.25 ± 0.44	14.33 ± 5.94	19.40 ± 15.72	24.90 ± 7.58
Ba	24.68 ± 4.10	22.68 ± 4.77	16.78 ± 4.74	15.93 ± 4.35	9.80 ± 1.39	31.53 ± 9.11
Pb	0.23 ± 0.05	0.13 ± 0.05	0.18 ± 0.10	3.98 ± 1.89	2.53 ± 0.50	1.35 ± 0.57

Table S4.7 Bacterial (mainly Micrococcales) and fungal (mainly Ascomycota) OTUs detected in all 3 strawberry genotypes and in all plant-soil compartments.

OTU	**Phylum**	**Order**	**Family**	**Genus**	**Species**
Otu000001	Actinobacteria	Micrococcales	Micrococcaceae	*Pseudarthrobacter*	-
Otu000003	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	-
Otu000005	Actinobacteria	Micrococcales	Micrococcaceae	*Paenarthrobacter*	-
Otu000007	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	*Limnohabitans*	-
Otu000010	Actinobacteria	Micrococcales	Micrococcaceae	-	-
Otu000009	Actinobacteria	Micrococcales	Micrococcaceae	*Paenarthrobacter*	-
Otu000014	Actinobacteria	Micrococcales	Micrococcaceae	*Paenarthrobacter*	-
Otu000016	Actinobacteria	Micrococcales	Micrococcaceae	*Paenarthrobacter*	-
Otu000024	Actinobacteria	Micrococcales	Micrococcaceae	*Paenarthrobacter*	-
Otu000017	Actinobacteria	Micrococcales	Micrococcaceae	-	-
Otu000018	Actinobacteria	Micrococcales	Micrococcaceae	-	-
Otu000046	Actinobacteria	Micrococcales	Micrococcaceae	*Paenarthrobacter*	-
Otu000043	Actinobacteria	Micrococcales	Micrococcaceae	*Paenarthrobacter*	-

Otu000060	Actinobacteria	Micrococcales	Micrococcaceae	-	-
Otu000032	Actinobacteria	Micrococcales	Micrococcaceae	*Paenarthrobacter*	-
Otu000066	Actinobacteria	Micrococcales	Microbacteriaceae	-	-
Otu000084	Actinobacteria	Micrococcales	Micrococcaceae	-	-
Otu000090	Actinobacteria	Micrococcales	Micrococcaceae	-	-
Otu000120	Actinobacteria	Micrococcales	Micrococcaceae	-	-
Otu000100	Actinobacteria	Micrococcales	-	-	-
Otu000171	Actinobacteria	Micrococcales	Micrococcaceae	-	-
Otu000193	Actinobacteria	Micrococcales	Microbacteriaceae	-	-
Otu000279	Actinobacteria	Micrococcales	Micrococcaceae	-	-
Otu000241	Actinobacteria	Micrococcales	Micrococcaceae	*Paenarthrobacter*	-
Otu0001	Ascomycota	Capnodiales	-	-	*Capnodiales unclassified*
Otu0002	Ascomycota	Helotiales	-	*Cadophora*	*Cadophora luteo-olivacea*
Otu0003	Ascomycota	Helotiales	Sclerotiniaceae	*Botrytis*	*Botrytis carolinian a*
Otu0007	Ascomycota	Glomerellales	Plectosphaerellacea e	*Plectosphaerella*	*Plectospha erella cucumeri na*
Otu0008	Fungi_unclassified	-	-	-	-
Otu0011	Ascomycota	Saccharomycetale s	Saccharomycetaceae	*Saccharomyces*	*Saccharo myces cerevisiae*
Otu0017	Ascomycota	Dothideales	Aureobasidiaceae	*Aureobasidium*	*Aureobasi dium pullulans*
Otu0018	Ascomycota	Capnodiales	Cladosporiaceae	*Cladosporium*	*Cladospor ium sphaerosp ermum*
Otu0020	Ascomycota	Pleosporales	Didymellaceae	*Ascochyta*	*Ascochyta rabiei*
Otu0022	Basidiomycota	Tremellales	Bulleribasidiaceae	*Vishniacozyma*	*Vishniaco zyma victoriae*
Otu0025	Ascomycota	Pleosporales	Pleosporaceae	*Alternaria*	*Alternaria alternata*
Otu0043	Basidiomycota	Holtermanniales		*Holtermanniella*	*Holterma nniella takashima e*
Otu0045	Ascomycota	Pleosporales	Pleosporaceae	*Alternaria*	-
Otu0099	Ascomycota	Hypocreales	Cordycipitaceae	*Lecanicillium*	*Lecanicilli um muscariu m*
Otu0135	Basidiomycota	Tremellales	Bulleribasidiaceae	*Vishniacozyma*	*Vishniaco zyma heinaeyen sis*

Table S4.8 Number of OTUs belonging to particular bacterial or fungal functions assigned based on FAPROTAX and FUNguild, respectively.

BACTERIA - FUNCTIONS	OTUs
Aromatic compound degradation	99
Cellulolysis	31
Chemoheterotrophy	2389
Chitynolysis	3
Hydrocarbon degradation	41
Intracelluar parasites	235
Iron respiration	2
Manganese oxidation	12
Methanol oxidation	444
Multifunctions	231
Nitrate reduction	40
Nitrite oxidation	24
Nitrogen fixation	19
Photoautotrophy	27
Photoheterotrophy	8
Predatory/exoparasitic	233
Sulfate respiration	27
Sulfur compound oxidation	82
Ureolysis	126
Xylanolysis	2
Total assigned functions	*20*
FUNGI - FUNCTION	
Animal endosymbiont-saprotroph	1
Animal parasite-fungal parasite	1
Animal pathogen	34
Arbuscular mycorrhizal[a]	6
Ectomycorrhizal[a]	5
Endophyte[a]	57
Endophyte-plant pathogen	7
Endophyte-saprotroph	9
Ericoid mycorrhizal (dark septate endophyte)[a]	22
Fungal parasite[a]	13
Lichen parasite	1
Lichenized	1
Lichenized-saprotroph	1
Multifunctions	55
Plant pathogen[b]	136
Saprotroph	357
Total assigned functions	*16*

Fungal OTU classification: a= benficial, b=pathogenic.

Table S4.9 List of fungal OTUs identified at the species level, for which biological control or plant growth promoting activities have been described in literature.

Activities	Fungal species
BCA against fungal pathogens	*Acremonium alternatum* (Raps and Vidal, 1998)
	Aureobasidium pullulans (Mari et al., 2012)
	Candida sake (Marìn et al., 2016)
	Chaetomium globosum (Shanthiyaa et al., 2013)
	Chaetomium cupreum (Soytong et al., 2001)
	Clonostachys rosea (Ravnskov et al., 2006)
	Coprinopsis urticicola (Gholami et al., 2019)
	Debaryomyces hansenii (Gil-Serna et al., 2011)
	Mortierella hyaline (Gholami et al., 2019)
	Papiliotrema flavescens (Schisler et al., 2019)
	Rhodotorula babjevae (Sandberg, 2019)
	Ceriporia lacerata (Yin et al., 2018)
	Chaetomium globosum (Khan et al., 2012)
	Penicillium adametzii (Szwajkowska-Michalek et al., 2012)
	Saccharomyces cerevisiae (Lopes et al., 2015)
Induction of systemic resistance	*Trichoderma harzianum* (Adnan et al., 2019)
Entomopathogenic activity	*Beauveria bassiana* (Zimmermann, 2007)
	Lecanicillium muscarium (Ownley et al., 2010)
Phytase production	*Chaetomium globosum* (Shanthiyaa et al., 2013; Soytong et al., 2001)
Improvement of Soil Quality by Production of Chitins and Phytohormones	*Chaetomium globosum* (Shanthiyaa et al., 2013; Soytong et al., 2001)
	Aspergillus fumigatus (Khan et al., 2011)
	Cladosporium sphaerospermum Hamayun et al., 2010)
	Mortierella elongate (Li et al., 2018)
	Fusarium proliferatum (Bilal et al., 2018)
Plant Growth Promotion by enhancement of mineral nutrients use efficiency	*Periconia macrospinosa* (Yakti et al., 2018)
Improvement of plant Growth under saline stress by enhancement of antioxidative defense system	*Trichoderma longibrachiatum* (Zhang et al., 2016)

Table S4.10 Bacterial isolates screened for Plant Growth Promoting traits.

CV	N°	Organ of isolation	Acetoin production	IAA production	Siderophores	Botrytis antagonism	Xf antagonism	NH4+ production	accd activity	Species
monterey	21	root	+	-	Carboxilate t	-	+	-	0	*Acinetobacter calcoaceticus*
monterey	39	leaf	-	+	NO	-	++	+	0	*Agrobacterium rubi*
monterey	12	soil	-	-	NO	-	-	-	0	*Arthrobacter sp.*
darselect	7	rhizosphere	+++	-	NO	-	++	-	1,59±2,9	*Bacillus pumilus*
monterey	2	leaf	++	-	NO	-	+	+	0	*Bacillus pumilus*
darselect	3	leaf	-	-	NO	+	++	-	0	*Brevibacillus brevis*
monterey	14	soil	+	-	Hydroxamate	-	+	-	0	*Chryseobacterium sp.*
darselect	20	leaf	+	-	NO	-	-	-	0	*Curtobacterium sp.*
monterey	25	rhizosphere	+	-	NO	-	-	-	0	*Ensifer adhaerens*
elsanta	36	rhizosphere	-	-	NO	-	-	-	0	*Enterobacter sp.*
monterey	48	leaf	-	++	NO	-	-	-	0	*Frigoribacterium faeni*
elsanta	31	leaf	-	-	NO	-	-	+	0	*Frigoribacterium sp.*
elsanta	16	leaf	-	-	NO	-	-	-	0	*Frigoribacterium sp.*
elsanta	15	rhizosphere	-	-	Carboxilate t	-	-	-	0	*Massilia sp.*
elsanta	10	rhizosphere	-	-	Carboxilate t	-	+	+	0	*Massilia sp.*
elsanta	9	soil	-	-	NO	-	-	+	0	*Massilia sp.*
elsanta	8	rhizosphere	-	-	NO	-	-	-	0	*Massilia sp.*
monterey	29	rhizosphere	-	-	Hydroxamate	+	-	-	0	*Massilia sp.*
monterey	26	rhizosphere	+	-	NO	+	-	-	0	*Massilia sp.*
darselect	22	soil	+	-	Carboxilate t	-	-	-	0	*Microbacterium sp.*
elsanta	5	rhizosphere	-	-	NO	-	-	-	0	*Microbacterium sp.*
monterey	13	soil	+	-	NO	-	-	-	0	*Pantoea agglomerans*
elsanta	27	root	-	-	NO	-	-	++	0	*Providencia rettgeri*
darselect	12	root	-	-	NO	-	-	-	22,05±1,32	*Pseudomonas brassicaerearum*
elsanta	12	rhizosphere	+	+	NO	-	-	+	0	*Pseudomonas fluorescens*
monterey	27	root	+	-	Carboxilate t	-	++	+	0	*Pseudomonas fluorescens*
darselect	17	soil	-	+	NO	-	-	-	64±25,61	*Pseudomonas fluorescens*
elsanta	39	rhizosphere	-	++	NO	-	-	+	33,68±6,37	*Pseudomonas jessenei*
elsanta	3	soil	+	-	Carboxilate t	-	-	-	0	*Pseudomonas migulae*
darselect	27	soil	+	+	Carboxilate t	-	-	-	0	*Pseudomonas putida*
darselect	24	soil	+	-	Carboxilate t	-	-	++	70,85±32,11	*Pseudomonas reinekei*
darselect	26	soil	-	+	Carboxilate t	-	-	-	0	*Pseudomonas silesiensis*
elsanta	21	soil	-	-	NO	-	-	+	0	*Pseudomonas sp.*
monterey	31	soil	-	-	Carboxilate t	-	-	+	0	*Pseudomonas sp.*
monterey	18	soil	-	-	NO	-	-	-	0	*Pseudomonas sp.*
monterey	1	root	-	-	Carboxilate t	-	-	+	0	*Pseudomonas trivialis*
monterey	49	rhizosphere	-	-	NO	-		+	0	*Psychrobacillus*
monterey	35	leaf	-	-	NO	-	++	-	0	*Rhizobium soli*
monterey	44	leaf	-	-	NO	-	-	+	0	*Sphingomonas sp.*
monterey	23	soil	-	-	NO	-	++	+	0	*Stenotrophomonas rhizophila*
darselect	18	leaf	-	-	NO	-	-	-	0	*Vagococcus fluvialis*
elsanta	35	root	-	-	NO	-	-	-	0	*Vagococcus sp.*
monterey	40	leaf	-	+++	NO	-	-	-	0	*Vagococcus sp.*

Table S4.11 Quality parameters evaluated on harvested strawberry fruits.

Cultivar	Diameter (mm)	Texture (N)	pH	SSC (°Brix)	Titratable acidity (citric acid eqs, mg/ml)
Darselect	34.57±1.99	2.29±0.20	3.75±0.03	6.37±1.23	3.71±1.88
Elsanta	33.03±3.06	2.20±0.46	3.68±0.11	11.55±3.23	3.94±1.22
Monterey	31.26±4.82	3.05±1.28	3.61±0.05	5.67±2.39	2.70±1.28

Table S4.12 Nitrogen fixing bacterial genera detected in cultivated strawberry, wild strawberries and legume plants.

Bacterium genera	Cultivated Strawberry	Wild Strawberry	Legumes
Allorhizobium	✓ (71 OTUs)	×	✓
Neorhizobium		×	✓
Pararhizobium		×	✓
Rhizobium		✓ (3 OTUs)	✓
Aminobacter	✓ (1 OTU)	✓ (1 OTU)	✓
Azorhizobium	×	×	✓
Bradyrhizobium	✓ (22 OTUs)	✓ (6 OTUs)	✓
Burkholderia Paraburkholderia	✓ (48 OTUs)	✓ (5 OTU)	✓
		×	✓
Cupriavidus	✓ (1 OTU)	×	✓
Devosia	✓ (83 OTUs)	✓ (3 OTU)	✓
Ensifer	✓ (4 OTUs)	×	✓
Mesorhizobium	✓ (30 OTUs)	✓ (1OTU)	✓
Methylobacterium	✓(6 OTUs)	✓ (2 OTU)	✓
Microvirga	✓(3 OTUs)	×	✓
Ochrobactrum	×	×	✓
Phyllobacterium	✓(1 OTU)	×	✓
Shinella	✓(29 OTUs)	×	✓

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5. Exploring the potential of native bacteria for strawberry plants growth promotion under biotic and abiotic stress conditions

Abstract:

Strawberry production is challenged by several biotic and abiotic stresses, such as the angular leaf spot (ALS) disease caused by *Xanthomonas fragariae,* and high soil salinity and drought.

In the last decades, the development of commercial products containing combinations of different Plant Growth Promoting (PGP) microorganisms has been one of the main focuses of the agricultural research. However, their efficacy could be hindered by competition or incompatibility occurring among the different strains or with the indigenous plant microbiota. Therefore, the application of beneficial microorganisms on the same host species from which they were isolated might increase their positive effect, promoting their utilization for a sustainable agriculture.

In this work, native bacteria isolated from different strawberry cultivars were screened for their PGP abilities, in both normal and stressor (*X. fragariae* infection and salinity) conditions. Biometric parameters monitoring of inoculated plants allowed the selection of a restricted group of bacteria (*Pseudomonas fluorescens* m27, *Stenotrophomonas rhizophila* m23, *Agrobacterium rubi* m39) whose application showed a significant increase of plant growth performances and resistance to stress. Their beneficial potential was successively tested in coordinated inoculations with the selective application of different strains on different plant organs. Simultaneous strains inoculation could be hypothesized to be more effective than single strain application, due to the additive beneficial effect of several coexisting PGP traits. However, our results showed that such coordinated inoculum reduced plant growth and fruit quality in respect to single strain treatment. Further research should aim at understanding the complex mechanisms influencing plant-microbes and microbes-microbes networks. Indeed, unsuccess of the coordinated inoculum might be due to plant microbiota imbalance due to a high PGP bacteria inoculation, which might require more time for plant adaptation and thus beneficial response.

5.1 Introduction

Strawberry is a widely consumed berry fruit, appreciated by consumers for its organoleptic and nutraceutical properties (Husaini et al., 2015). Strawberry is characterized by a large consumers market in the world main economies, having China (6,48 bn US$), Europe (3,49 bn US$) and United States (3,47 bn US$) the biggest market share (FAOSTAT). Strawberry cultivation might be affected by several pests and diseases, that might cause considerable economic losses. Among others, *Xanthomonas fragariae*, which causes the angular leaf spot (ALS), is a bacterium classified as quarantine pathogen by the European and Mediterranean Plant Protection Organization (EPPO). Typically, ALS symptoms appear on leaves, as water-soaked lesions. As the disease advances, leaves become reddish, the whole plant might collapse and up to 10% of strawberry yield might be lost (Maas et al., 1995). In horticulture, management of bacterial diseases generally involves preventive application either of copper- or antibiotics-based products (Sundin et al., 2016). However, over time both these practices lead to bacterial resistance to the active substance and to their subsequent inefficient application. Another serious stress affecting strawberry cultivation is soil salinity, which results in a general water deficit in the plant. Strawberry plants are extremely sensitive to it although several tolerance degrees exist among different cultivars (Gulen et al., 2015). Salinity affects leaf number, leaf area, plant dry weight, root and shoot growth, accelerating leaf senescence, reducing the photosynthetic ability of the plant. Overall, this results in reduced fruit production and altered quality.

The plant associated microbiota is able to express several functional traits able to benefit the associated host, such as the protection from pests and abiotic stresses with the consequent promotion of plant growth (Bulgarelli et al., 2013; Sangiorgio et al., 2020). Furthermore, the microbiota associated to small berry fruits was also demonstrated to be able to affect fruit quality and aroma (Todeschini et a., 2018; Sangiorgio et al., 2021, 2022). Altogether, these abilities might be exploited to face the present agricultural challenges, providing a sustainable alternative to the use of antibiotics or chemical fertilizers (Sessitsch et al., 2019).

Although, over at least three decades, the scientific community has been working on the development of efficient microorganisms-based inoculants, generally, once applied in the field, they lack in consistency (Montesinos, 2003). One of the main reasons relies in the inability of the introduced microbe to efficiently colonize the plant organ and to collaborate with the natural microbiota (Compant et al., 2019).

Ideally, a selection of Plant Growth promoting (PGP) microorganisms which are part of the plant core microbiota, should be utilized in single or in mixed inoculation for efficient bioinoculants development with the aim to exploit their plant colonization and persistence ability (Santiago et al., 2017; Banerjee et al., 2017; May et al., 2021).
The present study aimed to explore the *in vivo* growth promoting effect of bacteria isolated from three strawberry cultivars and applied on the same host both individually or in a coordinated bioinoculant, based on the contemporary application of different strains in different plant organs. First, the efficacy of the fifteen selected bacterial isolates applied either on roots or leaves, was tested both under biotic and abiotic stresses, respectively during *X. fragariae* pathogenic infection and under induced salinity stress. Afterward, the most performant strains were selected and the efficiency of the application of a coordinated bioinoculant on strawberry plant growth performances was determined.

5.2 Materials and Methods

5.2.1 Isolation, characterization, and species identification of bacterial strain

Overall, fifteen bacterial isolates were tested *in vivo* for their plant growth promoting activity on strawberry. Thirteen out of 15 bacterial strains derived from a previous work (see chapter 4), in which they were isolated from soil, rhizosphere, roots and leaves of strawberry plants and functionally characterized for PGP properties (Table 5.1). Additionally, in this work, 12 epiphytes were isolated and characterized from strawberry fruits of 'Monterey', 'Elsanta', 'Darselect' cultivars (Table S5.1). On the basis of their PGP abilities, two of them (Table 5.1), *Rhodococcus* spp. m38 and *Pantoea agglomerans* d15 were selected as most appropriate inoculant candidates. The species, the *in vitro* functional characterization and the organ and cultivar of isolation of the 15 bacterial strains selected for root and leaves inoculation are listed in Table 5.1.
Procedure for isolation, and taxonomical and functional characterization of bacteria isolated from strawberry fruits is here briefly described. Strawberry fruits were washed in sterile 10 mM $MgSO_4$ solution. Washings of $MgSO_4$ were serially diluted and plated on Luria Bertani (LB) agar medium (Sigma Aldrich) supplemented with cycloheximide (100 μg ml^{-1}) to prevent fungal growth. Plates were incubated at 27°C for 24h. Colonies were phenotypically characterized, and for each phenotype in a repetition, a single colony was randomly collected from the highest dilution plates. The isolates were then stored at -80°C in LB broth supplemented with 20% v/v

glycerol. From each bacterial isolate, DNA using GenElute Bacterial Genomic DNA kit following manufacturer's instructions was extracted. For samples species identification, 16S rRNA gene was amplified using Lac16Sfor (5'-AATGAGAGTTTGATCCTGGCT-3') and Lac16Srev (5'-GAGGTGATCCAGCCGCAGGTT-3') primers, and sequenced at Biofab Research Srl. The sequences obtained were checked for quality and then matched for homology with those available on BLAST suite (NCBI) at date January 2021. Bacteria isolated from strawberry fruits were then functionally characterized for acetoin, IAA, ammonia and siderophores production, ACC deaminase activity, *X. fragariae* and *B. cinerea* inhibition as described in Perpetuini et al., 2019.

5.2.2 Bacterial inoculation and treated plant material

Strawberry bare roots of cultivar Monterey were planted in 9x9x13cm black pots filled with blond sphagnum peat moss soil (Vigorplant s.r.l, Lodi). Plants were kept in controlled conditions with 16h of continuous light and 80% relative humidity.

PGP Bacteria (PGPB) inoculant candidates listed in Table 1 and *Xanthomonas fragariae* were grown overnight in LB and Wilbrink Liquid Medium (prepared as described in EPPO, 2006), respectively. Cells were pelleted and resuspended in 10 mM $MgSO_4$. 10 ml of suspension for each bacterial strain were prepared and adjusted to a 10^8 cfu/ml concentration.

In the first experiment, *in vivo* plant growth promotion of the 15 bacterial candidates was tested on leaves and roots of strawberry plants, both under unstressed and stressed conditions in order to select the best suitable candidates for further experiments. Five plant replicates were considered as control, indeed neither a stress nor a PGPB was applied. For roots inoculation, strawberry plants were individually inoculated with the seven selected bacterial strains showing IAA production activity (Table 5.1), i.e. *Agrobacterium rubi* m39, *Pseudomonas fluorescens* d17, *P. jessenei* e39, *Pantoea agglomerans* d15, *P. silensis* d26, *P. putida* d27 and *P. fluorescens* e12. Additionally, five plant replicates on which saline stress was imposed (without the application of any PGPB) were considered as NaCl control (NaCl). Five replicates for each strain inoculation and stress/unstress condition were prepared, for a total of 75 plants (including NaCl controls).

High salinity condition was imposed as stress to roots by substituting regular irrigation with tap water supplemented with 35mM NaCl.

For leaves, the application in single with the nine selected bacterial strains, i.e. *Brevibacillus brevis* d3, *Massilia* sp. e10, *Agrobacterium rubi* m39, *Rhizobium soli* m35, *P. fluorescens* m27, *Stenotrophomonas rhizophila* m23, *Bacillus pumilus* m2, *Rhodococcus* spp. m38, *Vagococcus* m40, showing antagonistic activity against *X. fragariae* was performed, both under stressed or unstressed conditions. The stress imposed on leaves consisted in the application of *X. fragariae* pathogen on the same organ, which was dosed 24 hours after plant inoculation with the selected PGPB. Additionally, five plant replicates on which *Xanthomonas fragariae* was applied (without the application of any bacterial antagonist or chemical product) were considered as *X. fragariae* control (Xf) and five plants treated with Streptomycin (100 mg/L) were also included as positive controls, because the product is known to be highly effective against *X. fragariae*. Leaves of strawberry plants were sprayed with a bacterial suspension till run-off. Five replicates for each strain inoculation and stress/unstress condition were prepared, for a total of 100 plants (including Xf and streptomycin controls).

Each inoculation to which the plants were subjected, was performed at the stage of three true leaves (after one month of growth).

For the second experiment, bacterial candidates showing the best performance during the first *in vivo* test, were selected for individual or mixed application to healthy strawberry plants. In particular, the strains able to improve *in vivo* strawberry plants fitness, *A. rubi* m39, *S. rhizophila* m23 and *P. fluorescens* m27, were inoculated with same concentrations and procedure as described above. The three bacterial strains were applied both individually and in a cooperative inoculum on strawberry roots, leaves and flowers, respecting the organ-specific capabilities demonstrated in the first experiment. Indeed, *A. rubi* m39 was applied on roots, *S. rhizophila* m23 on leaves and *P. fluorescens* m27 on flowers. Five plant replicates for each treatment were prepared. Additionally, five untreated plants were prepared and considered as control.

5.2.3 Growth parameters, disease incidence, disease index and fruit quality

One month after bacterial inoculation, standard plants biometric parameters were measured. In particular, fresh and dry weight of leaves and roots. Leaves and roots were dried for 3 days at 65°C, and then weighed. Furthermore, root length was measured. Chlorophyll content recording the Soil Plant Analysis Development (SPAD index) with a portable chlorophyll meter SPAD-502 (Konica- Minolta Corporation, Ltd., Osaka, Japan) was measured. Number of leaves and flowers was counted over

one month at 5, 8, 13, 16, 19 and 27 dai (i.e. days after bacterial inoculation) for leaves and at 8, 13, 16, 19 and 27 dai for flowers. For each observation day (T_x), leaves or flower growth was expressed as the number of leaves or flowers at ($T_x - T_0 / T_0$)*100, where T_0 was the first observation day, i.e. the inoculation day. Plant productivity was expressed as the total sum of flowers/fruits at the end of the cycle. Additionally, the development of *X. fragariae* symptoms over one month after inoculation was monitored. Disease index was calculated as the ratio symptoms levels/number total plants, whereas the disease incidence as the ratio symptomatic plants/number total plants. Disease symptoms scale used was reported in Table S5.2.

Soluble solids content (SSC) was measured on fruit juice with a digital refractometer (Atago-PAL1, Japan) and expressed as °Brix. Fruits color was determined using a CR-400 Chroma meter Colorimeter (Konica Minolta, Japan), whereas fruit hardiness using a Durofel device (Setop, France).

5.2.4 Statistical analysis

Past software (Version 4.0) (Hammer et al., 2001) was used for basic statistical functions. To investigate whether the different bacterial treatments differ from the controls, the Student's t-test was computed. The statistically significant differences evidenced in all analysis was evidenced as P value < 0.05. R studio (Version 1.1.463) and the package ggplot2 (Wickham, 2016) were used for plot representations.

5.3 Results

5.3.1 Functional characterization and selection of PGPB isolated from strawberry for in vivo inoculation

In a previous work, bacteria isolated from bulk soil, rhizosphere, roots and leaves of three strawberry cultivars were screened for their *in vitro* plant growth promoting activities: acetoin, indol-acetic-3-acid (IAA), siderophores and ammonia (NH_4^+) production, antagonism against *Botrytis cinerea* and *X. fragariae* (see chapter 4), and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity were tested. Similarly, in this work 12 bacterial isolates growing epiphytically on fruits of three strawberry cultivars were isolated, identified and functionally characterized (Table S5.1). Altogether, 5 strains from fruits of cultivars 'Monterey', 2 from 'Darselect' cultivar and 5 from 'Elsanta' cultivar were isolated. Among them, only *Pantoea*

agglomerans d15 strain was able to produce IAA, whereas three strains expressed ACC deaminase activity: *Pseudomonas fluorescens* d17 strains, *Pseudomonas jessenei* e39 and *P. agglomerans* d15 *strain. Bacillus pumilus m2*, *Frigoribacterium* spp. e25 and *Pseudoarthrobacter* spp. e4 strains were able to produce acetoin (Table S5.1). *Rhodococcus* spp. m38 and *Pantoea agglomerans* m8 strains were the only strains to be able to antagonize *X. fragariae*.

Beneficial microbes for strawberry root applications were selected on the base of their ability to produce IAA. Indeed, IAA production by rhizospheric microorganisms is a trait that might stimulate and facilitate plant growth (Hayat et al., 2013). Contrarily, for the sole leaves treatment, they were selected for their *in vitro* activity as Biological Control Agents (BCA) of *X. fragariae* and for possessing at least one other PGP functionality (Table 5.1).

Therefore, given the PGP capability, 13 bacteria were selected from the collection of bacterial strains isolated from bulk soil, roots, rhizosphere and leaves of strawberry plants (Table 5.1) (see chapter 4) and 2 bacterial strains were selected from those isolated from strawberry fruits: the IAA producer d15 strain and the *X. fragariae* antagonist m38 strain (Table 5.1; Table S5.1).

Table 5.1. Bacterial strains selected for root and leaves inoculation. In vitro functional characterization was evidenced. Legend: +, normal activity; ++, high activity; -, no activity.

Strain	Species	Cultivar of isolations	Organ of Isolation	Acetoin production	IAA production	Siderophore	Botrytis antagonism	X. fragariae antagonism	NH4+ production	ACC deaminase activity	Organ Of application	Reference
d3	*Brevibacillus brevis*	Darselect	Leaf	-	-	NO	+	++	-	0		
e10	*Massilia* sp.	Elsanta	Rhizosphere	-	-	Carboxilate type siderophores	-	+	+	0		
m35	*Rhizobium soli*	Monterey	Leaf	-	-	NO	-	++	-	0		
m27	*Pseudomonas fluorescens*	Monterey	Root	+	-	Carboxilate type siderophores	-	++	+	0	leaf	See chapter 4
m23	*Stenotrophomonas rhizophila*	Monterey	Soil	-	-	NO	-	++	+	0		
m2	*Bacillus pumilus*	Monterey	Leaf	++	-	NO	-	+	+	0		
m38	*Rhodococcus*	Monterey	Fruit	-	-	NO	-	+	+	0		This work
m40	*Vagococcus*	Monterey	Leaf	-	+	NO	-	+	-	0		
m39	*Agrobacterium rubi*	Monterey	Leaf	-	+	NO	-	++	+	0	leaf/root	See chapter 4
d17	*Pseudomonas fluorescens*	Darselect	Soil	-	+	NO	-	-	-	64±25,61		
e39	*Pseudomonas jessenei*	Elsanta	Rhizosphere	-	++	NO	-	-	+	33,68±6,37		
d15	*Pantoea agglomerans*	Darselect	Fruit	-	+	NO	-	-	++	1,5±6,23		This work
d26	*Pseudomonas siliensis*	Darselect	Soil	-	+	Carboxilate type siderophores	-	-	-	0	root	
d27	*Pseudomonas putida*	Darselect	Soil	+	+	Carboxilate type siderophores	-	-	-	0		See chapter 4
e12	*Pseudomonas fluorescens*	Elsanta	Rhizospere	+	+	NO	-	-	+	0		

5.3.2 Plant growth promotion of inoculated plants under stressed or unstressed strawberries

Roots inoculation

Roots of strawberry plants were singularly inoculated with seven different bacterial strains, both under unstressed (Figure 5.1a) or saline stress (Figure 5.1b) conditions. The strains used for roots inoculation were *Pseudomonas fluorescens* d17, *P. jessenei* e39, *Pantoea agglomerans* d15, *P. siliensis* d26, *P . putida* d27, *P. fluorescens* e12, *Agrobacterium rubi* m39. Almost all of them, except for *A. rubi* m39, were able to increase the single and total fresh weight in leaves. In particular, plants inoculated with e39 significantly increased plant height and weight parameters. Altogether, *P. agglomerans* d15 was the less performative strain, in particular due to its depressive effect on root development. Interestingly, it has been showed that m39 strain was the only strain able to increase plant productivity of 43 % (i.e. flower and fruit production) (Figure 5.1 a).

When saline stress on roots was induced, a more evident beneficial activity of the inoculated bacteria was observed. In particular, plants inoculated with d15, d27 (*Pseudomonas putida*), e12 (*P. fluorescens*) and m39 (*Agrobacterium rubi*) showed an increase of circa 33, 100, 150 and 200% in plant productivity (in terms of flowers and fruits produced), respectively (Figure 5.1b). Leaves growth of differently inoculated plants, both in unstressed or stressed conditions, was recorded over time (Figure 5.2). Overall leaves growth of unstressed inoculated plants was similar to that observed in the control plants (Figure 5.2a). However, under unstressed conditions, a general increase of growth after 16 dai can be observed (Figure 5.2a). In particular, at 19 and 27 dai, as well as at 5 dai, the growth of d17 inoculated plants significantly increased in respect to the control (Figure 5.2a). Additionally, at 27 dai e39 and e12 inoculated plants showed a significantly higher leaves growth in respect to uninoculated plants (Figure 5.2a). Remarkably, under saline conditions, leaves of plants inoculated with bacteria grew less than the control ones (Figure 5.2b). In particular, after 5 days from inoculation with e39 strain, plants showed a significantly lower leaves growth ($p<0.05$) (Figure 5.2b). Similarly, flower growth was recorded over time. Under unstressed conditions, no clear tendency could be found among differently treated plants (Figure 5.2a). However, under saline stress, the application of m39 and e12 strains lead to flower growth picks over several observation days (Figure 5.2b).

Given the ability of m39 to promote plant growth in both conditions, it was selected as candidate for further experiments.

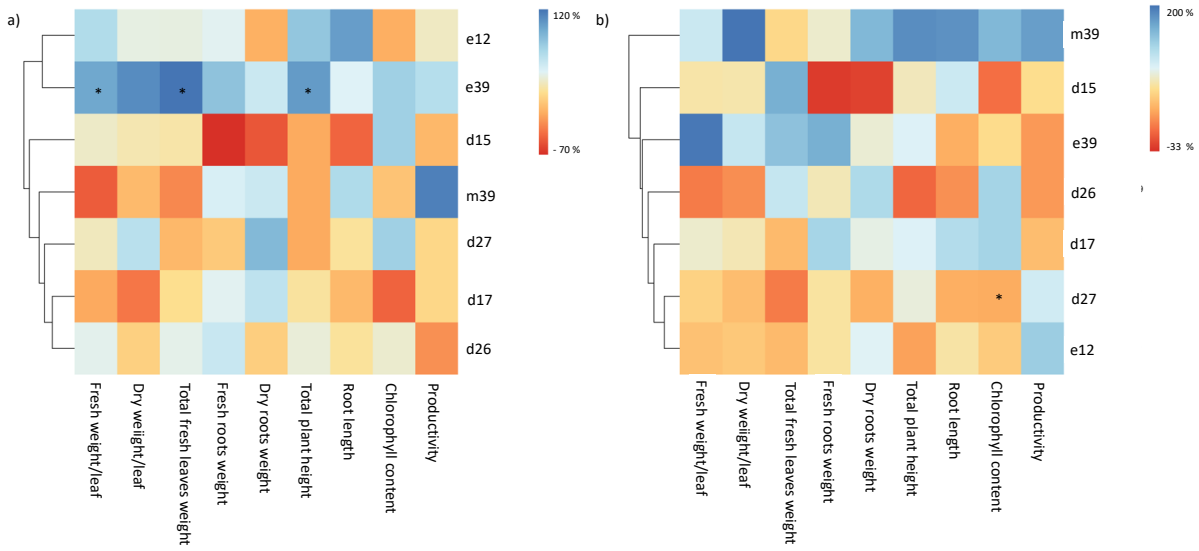

Figure 5.*1* – Biometrics parameters of roots inoculated plants under no stress (a) or saline stress (b) conditions are expressed as percentage increase/decrease in respect to unstressed or stressed water inoculated plants. Color scale ranges from red (lower values in respect to the control) to blue (higher values in respect to the control). Asterisks indicate parameters significantly different from the control, according to Student's t-test at p-value <0.05.

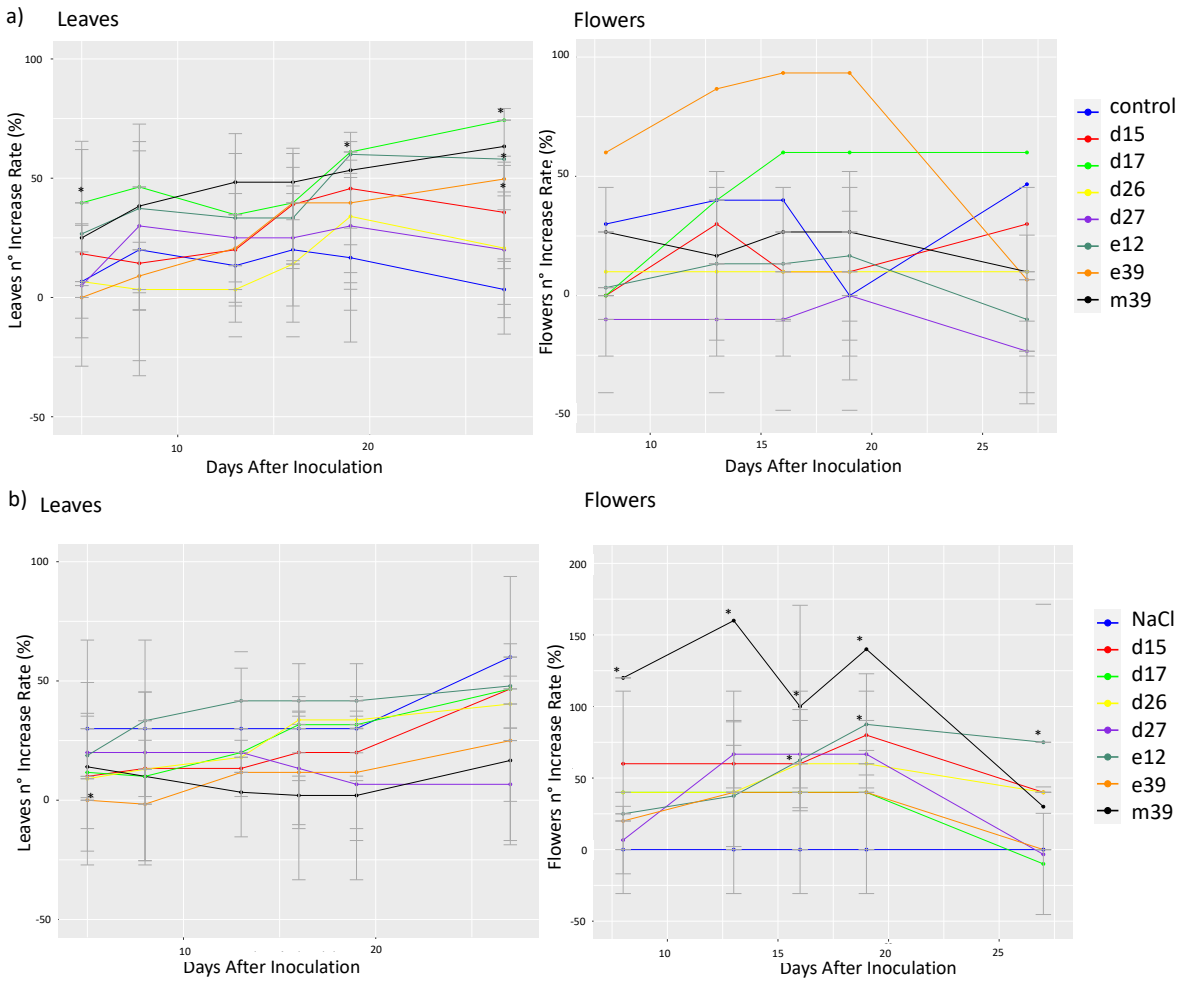

Figure 5.2 – Leaves and flowers growth over time of inoculated plants under no stress (a) or saline stress (b) conditions. Asterisks indicates significant difference in respect to unstressed or stressed water inoculated plants.

Leaves application

Strawberry leaves were singularly inoculated both under unstressed (Figure 5.3a) and stressed (Figure 5.3b) conditions with 9 different PGP bacterial strains. The strains used for leaves application were *Brevibacillus brevis* d3, *Massilia* spp. e10, *Agrobacterium rubi* m39, *Rhizobium soli* m35, *Pseduomonas fluorescens* m27, *Stenotrophomonas rhizophila* m23, *Bacillus pumilus* m2, *Rhodococcus* m38, *Vagococcus* m40. Under normal conditions (Figure 5.3a), leaves application of selected bacteria resulted in a general increase in plant productivity. In particular, *Massilia* spp. e10 and *S. rhizophila* m23 significantly increased plant productivity in respect to control plants. On the other hand, m23 inoculated plants were smaller, with less roots as well as leaves biomass. *Rhodococcus* spp. m38 significantly affected the fresh weight of single and total leaves and lead to plant extension. *Vagococcus* spp. m40 was in general the less performative strain,

showing a percentage decrease in fresh and dry weight/leaf, total fresh leaves weight, dry roots wight and chlorophyll content in respect to the control.

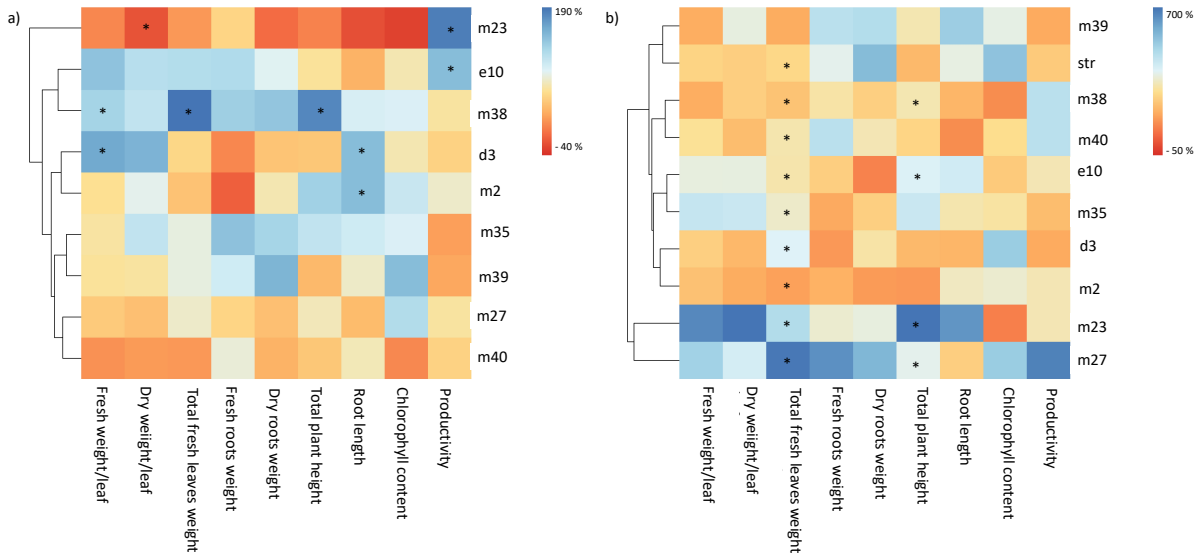

Figure 5.3 - Biometrics parameters of leaves inoculated plants under no stress (a) or pathogenic stress *X. fragariae* (b) conditions are expressed as percentage increase/decrease in respect to unstressed or stressed water inoculated plants. Color scale ranges from red (lower values in respect to the control) to blue (higher values in respect to the control). Asterisks indicate parameters significantly different from the control, according to Student's t-test at p-value <0.05.

In order to test the *in vivo* BCA ability of selected strains, they were applied following leaves inoculation with *Xanthomonas fragariae* and symptoms were monitored over 27 dai. *R. soli* m35 strains showed to be effective in controlling ALS disease, both by lowering the disease index of plants over time (Figure 5.4a) and the disease incidence at 27 dai (Figure 5. 4b). m35 strain was also able to significantly increase the total fresh leaves weight (+76% in respect to plants that were not inoculated with PGP bacteria) as for most part of the strains. However, in respect to the other applied strains, it did not increase plant productivity to such extend. As m35, *P. fluorescens* m27 application, reduced the disease incidence to 20% (Figure 5.4b), but at the same time it was able to increase plant productivity (+700%) and height (+21%) (Figure 5.3b). m2, m23 and m38 strains showed a disease incidence equal to that of the commercial antibiotic streptomycin (Figure 5.4b), whereas m39 and e10 did not protect strawberries from

infection, showing even a faster symptoms development over time in respect to the untreated plants (Figure 5.4a).

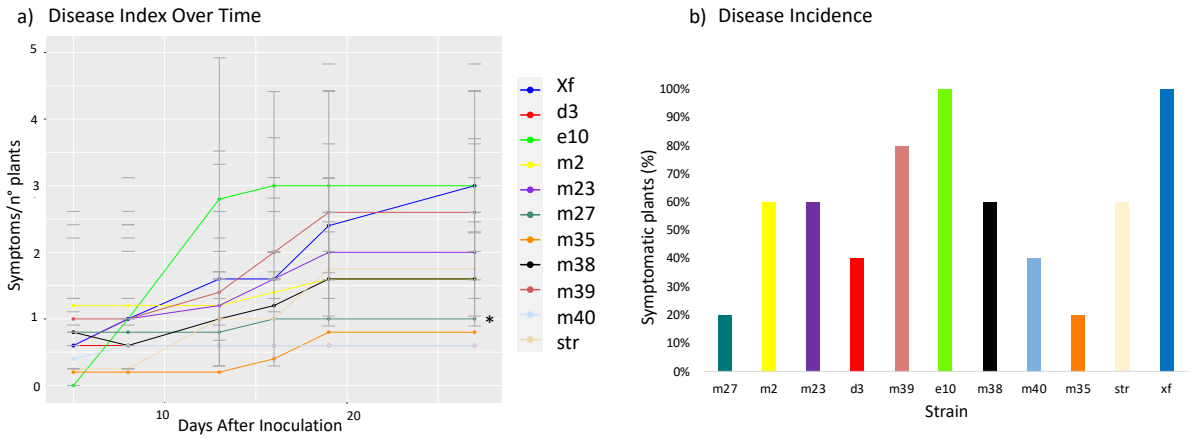

Figure 5.4 – Effect of bacterial inoculation on X. fragariae infected strawberry leaves, expressed as (a) disease index over time or (b) incidence (analyzed at 27 dai). * indicates significant difference (p<0.05) according to Tuckey pairwaise test, in respect to stressed water inoculated plants.

As for plants inoculated on roots with selected bacterial strains, plants inoculated on leaves, both in unstressed or stressed conditions, were monitored over time for their leaves and flowers growth rate (Figure 5.5). Overall, inoculated plants showed a higher leaves growth trend as the untreated one (Figure 5.5a,b). In particular, under unstressed conditions, m2, m23 and m38 strains induced a significantly higher leaves growth rate 19 and 27 dai, whereas plants inoculated with m27, significantly increased their leaves growth 13 and 27 dai (Figure 5.5a). In the case of plants stressed with the pathogen strain *X. fragariae*, at 19 and 27 dai all the applied PGP bacteria showed an increased leaves growth in respect to the control (Figure 5.5b). Flower growth rate recorder over time indicates that in unstressed conditions foliar application of m23, m27 and e10 is significantly higher over time (Figure 5.5a). Interestingly, at the end of the experiment, i.e. 27 dai, m2, m27, e10 and m38 applied strains lead to a significantly higher flower growth (Figure 5.5b).

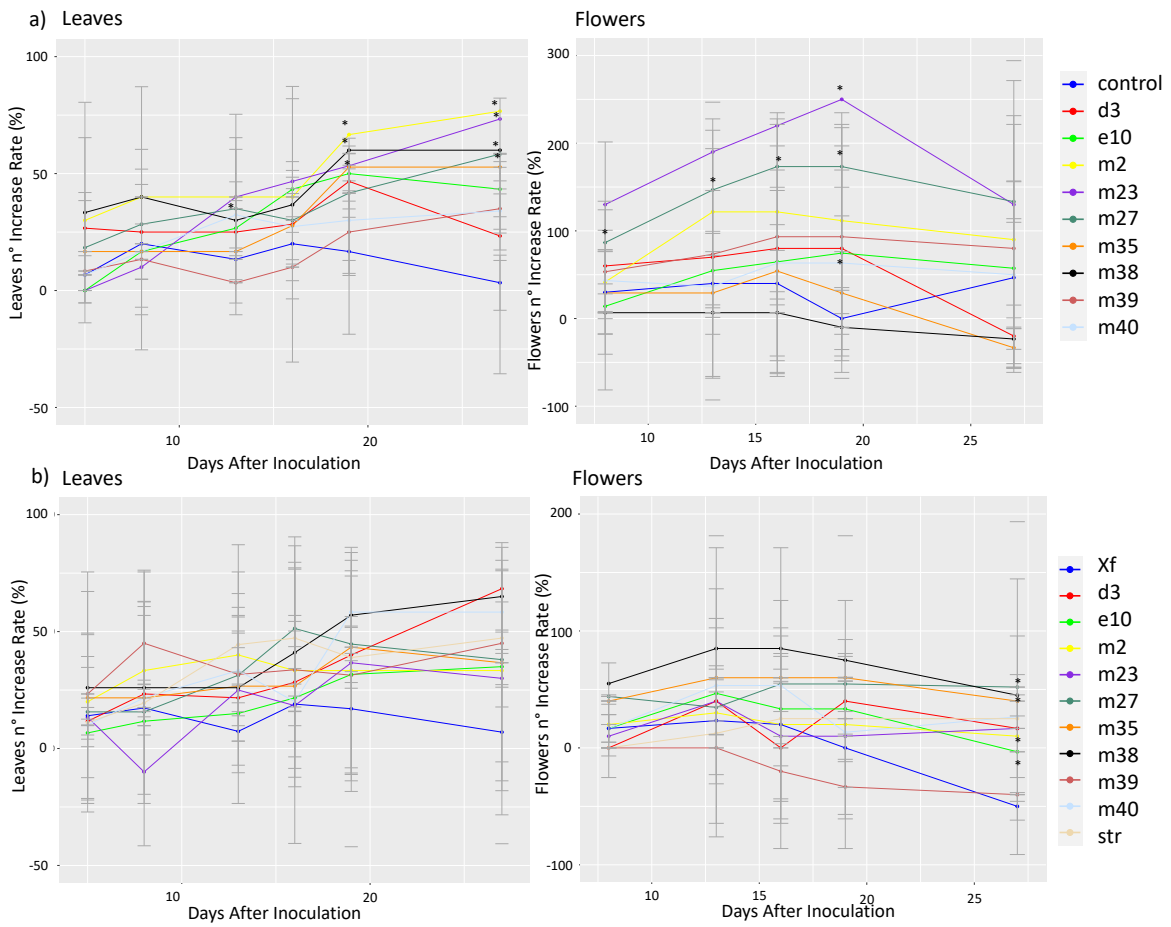

Figure 5.5 - Leaves and flowers growth over time of inoculated plants under no stress (a) or Xanthomonas fragariae stress (b) conditions. * indicates significant difference in respect to unstressed or stressed water inoculated plants.

Based on the obtained results, m23 and m27 strains were selected in order to be further applied on above-ground organs of strawberry plants, leaves and flowers,.

5.3.3 Plant growth promotion of individually- and mixed- inoculated selected bacterial strains

Simultaneous and coordinated application of selected PGP bacteria, each characterized by delivering a specific functional trait to the plant, might be more effective for plant growth promotion than single strain application. In particular, the selection of a particular plant district for the simultaneous application of a cooperative inoculant (CI) can be decisive, allowing to the PGP bacteria a range of action confined or that start from the area in which they are more effective. Therefore, the inoculum of m39, m23 and m27 strains, both individually or in a CI, on roots, leaves and flowers, respectively, was performed. m23 and m27 single application to leaves and flower, respectively, promoted plant productivity in normal condition confirming the results

obtained in the first experiment, even if with a different application of m27. Contrarily, the single root application of m39 strain on 'Monterey' cultivar plants caused a substantial decrease of plant productivity (Figure 5.6a and b). Furthermore, the CI resulted in a general decrease of plant growth parameters, except for plant productivity (Figure 5.6a) which remained unchanged in respect to the control. The CI determined also a statistically significant decrease in hardness of fruits. Plants inoculated on flowers with the CI or the sole m27 strain, presented heavier and bigger fruits in respect to the control, as well as a higher SSC content. Leaves inoculation with m23 strain resulted in harder fruits with a higher SSC content (Figure 5.6b).

The leaves growth was observed over time. The application of m39 strain on roots significantly promoted leaves growth at 27 dai, whereas the application of m27 strain on flowers resulted in a significant lower leaves growth in respect to the control, at 14 and 19 dai (Figure 5.6c)

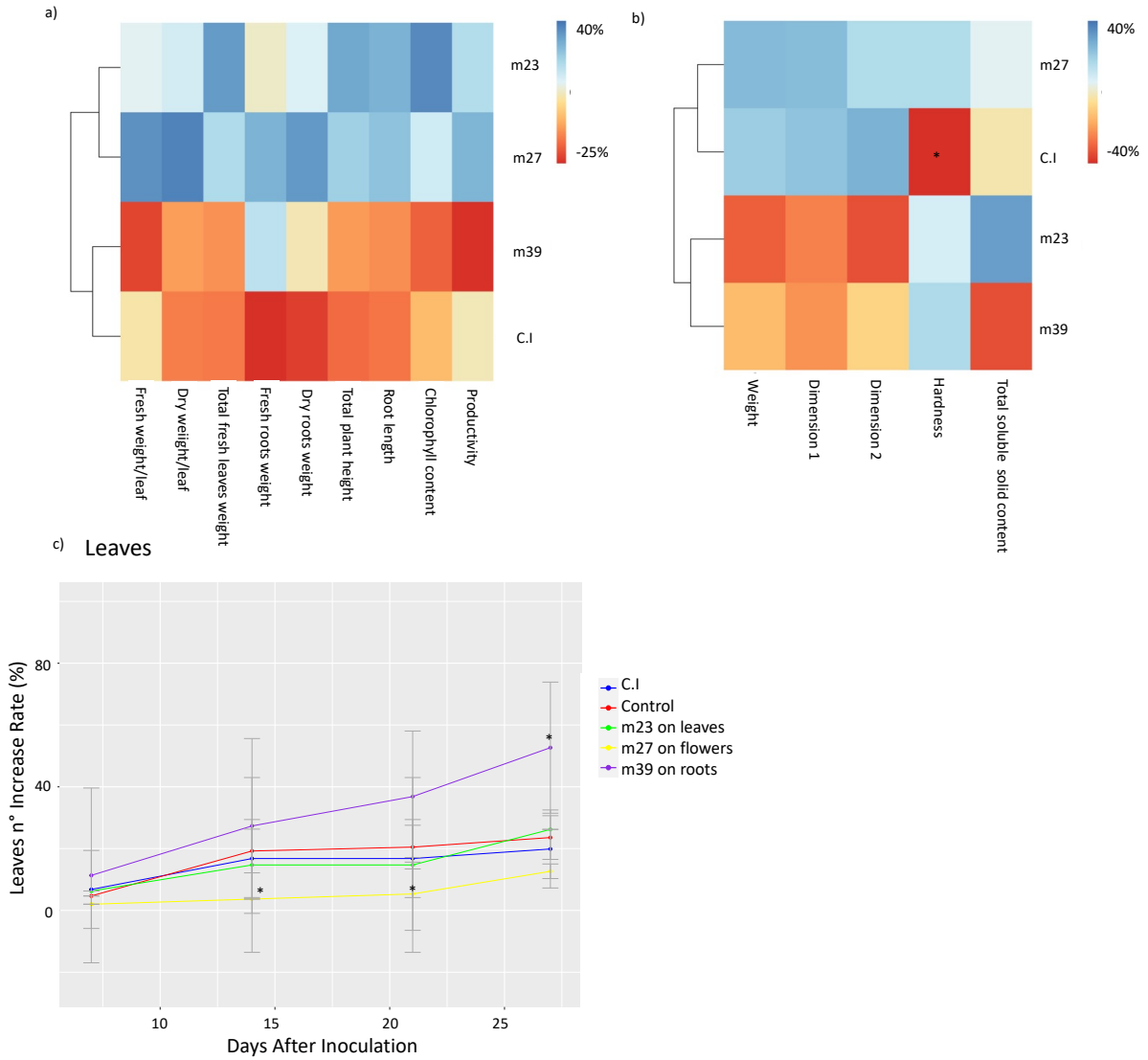

Figure 5.6 –Plant growth biometric parameters (a) and fruit quality parameters (b) heatmaps of individually and coordinated inoculated plants, expressed as percentage increase/decrease respect to the control plants. Leaves growth over time (c) and coordinated inoculated plants Color scale ranges from red (lower values in respect to the control) to blue (higher values in respect to the control). * indicates significant difference in respect to water inoculated plants.

5.4 Discussion and Conclusions

Strawberry is a highly popular fruit, characterized by a recognizable taste and aroma. Although strawberry is spread worldwide, its cultivation faces many challenges, such as biotic and abiotic stresses (Husaini and Xu, 2016), leading to considerable economic losses. Since decades, the existence of an association between plants and microorganisms is well known (Bulgarelli et al., 2013), and several of those microorganisms have been also characterized and applied for their ability to improve

plant growth. Indeed, the plant core microbiota has essential functions for the inhabited plant, contributing to its overall stability and fitness maintenance: traits implicated in plant hormonal balance, molecular communication, plant nutrition, drought tolerance and phytoprotection (Checcucci and Marchetti, 2020).

Even if in strawberry plants such microorganisms have been identified (Husaini et al., 2015), as for several other crops, PGPB and BCA can lack in consistency and effectiveness when applied in the field (Sessitsch et al., 2019). This is partly due to the fact that the applied inoculation is generally constituted by microbes not native of the plant to which they are applied, and thus not able to establish in the pre-existing microbiota (Mazzola et al., 2017). Furthermore, it is necessary to account for the area of action of PGPB used as bioinoculants: indeed, it is known that plants exhibit different ecological niches in their organs, which can select for specific microbiota composition (Bai et al., 2015). Thus, it is possible that microorganisms activity, such as that of plant growth promotion, can be more efficient in the plant organ from which they are originally isolated (Checcucci et al. 2016). Nevertheless, it is possible that plant endophytes show a particular or unexpected ability in a plant tissue other than that from which they are usually resident. Likewise, plant endophytes community can be shaped by different growth conditions (Compant et al., 2016), including biotic and abiotic factors.

In this work, we set up an *in vivo* system to determine which PGPB, among the one characterized and selected isolated from strawberry cultivar 'Monterey', were able to benefit plant fitness. Furthermore, the most performant strains, based on the results obtained in the first experiment, were mixed in a coordinated bioinoculant.

Initially, the strains used as roots bioinoculants, were those able to produce indol acetic acid (IAA). IAA is known to be the most common auxin in plants that positively influences root growth and morphology (Salisbury, 1994). In particular, it is largely known that IAA protects plants from abiotic stresses, such as drought and salinity, and triggers plant growth by an increased nutrient absorption from the soil (Etesami et al., 2018).

As expected, the application of PGPB producing IAA led to a general increase of plant growth and productivity, showing a more noticeable positive effect under saline stress condition. Therefore, it is possible to hypothesize that the consequent flowering picks observed might be due to the recently discovered auxin mediated flowering pathway (Dong et al., 2021). Different IAA producing bacterial strains provided variable levels

of growth promotion effect to the plant. This might be due to their different ability to produce the hormone *in vivo* and to efficiently colonize the plant (Asghar et al., 2002). Under unstressed conditions, application of *P. jessenii* e39 to strawberry roots caused a significant increase in fresh leaves weight and plant height. Interestingly, *P. jessenii* has been found to provide protection against a wide range of plant pathogens (Deora et al., 2010), but no plant growth promotion effect following its application has been observed yet. Under saline stress, e39 treated plants showed a significant decrease in leaves growth at 5 dai. This might be due to the fact that although the inoculum contained a beneficial bacterium, the application of an external factor can cause a physiological stress response in the plant. After the observation at 5 dai, leaves growth of e39 treated plants increased, probably due to the positive effect of ACC deaminase enzyme that systemically lowers ethylene levels (Glick, 2014). However, as for all the applied strains, leaves growth of inoculated plants was lower than that of control plants. *P. putida* d27 was among the less performative strains both in normal and stressed conditions. In particular, its application led to a significant decrease of the chlorophyll content (in saline conditions) which is in contrast with previous work with other *P. putida* strains that caused an increased chlorophyll content in salt and drought stressed plants (Kang et al., 2014).

When applied to roots, *A. rubi* strain m39, although initially isolated from the leaf district of the same cultivar, was the sole, among PGPB tested, able to enhance plant productivity (+200% in respect to the control) in both unstressed and stressed conditions. This capability seems to be shared with *A. rubi* A18 strain, isolated from sweet cherry and applied on strawberry plants. Indeed, it was found to promote plant growth and productivity in the inhospitable calcareous soils (Ipek et al., 2014). Although *A. rubi* m39 was not isolated from strawberry roots, its application as bioinoculant was able to determine a positive effect in both roots growth in stressed and unstressed condition. Contrarily, application of m39 on strawberry roots did not cause an increase in leaves growth in respect to the control plants. Similarly, when applied to leaves, m39 did not show any relevant PGP trait in any condition tested. These results seem to be in contrast with the hypothesis that bacteria isolated from a certain plant organ, when reinoculated onto the same district, are more performant than when applied in different areas. The bacterial diffusion inside the plants through xylematic vassels represents the main way used for tissues and organs colonization by endophytes (Frank et al., 2017). Therefore, we can hypotesize that the original

isolation of m39 strains from strawberry leaves does not compromise its ability to spread as needed in roots, where it carries out its PGP activity.

Bacterial strains to be applied on the leaves were selected based on their *in vitro* ability to limit *Xanthomonas fragariae* pathogenicity. *X. fragariae* is the most problematic bacterial disease of strawberries. Nowadays, it is widely widespread in Europe, North and South America, Iran and Ethiopia (EPPO). Although symptoms appear as lesions on leaf surface and fruit do not show any symptom, yield losses due to this pathogen may account up to 80% (Epstein, 1966). The bacteria selected were also characterized by possessing at least one PGP trait. Indeed, we can hypothesize that the development of a commercial product based on a bacterium showing both BCA and PGP activity could be an advantage when applying the product on an unevenly diseased field or when pathogen presence is unclear, delivering beneficial functions even to healthy plants. Following leaf bacterial application, it was possible to observe the significant increase of several different plant growth parameters. *Massilia* is a bacterial genus commonly found in soil and plant roots (Ofek et al., 2012). Several *Massilia* strains are characterized by plant growth promoting traits, such as phosphate solubilization (Zheng et al., 2017), or disease suppressive abilities against plant pathogens such as *Rhizoctonia solani* (Giuliano et al., 2018). In this work, under unstressed conditions, plant treatment with *Massilia* spp. e10 led to a significant increase in plant productivity, whereas when leaves were challenged with the bacterial pathogen *X. fragariae*, plants were significantly taller in respect to control ones. Another bacterium that efficiently promoted plant growth was *Rhodococcus* spp. m38, which significantly increased leaves fresh weight, and the plant height. Interestingly, *Rhodococcus* has been already found to promote the growth of *Pisum sativum* under toxic Cr^{6+} concentrations (Trivedi et al., 2007). However, in our work, *Rhodococcus* m38 was not able to promote strawberry plants growth under stressed conditions and thus was not considered for further experiments. In accordance with previous works (Nehra et al., 2016), *Brevibacillus brevis* d3 application was proven to increase plant fitness by enhancing root length and leaves fresh weight but was not able to control ALS *in vivo*, although total fresh leaves weight significantly increased in stressed conditions.

Among the bacteria applied on leaves, *S. rhizophila* m23 strain application lead to higher plants productivity under no stress conditions, although leaf dry weight was significantly reduced, and providing a good protection from *X. fragariae* disease. Additionally, under stressed conditions, m23 treated plants displayed a significantly higher total plant height. Indeed, *S. rhizophila* is a species commonly found in plants,

isolated by the authors in rhizosphere, but also associated to stems and leaves, and it has been found to exert biological control properties against fungal pathogens (Wolf et al., 2002) and to promote plant growth altering plants fungal microbiota (Schmidt et al., 2012). Besides, *P. fluorescens* m27 was selected for its ability to provide a significant good level of protection from *X. fragariae*, coupled with a higher plant productivity in respect to control plants. Several *P. fluorescens* strains, also non-native, have been proven to promote plant growth leading to flowering anticipation, fruit yield and vitamin content increase (Bakker et al., 2007; Todeschini et al., 2018) whereas no strain has yet been proven for its biological control activity against *X. fragariae*.

Overall, it is interesting to note that, in almost all cases, the same bacterial strain showed different plant growth promoting performances under unstressed or stressed conditions. For instance, application of m27 strain on *X. fragariae* infected leaves led to a significant increase in total fresh leaves weight and plant height, whereas no effect was observed in unstressed plants. Similarly, in stressed condition m39 treated plants showed a more expanded foliar biomass, whereas it decreased in respect to the control in unstressed plants. When in stress conditions, some strains showed a more evident beneficial effect on plant growth. Indeed, it was demonstrated that the beneficial role of PGPB bioinoculation is more relevant under abiotic and biotic stress, as if their abilities were activated and enhanced by unfavorable conditions. Indeed, microorganisms ubiquities and their adaptation characteristics developed during evolution allow them to adapt to the most stressful conditions, bringing these abilities to the plants they are associated with (Choudhary et al., 2016).

Under stressful conditions, plant microbiota effects can be reinforced by the presence of newly recruited beneficial microorganisms, gradually promoting microbial populations bearing specific functions within rhizosphere and plants vessels diffusion (Lemanceau et al., 2017). Indeed, under stressed conditions, for instance in nitrogen limiting conditions, plants might recruit specific beneficial bacteria to overcome shortages (Hassan and Mathesius, 2012). Therefore, the application of specific microbial bioinoculants can improve plant fitness if they are able to activate their potential when needed by the host plant.

Collaboration between plant and microbes depends on a large plethora of factors. In particular, partners genotype compatibility should receive prime consideration during the bacteria selection process, considering that host genotype has a significant effect on the composition of the associated bacterial community (Bulgarelli et al., 2015; Szymańska et al., 2021).

Furthermore, besides PGP microorganisms selection, bioinoculants preparation might become a particularly critical point (Szymańska et al., 2021). Indeed, bioinoculants efficiency analyzed under laboratory condition cannot correspond to that observed under fields condition, considering the adverse environmental conditions and the low compatibility of microorganisms towards native plant microbiota (Montesinos, 2003; Tabassum et al., 2017). Therefore, selecting Plant Growth Promoting microbes among the microbiota native to the plant, might easily promote plant growth and development because the genotype-genotype selection among partners has been already settled by evolution (Haney et al., 2015; Santhanam et al., 2015; Mazzola and Freilich, 2017). In line with what stated, the first experiment highlights a better performance of strains originally isolated from 'Monterey', the cultivar used in this work, confirming the highest efficiency demonstrated by native bacteria (Checcucci et al., 2018). However, the efficacy of the bacterial inoculations has not been tested on different strawberry cultivars. Therefore, further experiments would eventually confirm this hypothesis.

One of the main strategies used for microorganisms inoculation, is the combination of different microbes (including both bacteria and fungi kingdom) characterized by having different PGP abilities. The enhancement of plant growth following a co-inoculation is due to the synergic effect of different microorganisms, which increase the beneficial effect contributed by the others (Hillel and Jerry, 2005). Nevertheless, in this work, the results obtained from the contemporary application of the three selected strains in the three different plant organs on which they demonstrated a real beneficial effect on the overall plant growth, showed a decrease of strawberry biometric parameters in respect to those observed for single strain inoculations. Several hypothesis could be formulated: i) the plant could have the necessity to regain its balance following the addition of a high exogenous bacteria concentration, indeed the application of bacterial inoculants might affect the native microbial communitites resulting in a modulation of the PGPB applied, both in a positive or negative way (Castro-Sowinski et al., 2007); ii) the beneficial effect delivered by microbes-based bioinoculant might be observed in the following plant generation or after long time (Frank et al., 2017; Walkiewicz et al., 2020); iii) although the inoculated strains are part of the native plant microbiota, the initial application produces a change in its composition, at least in terms of taxa abundance. In particular, this can happen if the strains inoculated were applied where they are more efficient and not on the organs where they were initially isolated (Naiman et al., 2009; Kozdrój et al., 2004; Zhong et

al., 2009); iv) given that PGPB application might provoke endogenous changes in plant hormones levels (Kurepin et al., 2015; Tsukanova et al., 2017), the simultaneous application of PGPB might have caused an hormonal imbalance to the plant, stimulating plant detrimental reactions (not observed when PGPB were singularly inoculated).

Therefore, it will be worth to set future studies in which the application of PGP microorganisms in a coordinated bioinoculant will respect their native niche. Indeed, it is possible that, starting from the organ in which they are resident, they should head particular function towards other plant organs. Of course, it is necessary to account for natural endophytes, that are able to influence the movements of inoculated microbes inside plant and shape thee expression of their PGP potential (Maggini et al., 2017).

5.5 Supplementary Material

Table S1. Bacteria isolated from strawberry fruits with their respective functional characterization.

Cultivar	Code	acetoin production	IAA production	siderophores	Botrite antagonismo	Xf antagonismo	NH4+ production	accd activity (uM/h)	
darselect	9	++	-	NO	-	-	++	0	**Bacillus pumilus**
elsanta	25	+	-	NO	-	"+/-"	+	0	**Frigoribacterium sp.**
monterey	34	-	-	NO	-	-	+	6,09±6,90	**Methylobacterium sp.**
elsanta	38	-	-	NO	-	-	-	0	**Microbacterium sp.**
darselect	15	-	+	NO	-	-	++	1,5±6,23	**Pantoea agglomerans**
monterey	8	-	-	Carboxilate type siderophores	-	++	+	0	**Pantoea agglomerans**
elsanta	4	+	-	NO	-	-	-	0	**Pseudoarthrobacter sp.**
monterey	7	-	-	Hydroxamates siderophores	-	-	-	135,22±3,98	**Pseudomonas fluorescens**
elsanta	41	-	-	NO	-	-	"+/-"	0	**Psychrobacillus sp.**
monterey	42	-	-	NO	-		+	0	**Psychrobacillus sp.**
monterey	38	-	-	NO	-	+	+	0	**Rhodococcus sp.**
elsanta	1	-	-	NO	-	-	-	0	**Vagococcus sp.**

Table S2 – Visual *Xanthomonas fragariae* symptoms scale. 0=no symptoms, 5=severe symptoms

5.6 References

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6. Investigating the effect of cultural management on microbiome, quality and aroma of raspberry fruits

Abstract: Plant-associated microbes can shape plant phenotype, performance, and productivity. Cultivation methods, such as Integrated Pest Management (IPM) or organic production, can influence the plant microbiome structure and differences observed in the nutritional quality of differently cultivated fruits might be due to variations in the microbiome taxonomic and functional composition. Here, the influence of organic and IPM cultivation on quality, aroma and microbiome of raspberry (*Rubus idaeus* L.) fruits was evaluated. Differences in the fruit microbiome of organic and IPM raspberry were examined by next-generation sequencing and bacterial isolates characterization to highlight the potential contribution of the resident-microflora to fruit characteristics and aroma. The cultivation method strongly influenced fruit nutraceutical traits, aroma and epiphytic bacterial biocoenosis. Organic cultivation resulted in smaller fruits with a higher anthocyanidins content and lower titratable acidity content in comparison to IPM berries. Management practices also influenced the amounts of acids, ketones, aldehydes and monoterpenes, emitted by fruits. Our results suggest that the effects on fruit quality could be related to differences in the population of *Gluconobacter*, *Sphingomonas*, *Rosenbergiella*, *Brevibacillus* and *Methylobacterium* on fruit. Finally, changes in fruit aroma can be partly explained by volatile organic compounds (VOCs) emitted by key bacterial genera characterizing organic and IPM raspberry fruits.

6.1 Introduction

Raspberry (*Rubus idaeus* L.) is a highly valuable crop with a large agricultural potential, both in Europe and worldwide. Raspberry consumer demand, as well as its cultivation, have been steadily increasing in the past 20 years, with a further acceleration since 2010 (FAOSTAT). Russia is the top producing country, with a production volume of 165,800 tonnes accounting approx. for 20% of the world production (FAOSTAT). Raspberry is also widely cultivated in other European (Serbia, Poland) and north-American (Mexico, USA) countries (FAOSTAT; Girgenti et al., 2013).

Hedonic characteristics and nutraceutical properties of raspberry are the main drivers of consumer appreciation. In fact, raspberry is rich in health-beneficial compounds,

such as flavonols, catechins, ascorbic acid and ellagic acid derivatives (Stavang et al., 2015;Valentinuzzi et al., 2018). Additionally, more than 300 volatile organic compounds (VOCs) have been reported as constituents of raspberry aroma (Aprea et al., 2015), including alcohols, aldehydes, ketones, esters and terpenoids (Aprea et al., 2009; Estrada-Beltran et al., 2020). Among the main aroma-active compounds (i.e., those with low perception threshold compared to emission rates), C6 aldehydes (hexanal, hexenal isomers) and norisoprenoids (e.g. ionones) are responsible for grassy and floral notes of raspberry fruit aroma (Zhang et al., 2021).

The organic farming system consists of a low-input crop management (EC regulation No. 834/2007) excluding synthetic pesticides and fertilizers for plant protection and nutrition (Lupatini et al., 2017). In the last decade, organic food gained in popularity and the global market for certified organic products underwent a remarkable increase in sales (+72% 2009–2014) (Eyinade et al., 2021). Berry fruits have followed this trend, with a rise in the organic production from around 30 tonnes in 2012 to 70 tonnes in 2018 (EUROSTAT). However, the fulfilment of the increasing demand of organic raspberries faces several obstacles mining the growers profitability (Bodiroga and Sredojević, 2017). High costs are linked to the conversion of farms from integrated pest management (IPM) to the organic system and to obtaining of certifications, which are necessary for organic labelling, highly valued by consumers (Girgenti et al., 2013). Additionally, organic raspberry farming has been found to be characterized by lower yields (Bodiroga and Sredojević, 2017; Frias-Moreno et al., 2018). Furthermore, raspberry cultivation is affected by several pests and pathogens, including the insects *Resseliella theobaldi* (Diptera, Cecidomyiidae), *Lasioptera rubi* (Diptera, Cecidomyiidae), *Anthonomus rubi* (Coleoptera, Curculionidae), *Aphis idaei* (Hemiptera, Aphididae) and *Drosophila suzukii* (Diptera, Drosophilidae), the mite *Tetranychus urticae* (Acarina, Tetranychidae), the oomycete *Phytophtora fragariae* var. *rubi,* and the fungi *Leptosphaeria coniothyrium, Didymella applanata, Botrytis cinerea* and *Sphaerotheca macularis* (Barrel and Diemoz, 2011). Such pests and pathogens can compromise plant survival and/or fruit quality in the absence of adequate control measures (Dolan et al., 2016; Perpetuini et al., 2019). In organic systems, control inputs, besides being characterized by high prices, are few and specialized literature regarding appropriate control methods is lacking (Bodiroga and Sredojević, 2017). On the other hand, growers profitability for organic food products is generally higher than IPM ones (Bodiroga and Sredojević, 2017) due to the application of premium prices consumers are willing to pay (Hemmerling et al., 2015). Indeed, in developed countries, organic

food is considered safer and healthier (Orsini et al., 2016; Funk and Kennedy, 2016), as well as better for the climate and the environment (Seufert et al., 2017) when compared to IPM grown one. Several studies observed higher contents of polyphenols, vitamins, carotenoids and ellagic acid, as well as fruit dry matter, sucrose, malic acid and minerals, in organic berries (Di Vittori et al., 2018). However, other studies came to contrasting conclusions in establishing causalities between improved nutritional properties and organic cultivation system which might be due to the numerous variables interacting with the final fruit quality.

Plants are known to host a great diversity of microbes, whose composition and functionalities can shape plant phenotype, performances and productivity (Bulgarelli et al., 2013; Lemanceau et al., 2017). The increasing access to genomic data has allowed a more in-depth investigation of the microbial communities associated to the plant (Orozco-Mosqueda et al., 2018) and of their interaction with the host, pointing to a role of crop-associated microbes in agricultural performance and crop quality (Andreote et al., 2017; Sangiorgio et al., 2020). Although in the last few years several studies focused either on the microbial diversity present on various fruit species (Abdelfattah et al., 2018) or on the impact of root or foliar microbial applications on nutrient, flavonoid, organic acid contents and volatile compounds levels of fruits (Jiménez-Gómez et al., 2017), a deep understanding of the influence of native fruit epiphytic microbiomes on fruit quality and on fruit volatilome building is still lacking.

Cultivation methods can influence the composition and structure of the plant microbiome (Wasserman et al., 2019) and differences observed in the nutritional quality of differently grown fruit (Mditshwa et al., 2017) might be due to variations in the microbiome structure. Although dynamics of the soil microbiota under different cultivation methods have been extensively investigated (Ishaq et al., 2016; Suzuki et al., 2019; Bill et al., 2021), only a few studies focus on the influence of cultivation practices on fruit microbiomes (Wasserman et al., 2019). Thus, the aim of this work was to comprehensively dissect the effect of organic or IPM cultivation on fruit microbiome, fruit quality characteristics and aroma. Contextually, differences of organic and IPM raspberry fruit-associated microbiomes were examined by next-generation sequencing (NGS) and bacterial isolates characterization in order to highlight the potential contribution of the resident microflora to fruit quality and aroma.

6.2 Materials and Methods

6.2.1 Plant Material and Cultivation Method

Raspberry fruit of the primocane 'Enrosadira' were harvested at the end of September 2019 in two different farms both located in the Cesena area (Emilia-Romagna region, Italy). The organic orchard was cultivated according to European Union (EU) regulation EC 889/2008. An IPM orchard was cultivated according to Agrintesa guidelines which comply with the national regulations (Legislative Decree 50/2012). In the latter orchard, IPM Epik®, Karate Zeon®, Laser® and Signum® were applied for the control of pests and fungal pathogens, using dose and application methods as suggested by the producers. The organic farm did not apply any kind of fertilization, whereas in IPM orchard, plants were fertigated weekly from April to September according to standard practices: calcium nitrate (125 kg/ha), magnesium nitrate (600 kg/ha), ammonium nitrate (100 kg/ha), potassium nitrate (250 kg/ha), potassium phosphate (200 kg/ha), monopotassium phosphate (300 kg/ha), nitric acid (52%, 1000 L/ha), iron (25 kg/ha) and microelements (15 kg/ha) in variable percentages depending on the phenological state.

To assess fruit quality, aroma and microbiome, fruits were randomly harvested at full ripe stage (i.e., when fruits were easily detached from the receptacle), put in cold boxes and immediately brought to the laboratory. Fruit volatiles and NGS analysis were performed on the same batch of fruits, consisting in three replicates each constituted by a pool of six fruits. The material was kept together for 24 h and subsequently halved for analysis processing.

6.2.2. DNA Extraction and Next-Generation Sequencing for Microbiome Analysis

To perform metagenomic analysis, three biological repetitions, consisting in a pool of three raspberry fruits, were washed in 20 mL $MgSO_4$ 10 mM for 5 min under gentle agitation (70 rpm). Washing suspensions were frozen and stored at −80 °C. DNA was extracted according to the CTAB protocol (Maguire et al., 1994), using the pellet obtained from centrifuging the washing solutions at 13,000× *g* for 10 min. DNA quality and quantity were measured by spectrophotometric quantification with a NanoDrop ND-8000 V1.1.1 spectrophotometer (Thermo Fisher Scientific, Dreieich, Germany). Bacterial V3-V4 regions were amplified with 16S Amplicon polymerase chain reaction (PCR) Forward = 5_TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCA

G and Reverse = 5_GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCT AATCC primers according to Illumina protocols and subjected to automated sequencing by BioFab research (Rome, Italy).

6.2.3 Bioinformatic Analysis

BBMap version 38.79 was used to remove low-quality reads using a quality-trim left and right ends before mapping with an average phred score ≥ 25 (Bushnell, 2014). Sequences shorter than 170 bases were discarded. The sequences were analysed using the Qiime2 v. 2020.2.0 (Bolyen et al., 2019). Qiime2 dada2 plugin was used to length trimming, denoising, chimera and PhiX removal. SILVA 16S rRNA sequences database, release 312 (Quast et al., 2021), with a 97% identity criterion was used to assign taxonomy to features. Taxonomic composition of organic and IPM raspberry fruit microbiomes was visualized by means of Krona chart created using Krona web application (https://github.com/marbl/Krona/wiki, accessed on 20 January 2021).

6.2.4 Cultural Dependent Characterization of Fruit Microbiome

Raspberry fruit washing obtained as above were serially diluted and plated on Luria-Bertani agar medium (Sigma Aldrich, St. Louis, MO, USA) amended with cycloheximide (100 μg/mL) to prevent fungal growth. Plates were incubated at 27 °C for 24 h. Colonies were randomly collected from the plates at the highest dilution. Isolates were stored at −80 °C in LB broth supplemented with 20% glycerol (v/v). DNA was isolated from bacterial isolates using GenElute Bacterial Genomic DNA kit (Sigma Aldrich, St. Louis, MO, USA), following manufacturer's instructions. Genomic DNA patterns obtained by REP-PCR (Repetitive Element Palindromic PCR) and performed using primer BOXA1R (De Urraza et al., 2000), as described in (Gevers et al., 2001), were visualized on a polyacrylamide gel (Filippetti et al., 2005) in order to screen for bacterial isolates showing equal patterns and later perform identification and functional characterization only on one of them.

Bacterial isolates were identified by 16s rRNA sequencing as follows: 16s rRNA gene extraction was amplified using Lac16S-for (5′-AATGAGAGTTTGATCCTGGCT-3′) and Lac16Srev (5′-GAGGTGATCCAGCCGCAGGTT-3′) primers, as described in (Bringel et al., 2005). The amplification product was delivered to Biofab Research Srl

for sequencing. Sequences were compared with those available in http://www.ncbi.nml.nih.gov/BLAST, accessed on 15 January 2021.
Bacterial isolates were screened for several functional traits, according to the following protocols: indole-3-acetic-acid production (Ahmad et al., 2008); acetoin production (Blomqvist et al., 1993); ammonia production (Cappuccino and Sherman, 1992); siderophores production (Pérez-Miranda et al., 2007); 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity assay (Penrose and Glick, 2003).

6.2.5 Assessment of Fruit Quality Parameters

To measure soluble solids content (SSC) and fruit titratable acidity (TA), fruit juice was obtained homogenising berries and sieving them through a cloth. SSC of fruits was determined by digital refractometer (Atago-PAL1, Tokyo, Japan) and expressed as °Brix. Titratable acidity was determined manually by adding 0.1 M NaOH dropwise until reaching the titration end-point. Analyses were performed on nine replicates consisting of the juice from a pool of three berries each. Prior to homogenization, fruit colour was determined using a CR-400 Chroma meter Colorimeter (Konica Minolta, Tokyo, Japan) on each berry. Three measurements were performed on the same fruit.
To determine the anthocyanins content, 3 g of homogenized fruit were suspended in 25 mL of methanol and incubated 24 h at room temperature. Samples were then centrifuged for 5 min at 4000× *g* and the supernatant was stored at −20 °C. Anthocyanin content was determined on the supernatant by using high-performance liquid chromatography (HPLC, Waters 1525, Waters, Milford, MA) at 520 nm according to (Mattivi et al., 2006).
Quantification was performed with the external standard method with the standards calibration curves (different concentrations were used to build the curve: 1–100 μg mL^{-1}). Malvidin-3-glucoside chloride (Sigma Aldrich, St. Louis, MO, USA) was used as standard. The reliability of the quantification method was assessed by the R^2 value of the standard and assessing that the concentration of each quantified compound was within the concentration range of the standard. Anthocyanin identification was performed according to (de Ancos et al., 1999).

6.2.6 Fruit Volatile Analysis

Raspberry fruit volatiles were analysed by gas chromatography–mass spectrometry (GC–MS). Each replicate (n = 3) consisted of a pool of three berries. Berries were put in a glass vial, closed with a lid equipped with a pierceable silicon cap and stored at −80 °C. Samples were taken out from −80 °C and put at −20 °C for 20 min before equilibration. After adding 1-octanol 0.05% (*v*/*v* in H_2O) as internal standard, equilibration was performed for 20 min at 40 °C. Samples were then exposed to a 50/30-μm divinylbenzene/carboxen/polydimethyl siloxane (DVB Carboxen PDMS) Stable Flex 2 cm solid phase microextraction (SPME) fibre (Supelco, Bellefonte, PA, USA) for 40-min exposure. Afterwards, the SPME fibre was desorbed in a Shimadzu GC-MS-QP2010 Plus (Shimadzu, Tokyo, Japan) at 250 °C for 10 min in the split mode. The chromatographic separation of volatile compounds was performed on an RTX-WAX fused-silica capillary column (30 m × 0.25 mm i.d. × 0.25 μm) coated with polyethylene glycol (PEG) (Restek, Bellefonte, PA, USA). A three-step heating oven program was set: (1) 45 °C, 10 min; (2) 4 °C min^{-1} temperature increase to 200 °C; (3) 200 °C, 8 min. Helium was used as the carrier gas at a constant flow rate of 1 mL/min. A mass range from 33 to 400 *m*/*z* was scanned at a rate of 769 amu s^{-1}. Mass spectra and linear retention indices (calculated according to the retention times of linear C8–C20 standard alkanes) were used for the identification of volatile compounds, based on the NIST/EPA/NIH Mass Spectral Database (NIST 08, National Institute of Standards and Technology, Gaithersburg, MD, USA) and the ChemSpider information resource (http://www.chemspider.com, accessed on October 2020).

6.2.7 Bacterial Volatile Analysis by Proton-Transfer Reaction−Mass Spectrometry (PTR–MS) and in Silico Fruit Volatilome Assembly

Ten mL commercial raspberry juice (Bocon, Italy) diluted with distilled water to 75%, adjusted to pH 7, were inoculated with 100 μL of freshly grown bacterial culture and incubated for 24 h. The experimental set foresaw three replicates for each bacterial isolate. Headspace volatiles were measured as described in (Farneti et al., 2017) by using a proton-transfer reaction time of flight–mass spectrometer (PTR-ToF–MS) 8000 (Ionicon Analytik GmbH, Innsbruck, Austria). The drift tube conditions were as follows: 110 °C drift tube temperature, 2.8 mbar drift pressure, 428 V drift voltage, ion funnel (18 V). This leads to an E/N ratio of about 130 Townsend (Td), with E corresponding to the electric field strength and N to the gas number density (1 Td =

10–17 Vcm^{-2}). The sampling time per channel of ToF acquisition was 0.1 ns, amounting to 350,000 channels for a mass spectrum ranging up to $m/z = 400$. The sample headspace was withdrawn through PTR-MS inlet with 40 sccm flow for 60 cycles resulting in an analysis time of 60 s/sample. Pure nitrogen was flushed continuously through the vial to prevent pressure drop. Each measurement was conducted automatically after 25 min of sample incubation at 40 °C and 5 min between each measurement was applied in order to prevent memory effect. All steps of measurements were automated by an adapted GC autosampler (MPS Multipurpose Sampler, GERSTEL) coupled to PTR-ToF–MS. The analysis of PTR-ToF–MS spectra proceeded as described in (Farneti et al., 2017). The array of masses detected with PTR-ToF–MS was reduced by applying noise and correlation coefficient thresholds. The first removed peaks that were not significantly different from blank samples; the latter excluded peaks with over 99% correlation, which mostly correspond to isotopes of monoisotopic masses. PTR-ToF–MS outputs of the isolates were employed to build the in silico fruit volatilome. Each virtual fruit sample was obtained by the sum of m/z emissions of the corresponding isolates, weighted for their NGS genus relative abundance. Multiple isolates belonging to the same genus were averaged before weighting m/z profiles assembled in this way were visualized by principal component analysis (PCA).

6.2.8 Statistical Analysis

Past software (Version 4.0) (Hammer et al., 2001) was used for basic statistical functions and correlation canonical analysis. Student's *t*-test was computed to investigate whether single VOCs, VOCs classes and quality parameters of organic and IPM managed samples were significantly different. The significance level of all analysis was $p < 0.05$. R (Version 1.1.463), together with the external package "mixOmics" (Rohart et al., 2017), was used for PCA analysis and visualization employed in this work.

6.3 Results

6.3.1 Characterization of Raspberry Culture Dependent and Independent Bacteriome

The composition of bacterial communities of raspberry fruit cultivated with organic or IPM strategies were assessed by NGS analysis of 16S rRNA gene. Sufficient

sequencing effort was assessed (Figure S6.1). Community composition at the phylum level differed according to the cultivation method. Although both organic and IPM fruits were dominated by Bacilli and Alpha-proteobacteria, these phyla represented respectively 33% and 20% of total operational taxonomic units (OTUs) in IPM fruit, and 76 and 15% of total OTUs in organic fruits (Figure 6.1). Moreover, IPM fruits showed a higher bacterial biodiversity than organic ones (Shannon Index, H' = 3.6 and 2, respectively) and also higher taxa evenness (J' = 0.64 and 0.4, respectively; Table S6.1). The bacteriome of organic fruit was dominated by *Gluconobacter* spp. (73%), whereas IPM raspberries were colonized by several different taxa (*Brevibacillus* 14%, *Methylobacterium* 4%) (Figure 6.1). *Gluconobacter* spp. were absent in IPM fruits.

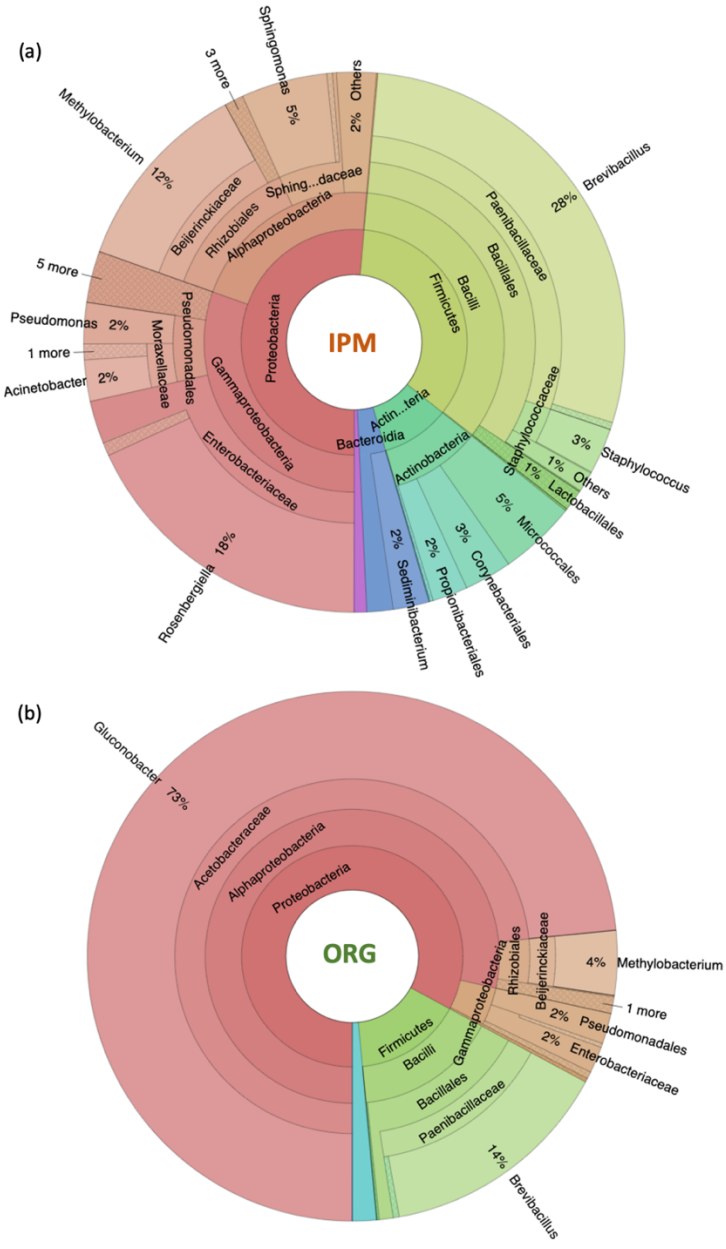

Figure 6.1. Krona chart showing bacterial community composition at phylum, order, class, family and genus level for (a) integrated pest management (IPM) and (b) organic raspberry fruits 'Enrosadira'.

To visualize differences among the bacterial microbiomes of the two cultivation methods, PCA was performed according to bacterial families (Figure 6.2a). Organic produced fruit had more even composition of the bacterial community with berries discriminated only by PC2 (13% of the total variance). Despite the high uniformity of organic fruit, the analysis also highlighted that the differences in bacterial families between berries produced with organic and IPM practices provided only a partial discrimination of berries. Acetobacteraceae and Enterobacteriaceae were the first two families presenting the highest loading values for PC1 (85% of the expected variance) and thus mainly responsible for fruit discrimination (Figure 6.2b).

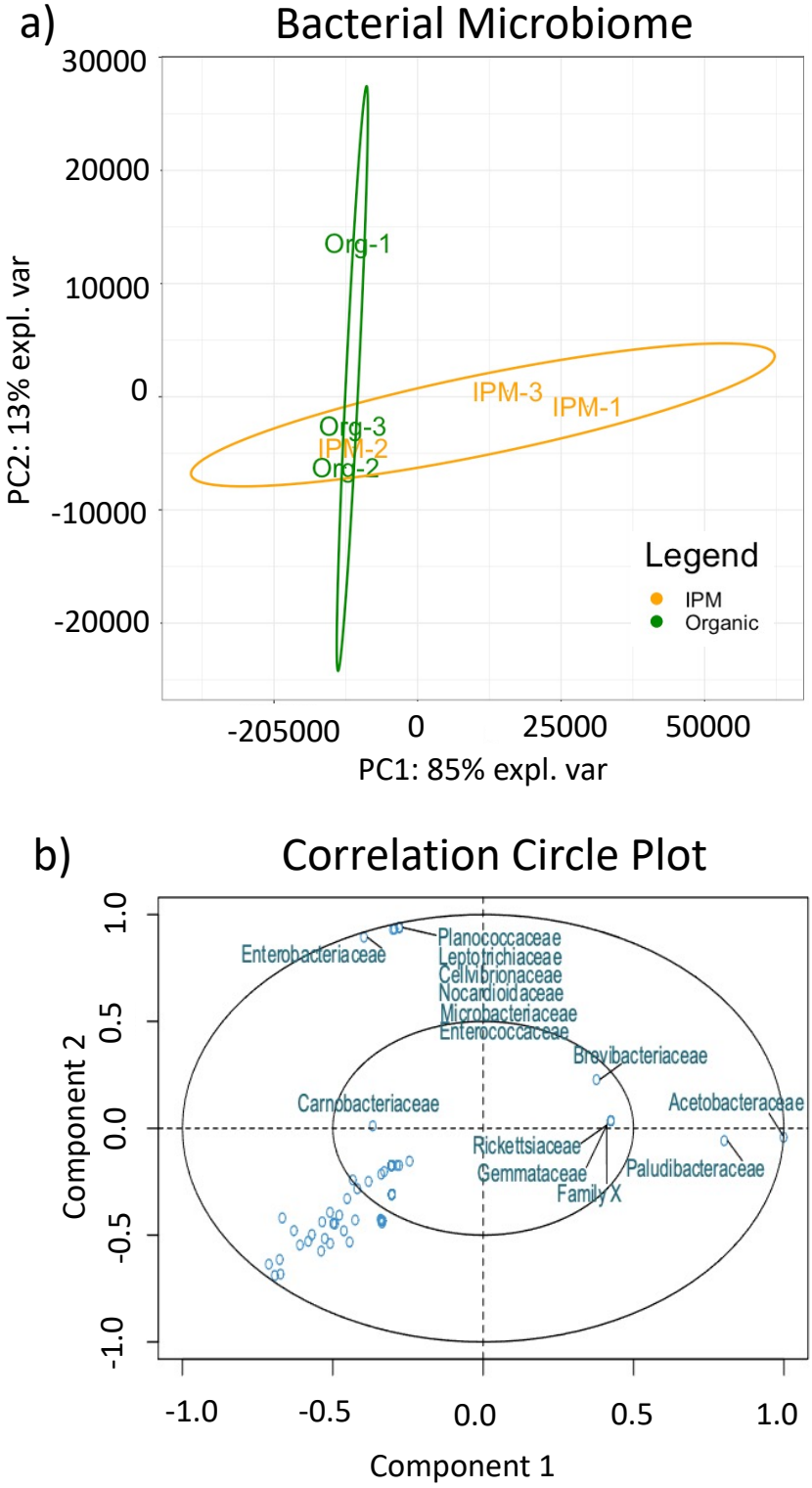

Figure 6.2. (a) Principal component analysis of bacterial families of raspberry fruits 'Enrosadira' cultivated with organic or IPM practices. Ellipses enclose confidence level = 95%. (b) Correlation circle plot with discriminating bacterial families is shown.

The total culturable bacterial population, expressed in log (colony-forming units (CFU)/fruit g), was higher in organic (6.1 ± 0.7) than IPM (5.6 ± 0.1). The analysis of culturable bacterial population indicated that IPM and organic fruit differentiated by few genera. *Rosenbergiella* was present only in IPM fruit, whereas *Tatumella*, *Klebsiella* and *Cronobacter* genera were found only in organic one (Table 6.1). IPM and organic unique bacterial isolates were screened for their in vitro plant growth promoting activities (Table 6.2). Indole-3-acetic-acid (IAA) producing bacteria, namely *Pantoea agglomerans* str. 2 and *Tatumella terrea*, were isolated only in organic fruit, whereas the sole acetoin-producing bacterium, *Enterobacter* spp. str. 2, was found on IPM fruit. All the isolates from organic fruit, but only one from IPM fruit, could release ammonia. Concerning siderophore production, *Klebsiella oxytoca* and *Enterobacter* spp. (str. 1 and 2) were the only species excreting this activity in organic and IPM fruit, respectively. Finally, none of the isolated strains neither in organic, nor IPM berries presented an ACC deaminase activity.

Table 6.1. Bacterial genera and species isolated from raspberry fruits 'Enrosadira' cultivated with organic or IPM practices.

Fruit Type	Bacterial Isolate
Both IPM and Organic	*Bacillus subtilis*, *Burkholderia* spp., *Cellulomonas* spp., *Enterobacter asburiae*, *Enterobacter* spp.- strain 1, *Erwinia aphidicola*, *Erwinia rhapontici*, *Erwinia toletana*, *Lactobacillus plantarum*, *Methylobacterium extorquens*, *Ochrobactrum intermedium*, *Ochrobactrum pseudogrignonense*, *Paenibacillus alvei*, *Paenibacillus macerans*, *Paenibacillus polymixa*, *Pantoea agglomerans*. - strain 1, *Pantoea ananatis*, *Pantoea rwadensis*, *Pseudomonas fluorescens* - strain 1, *Pseudomonas fluorescens* - strain 2, *Pseudomonas stutzeri*, *Sphingomonas camponoticapitis*, *Sphingomonas paucimobilis*,
IPM	*Rosenbergiella* spp. *Enterobacter* spp.- strain 2 and 3
Organic	*Pantoea agglomerans* - strain 2 *Tatumella punctata* *Tatumella terrea* *Cronobacter* spp. *Klebsiella oxytoca*

Table 6.2. Functional traits of bacteria strains isolated uniquely in organic or IPM berries.

Cultivation Method	Bacterium	IAA Production	Acetoin Production	NH_4^+ Production	Siderphores Production	ACC Deaminase Activity
IPM	*Rosenbergiella* spp.	−	−	−	−	−
	Enterobacter strain 2	−	+	−	+	−
	Enterobacter strain 3	−	−	+	+	−
ORG	*Pantoea agglomerans* strain 2	++	−	+	−	−
	Cronobacter spp.	−	−	+	−	−
	Klebsiella oxytoca	−	−	+	+	−
	Tatumella punctata	−	−	+	−	−
	Tatumella terrea	++	−	+	−	−

Qualitative rating of compound production/activity: − = not detected, + = detectable, ++ = high.

6.3.2. Fruit Quality Characteristics

Concerning sensorial quality traits, soluble solid content (expressed as °Brix) did not differ with respect to the cultivation method, whereas weight and titratable acidity (TA) were significantly lower in organically grown fruit (Figure 6.3a). Anthocyanins are the main compounds responsible for berry colour. Cyanidin-3-sophoroside was the most abundant anthocyanin in raspberry fruit, followed by cyanidin-3-glucoside (Figure 6.3b). Organic raspberries showed significantly higher concentration of all anthocyanins with the exception of cyanidin-3-glucorutinoside. The higher concentration of anthocyanins partially influenced berry colour, despite no significant differences were observed in any of the CIE coordinates (L × C × h). Canonical correlation analysis (CCA) was performed to investigate possible correlations between the bacterial genera harboured on fruit and the quality parameters (Figure 4). *Gluconobacter* positively correlated with total anthocyanin content, *Methylobacterium* and *Sphingomonas* with titratable acidity, whereas *Brevibacillus* and *Rosenbergiella* genera with soluble solid content.

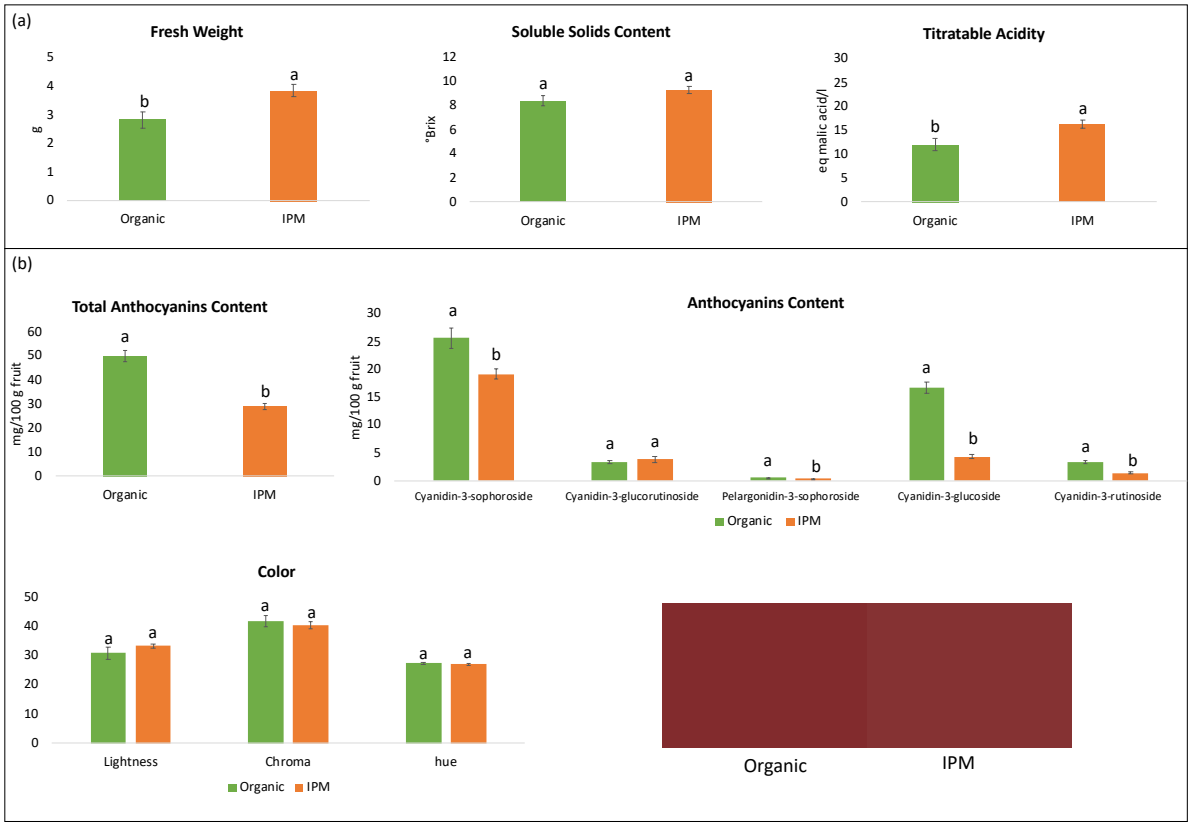

Figure 6.3. Main quality parameters (a), anthocyanidins content and color values (b) of raspberry fruit 'Enrosadira' with boxes representing the real color of fruits. Bars represent the mean ± SE. Different letters indicate significant differences between cultivation methods according to Student's t-test at p-value < 0.05.

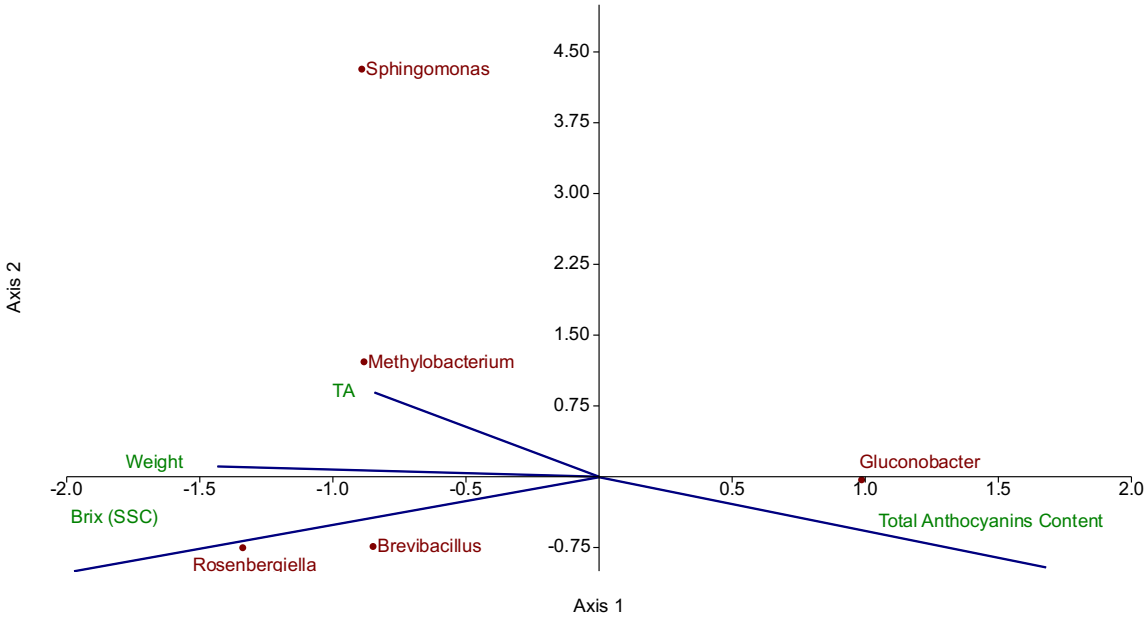

Figure 6.4. Canonical correlation analysis of bacterial genera discriminating organic from IPM grown fruit and quality parameters. Bacterial genera are coloured in brown, quality parameters in green.

6.3.3 Characterization of the Volatilome of Organic and Integrated Pest Management (IPM) Raspberry Fruits

The GC–MS raspberry volatile profile revealed 32 and 37 VOCs released by organic and IPM fruits, respectively. Single VOCs were grouped according to their chemical characteristics in the following classes: alkanes, acids, mono- and sesquiterpenes, esters, ketones, alcohols and aldehydes (Figure 6.5; Table 6.3).

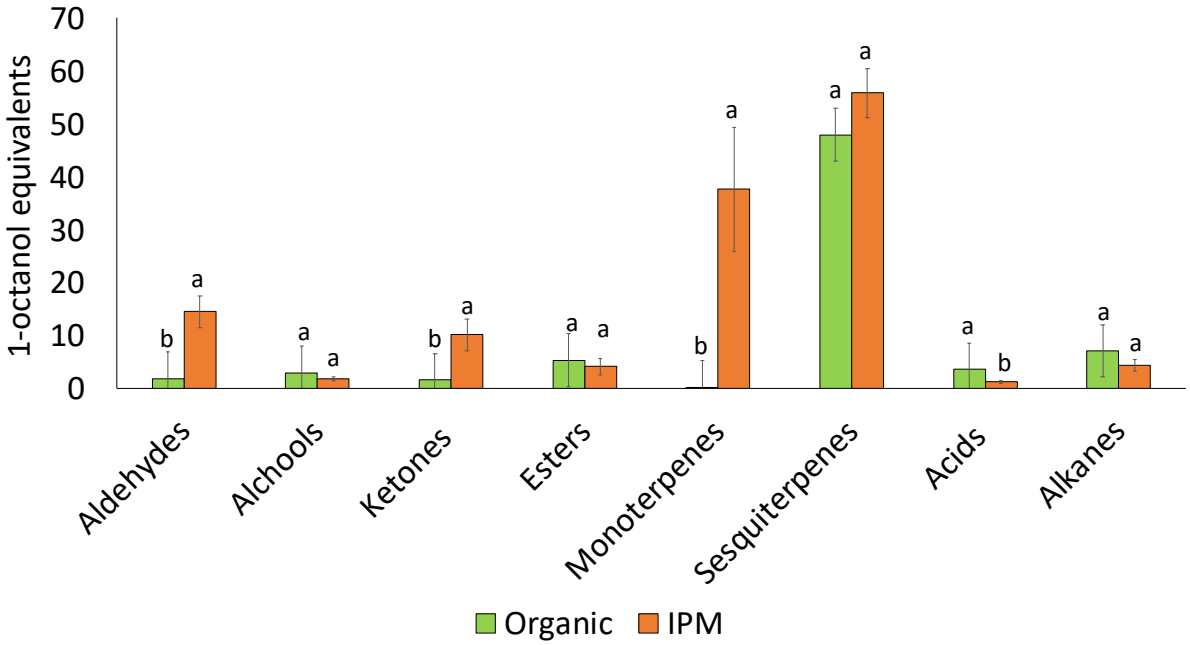

Figure 6.5. Abundance of volatile organic compound classes are showed for organic and IPM grown raspberry fruits 'Enrosadira'. Bars represent the mean ± SE. Different letter indicate significant differences between cultivation methods according to Student's *t*-test at *p*-value < 0.05.

Table 6.3. Volatile organic compounds emitted by raspberry fruit 'Enrosadira' cultivated with organic (Org) or integrated (IPM) practices. Data are reported as mean ± SE. Values labelled with different letters indicates significant differences between the cultivation methods according to Student's *t*-test at *p*-value < 0.05. nd = not detected.

Class	Compound	Retention Time (min)	Linear Retention Index	Mean of Volatile Compounds Concentration (1-octanol Equivalents)	
				ORG	IPM
Aldehydes	(E)-2-Hexenal	11.89	1192	0.25 ± 0.25 [a]	6.82 ± 2.35 [a]
	Hexanal	5.34	1067	nd	5.65 ± 0.75
	Octanal	15.95	1274	0.47 ± 0.07 [b]	0.76 ± 0.11 [a]
	Tetradecanal	37.71	1942	0.63 ± 0.14 [a]	0.44 ± 0.22 [a]
	5-Methylfurfural	27.51	1578	nd	0.81 ± 0.03
	Nonanal	20.46	1376	0.45 ± 0.03	nd
Alchools	6-Methyl-1-heptanol	18.11	1319	0.06 ± 0.03 [a]	0.03 ± 0.03 [a]
	1-Pentanol	14.27	1241	0.14 ± 0.07	nd
	2-Nonanol	29.29	1508	0.10 ± 0.01 [a]	0.05 ± 0.05 [a]
	2-Heptanol	17.82	1312	0.84 ± 0.14 [a]	0.43 ± 0.08 [b]
	β -Linalool	26.13	1535	0.55 ± 0.14 [a]	0.05 ± 0.05 [a]
	Isogeraniol	40.33	2039	0.21 ± 0.11 [a]	0.33 ± 0.24 [a]
	Nonanol	29.56	1645	nd	0.18 ± 0.09
	Trans-geraniol	34.71	1827	0.99 ± 0.08 [a]	0.67 ± 0.09 [b]
Ketones	Acetoin	15.23	1259	0.31 ± 0.11	nd
	2-Heptanone	9.99	1136	0.60 ± 0.20 [a]	0.45 ± 0.16 [a]
	2-Nonanone	20.27	1372	0.38 ± 0.10 [b]	1.08 ± 0.27 [a]
	2-Undecanone	27.58	1581	0.18 ± 0.04 [b]	1.15 ± 0.24 [a]
	Pentan-2-one	3.91	1012	nd	7.35 ± 2.35
Esters	1-Octyl acetate	23.71	1463	3.05 ± 0.19 [a]	0.75 ± 0.15 [b]
	Ethyl hexanoate	13.47	1225	0.71 ± 0.08 [a]	2.26 ± 1.13 [a]
	Isoamyl Acetate	6.97	1130	0.84 ± 0.24	nd
	Methyl Salicylate	32.75	1756	0.66 ± 0.33 [a]	1.02 ± 0.31[a]
Sesquiterpenes	β -Caryophyllene	27.21	1569	5.48 ± 1.38	nd
	α -Humulene	29.39	1640	1.34 ± 0.43	nd
	α -Ionone	34.5	1819	13.99 ± 1.16 [a]	13.35 ± 0.80 [a]
	β -Ionone	36.79	1906	19.86 ± 1.97 [b]	35.00 ± 2.49 [a]
	Dihydro α-ionone	33.59	1786	1.57 ± 0.09 [a]	0.96 ± 0.24 [b]
	Dihydro β-ionone	34.05	1802	5.63 ± 0.86 [a]	6.53 ± 1.33 [a]
Monoterpenes	2-Carene	15.47	1264	0.07 ± 0.03 [a]	0.38 ± 0.24 [a]
	2- β -Pinene	6.19	1092	nd	0.54 ± 0.16
	α -Phellanderene	8.79	1124	0.01 ± 0.01 [a]	15.33 ± 4.72 [a]
	α -Terpinene	9.65	1133	nd	0.86 ± 0.43
	β -Myrcene	9.18	1128	nd	4.91 ± 1.90
	β -Phellandrene	11.32	1177	nd	9.65 ± 2.96
	γ-Terpinene	13.65	1229	nd	0.67 ± 0.37
	Limonene	10.85	1164	0.08 ± 0.01 [b]	2.54 ± 0.79 [a]
	o-Cymene	14.81	1251	nd	2.69 ± 0.90
Acids	Acetic acid	22.28	1424	2.38 ± 0.62 [a]	1.02 ± 0.22 [a]
	Nonanoic acid	42.45	2119	0.41 ± 0.18 [a]	0.08 ± 0.08 [a]
	Octanoic acid	39.94	2025	0.78 ± 0.15	nd
Alkanes	Tridecane	17.38	1302	7.02 ± 3.55 [a]	0.02 ± 0.02 [a]

Z-3-Heptene	7.55	1131	nd	2.34 ± 0.58
E-3-Heptene	7.77	1113	nd	1.89 ± 0.56

The cultivation method had a significant effect on the overall amount of acids, ketones, aldehydes and monoterpenes emitted by fruit, being the latter almost absent in organic fruit. IPM fruit had a generally higher VOCs emission and the volatilome was dominated by terpenes accounting for half of the total VOCs emission. Hexanal, 5-methylfurfural, nonanol, pentan-2-one, 2β-pinene, α-terpinene, β-myrcene, β-phellandrene, γ-terpinene, *o*-cymene, Z-3-heptene and E-3-heptene were not detected in organic fruits, whereas nonanal, 1-pentanol, acetoin, isoamyl acetate, β-caryophyllene, α-humulene and octanoic acid were missing in IPM grown berries. Although present in both treatments, limonene, β-ionone, 2-nonanone and octanal were more intensely emitted in IPM samples, whereas the emission of 1-octyl acetate, 2-heptanol and *trans*-geraniol was higher in organic fruits. PCA performed on the relative emission of single VOCs showed a clear separation of organic and IPM fruit volatilomes along components 1 and 2, explaining 85% and 9% of the total variance, respectively (Figure 6.6a). The four most important VOCs responsible for data clustering were α- and β-phellandrene, pentan-2-one and β-caryophyllene (Figure 6.6b).

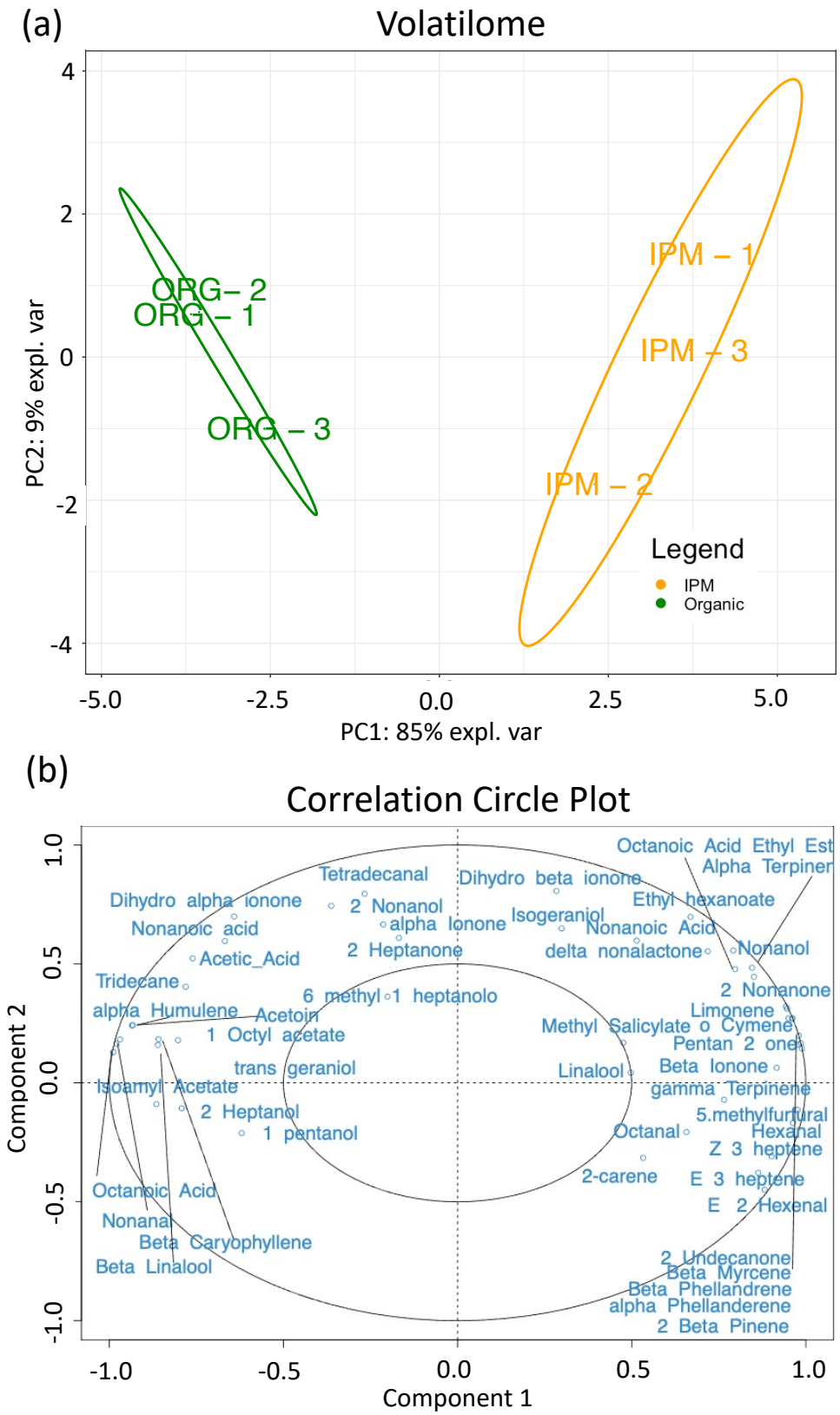

Figure 6.6. (a) Principal Component analysis of volatile organic compounds (VOCs) of raspberry fruits 'Enrosadira' cultivated with organic or IPM practices. Ellipses enclose confidence level = 95%. (b) Correlation circle plot with discriminating VOCs is shown.

Canonical correlation analysis was performed to investigate correlation between VOC classes and key bacterial genera (Figure 6.7). Bacterial genera abundance in organic and IPM fruits was averaged and a threshold of 3% was imposed to select key bacterial genera. *Gluconobacter* correlated with acids and alcohols, *Brevibacillus*, *Methylobacterium* and *Sphingomonas* genera were associated to aldehydes, whereas *Rosenbergiella* correlated with ketones and monoterpenes.

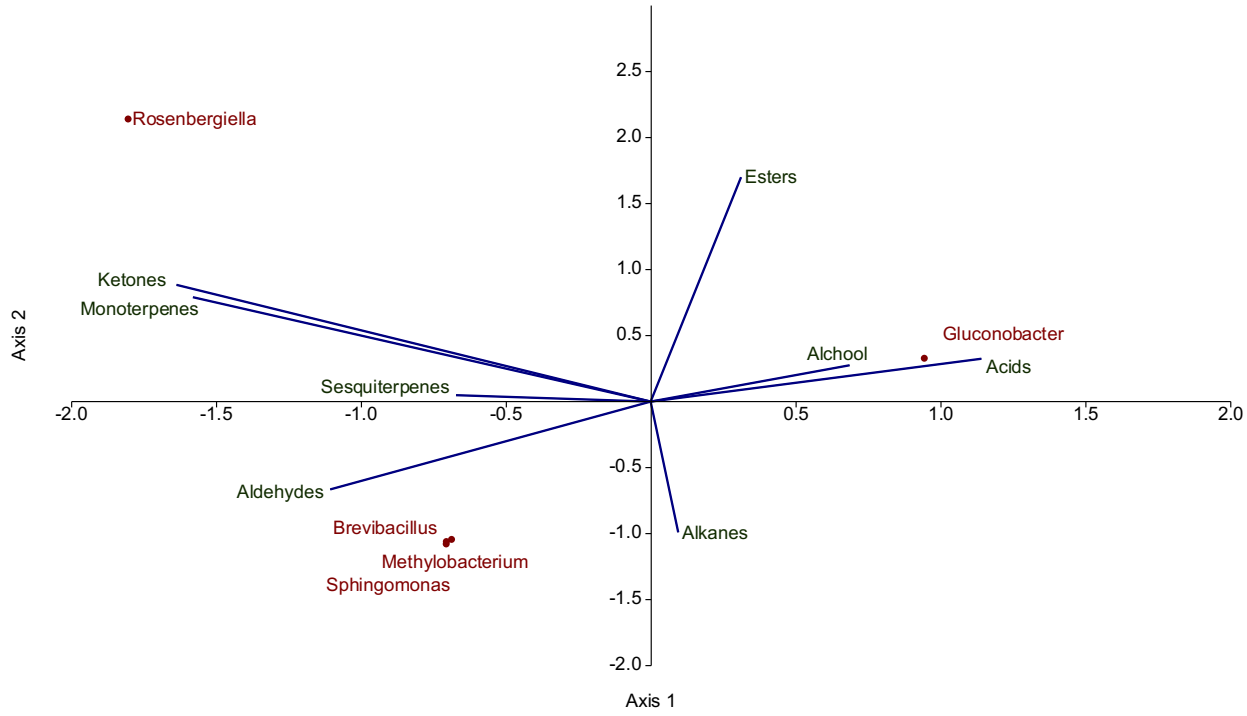

Figure 6.7. Canonical correlation analysis of bacterial genera discriminating organic from IMP grown fruit and volatile organic compounds classes. Bacterial genera are coloured in brown, volatile organic compounds classes in green.

6.3.4 In Silico Fruit Volatilome Assembly

To estimate the role of bacterial organic volatile compounds (bVOCs) on fruit volatilome, the emission of bVOCs by the bacterial species isolated from raspberry fruit was analysed by PTR-MS. To mimic the fruit environment, bacterial isolates were grown on filter sterilized raspberry juice. The m/z emissions of each bacterial isolate, weighted by the relative abundance of its genus calculated from NGS data, were used to construct an in silico-assembled bacterial volatilome for organic and IPM grown fruit. PCA showed a clear discrimination of organic and IPM berries according to the volatilome of fruit associated bacterial community (Figure 6.8a). To identify the most relevant bVOCs among the 118 masses of the dataset, the loading values of Principal

Component 1 (77% of explained variance) were extracted. The m/z fragments that showed the highest loading values were m/z 107.0349, m/z 33.033, m/z 97.0274, m/z 62.0216, m/z 59.049, m/z 69.033 and m/z 87.08. m/z 33.033, m/z 69.033, m/z 97.024 and m/z 107.0349 were putatively identified as methanol, furan, furfural and benzaldehyde, respectively, whereas m/z 59.049 and m/z 87.08 may attributed to linear aldehydes and ketones (C3 and C5, respectively) (Figure 6.8b).

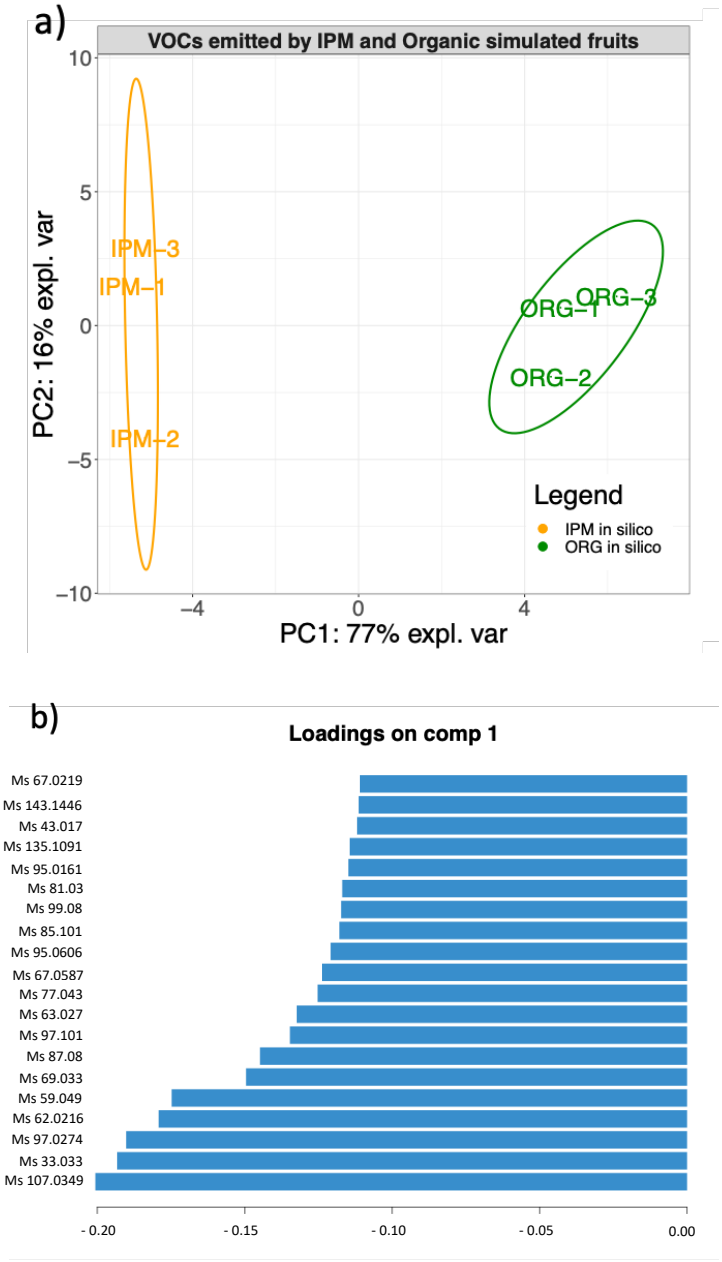

Figure 6.8. Characterization of volatile organic compound profiles, produced by assembling the emissions of single bacterial isolates from organic or IPM grown raspberries. Diagram shows (a) Principal Component (PC) 1 and 2 of principal component analysis (PCA) of the simulated fruits. Ellipses enclose confidence level = 95%. (b) loadings of the 20 m/z fragments showing highest correlation with PC1 in the PCA analysis.

6.4 Discussion

6.4.1 Diversity of Fruit-Associated Microbiomes

Several studies demonstrated the influence of management practices on soil microbial composition and functions (Hartman et al., 2018). In recent years, the microbial communities in the phyllosphere have gained increasing attention (Remus-Emsermann et al., 2018). Nonetheless, only a few studies have focused on the microbial communities hosted by fruits grown with different cultivation methods, mainly with the purpose of assessing risks to human health (Wassermann et al., 2019) or managing post-harvest crop storage (Wisniewski and Droby, 2019).

In this work, NGS analysis highlighted that IPM raspberry fruits were characterized by a higher bacterial biodiversity in comparison to organically grown ones, with *Brevibacillus* (28% relative OTU abundance), *Methylobacterium* (12%), *Rosenbergiella* (18%) and *Sphingomonas* (5%) being the most abundant genera. *Rosenbergiella* and *Sphingomonas* spp. were almost absent in organic fruits. *Rosenbergiella* belongs to the family Enterobacteriaceae and has been detected in several plant species (Lenaerts et al., 2014). *Sphingomonas* has been found in natural environments, such as soil and plants, and has been proven to possess multifaceted positive functions (Asaf et al., 2020).

The bacterial community of organic berries was dominated by the genus *Gluconobacter* (73%), followed by *Brevibacillus* (14%) and *Methylobacterium* (4%) (Figure 6.1). *Gluconobacter* was only found on organic fruits. This genus includes acetic acid bacteria known to live on a wide variety of fruits, and encompasses saprophytic, symbiotic, and pathogenic species (Hommel, 2014). In particular, some *Gluconobacter* strains are known to cause postharvest fruit losses, by causing rotting and browning (Van Keer et al., 1981).

Farming practices such as fertilizer or pesticide application shape microbiome compositions by modifying nutrient availability and ecological niches (Kemperman et al., 2013). Although different research works suggest that organic cultivation promotes microbial biodiversity in the soil and on fruit surface (Kremen et al., 2012; Lori et al., 2017; Wasserman et al., 2019), our results indicate that, in raspberry, IPM cultivation promoted bacterial biodiversity on fruit. It can be hypothesized that in crop systems with low inputs and disturbances, such as that of organic farming, microbial communities are dominated by few, highly adapted bacterial species able to outcompete potential generalist colonizers, thus becoming predominant. Indeed, the

natural habitats of *Gluconobacter* spp., the prevalent bacterial genera on organic fruits, are flowers and fruits (Gupta et al., 2001).

On the other hand, chemical pesticide application affects microorganisms in complex communities, opening ecological niches otherwise occupied by competing microorganisms (Cernava et al., 2019), with microbial community dynamics depending on the chemical nature, rates and frequency of treatments.

6.4.2 Quality Parameters of Organic and IPM Raspberry Fruits

Several studies report the influence of the cultivation method on the quality of fruits and berries (Crecente-Campo et al., 2012; Ponder and Hallmann; 2019; Dastogeer et al., 2020). As raspberry is a highly perishable product, the identification of the optimal maturity stage is crucial for fruit quality at consumption. Visual assessment of fruit colour or ease of fruit detachment from the receptacle are the parameters mostly used in raspberry industry to determine harvesting time. In this work, organic and IPM fruits were harvested at the same levels of detachment force, but they differed for several qualitative parameters (Figure 6.3). IPM fruits were characterized by higher weight and titratable acidity, whereas organic ones contained more anthocyanidins. During the raspberry maturation process, acidity shows a steady increase in the early stages, followed by a substantial decrease during ripening. Anthocyanidins content and fruit weight increase during maturation. Finally, variations in soluble solid content are negligible at the maturation stages close to harvest (i.e., physiological maturity) (Stavang et al., 2015). Therefore, the differences observed in the quality parameters may be related to a more advanced ripening of organic berries than IPM ones, in spite of similar detachment forces.

Exogenous IAA application on berries has been observed to stimulate ethylene emission, thus promoting fruit ripening (Bernales et al., 2019). IAA-producing bacteria, such as *P. agglomerans* and *T. terrea*, could be isolated only from organic fruit (Table 6.2). In this view, the difference in maturation could be related to the bacteria residing on the fruit, or on the fruitlet during development. An increase in ethylene production and respiration rate has been detected from the white fruit stage until full maturity (Fuentes et al., 2015; Bernales et al., 2019) and induction of anthocyanin formation was promoted after ethylene application (Iannetta et al., 1999). We suggest that the promotion of maturation by IAA producing bacteria exceeded the negative effect of auxin on anthocyanin content. In fact, IAA has been reported to have a

negative correlation with anthocyanin contents indicating that anthocyanin accumulation starts when IAA content decreases(Teribis et al., 2016). Moreover, in raspberry, exogenous application of a high concentration of IAA (100 μM) at fruit with equal ripening stage was found to depress anthocyanins accumulation (Moro et al., 2017). However, previous studies highlighted that organic cultivation promote anthocyanin content in berries (Faller and Fialho, 2009; Crecente-Campo et al., 2012; Ponder and Hallman, 2019; Anjos et al., 2020). Thus, other factors may have contributed to the increase of anthocyanin. It is generally assumed that organic plants contain more secondary metabolites, as a form of protection against the stress factors remediable in IPM crops by cultural inputs, i.e., fertilizers and pesticides (Rembiałkowska, 2007). In horticultural plants, an increase of total nitrogen generally depresses anthocyanin accumulation (Wang et al., 2018a,b). However, in strawberry cell culture, the percentage of NH_4^+ in the total nitrogen concentration directly correlated with changes in cell density and anthocyanin content (Sato et al., 1996). Similarly, in purple basil, NH_4^+ availability positively correlated with the contents of anthocyanins (Prinsi et al., 2020). All the bacterial species uniquely isolated in organic fruit can produce NH_4^+. Thus, the higher availability of this cation in fruits might have increased anthocyanin in organically produced berries.

Fruit acidity is an important component of organoleptic quality (Etienne et al., 2013) and in berries it is influenced by several environmental and plant factors (i.e., plant age and genotype, temperature, harvest season and orchard location) (Di Vittori et al., 2018). In previous studies on raspberry 'Tulameen' and 'Kweli', higher titratable acidity values were found in organic raspberries versus IPM grown ones (Anjos et al., 2020). Contrastingly, our results highlighted lower acidity values in organic fruits. Several studies suggest a role of bacteria on fruit acidity. *Bacillus* sp. inoculated on flowers and leaves of sour cherry affected sugar content and titratable acidity of fruits (Arikan and Pirlak, 2016). Similarly, application of *Pseudomonas fluorescens* on strawberry plantlets altered fruit acidity (Tpdeschini et al., 2018). In our work, canonical correspondence analysis reveals possible interaction between titratable acidity of fruits and *Methylobacterium* and *Sphingomonas* genera (Figure 6.4), more abundant in IPM fruits. *Methylobacterium* spp. is mainly found on the phyllosphere where it is able to use methanol as the sole carbon source, whereas several *Sphingomonas* spp. strains have recently gained attention for their ability to produce gibberellins (Kutschera, 2007; Khan et al., 2014; Asaf et al., 2020), together with ACC deaminase-mediated reduction of ethylene in the host (Federov et al., 2013). The

particular combination of hormones may delay late ripening processes, such as degradation of organic acids. Additionally, the direct contribution of *Methylobacterium* or *Rosenbergiella* spp. (Guo and Lidstrom, 2008; Laviad-Shitrit et al., 2020) to fruit organic acid content cannot be ruled out.

For raspberries, the increase of fruit weight is an important factor to reduce production costs, especially labour (Graham and Simpson, 2018). Although plant nutrition is one of the main factors directly influencing berry weight (Di Vittori, 2018), application of plant growth promoting bacteria on fruits also contributed to the increase of berry weight and size (Todeschini et al., 2018). In our study, fruit weight was significantly higher in IPM fruits and correlated to *Brevibacillus* and *Rosenbergiella* abundance (Figure 6.4). *Brevibacillus* spp. has been recorded in very diverse environmental habitats and several strains successfully promoted plant growth (Ray et al., 2020). For instance, application of *Brevibacillus* spp. on eggplant and pepper led to an increased number of fruits per plant as well as fruit size, weight, and yield (Ratòn et al., 2014). *Rosenbergiella* is a novel genus initially identified in floral nectar of almond (Halpern et al., 2013), but it has been found in the flowers of several plant species (Laviad-Shitrit et al., 2020), pollen and insects associated with the flower (Vannette, 2020). This bacterium has been isolated also from fruit pericarp (Dong et al., 2019), suggesting that its population on the flower could be transmitted also on fruit during development and possibly to seeds (Torres-Cortès et al., 2018). In seeds, *Rosenbergiella* seems to promote germination by the production of IAA (Caneschi et al., 2018). Although the *Rosenbergiella* spp. strain, isolated in this work from fully ripe fruit, did not show IAA production, this genus is known to include IAA-releasing species which may promote plant and fruit growth (Liu et al., 2020). IAA has also an important role in fruit development which was demonstrated by the overexpression of the auxin synthesis-related gene (DefH9-iaaM) in transgenic plants, that showed increased fruit size and number (Fuentes et al., 2019). Altogether, the observed differences in fruit quality, size and yield may originate indirectly as a result of bacterial influence on plant growth and fitness at different phenological stages or on different organs.

6.4.3 Volatilome and Bacterial Contribution to Fruit Aroma

Raspberry aroma is a key component for consumers' perception of sensory quality (Aprea et al., 2015). Monoterpenes are the largest class of compounds, among which terpinen-4-ol, geraniol, linalool, limonene, nerol, *p*-cymene, terpinolene, α- and β-

phellandrene, γ-terpinene and α- and β-pinene are the most frequently reported (Aprea et al., 2015). However, only few of the several VOCs identified in the literature are recognized as important descriptors of raspberry aroma, namely 4-(4-hydroxyphenyl)-2-butanone (also known as raspberry ketone), which contributes to the pure raspberry aroma; α-ionone and β-ionone (whose odour is described as violet-like), which are responsible for the overall fruit aroma (Larsen et al., 1991). In this work, raspberry ketone could not be detected because its linear retention index (LRI) lies outside the detection limit of this work.

Interestingly, IPM fruits were richer in β-ionone and 5-methylfurfural. The first compound is characterized by sweet and fruity odour, with seedy nuances, whereas the second one typically has almond and cherry notes. These VOCs possibly conferred more intense floral and fruity notes to the aroma of IPM fruits, which were also characterized by the presence of hexanal. The presence of this compound, a green-odour leafy compound, conferring grassy notes to fruit aroma, corroborates the hypothesis that IPM strategy delayed ripening of berries. A higher content of 2-heptanol and *trans*-geraniol in organic raspberries was observed. Interestingly, both compounds are known for being emitted by flowers of many species and to be effective insect repellents (Barnard and Xue, 2004; Ukeh and Umoetok, 2011), thus suggesting that they have been produced as a defense-related response in organic fruit where pest attacks are less controlled. During post-harvest handling, raspberry fruits are highly susceptible, to fungal diseases, in particular grey mould caused by *B. cinerea* (Cappellin et al., 2013). Remarkably, in this work IPM fruits were lacking β-caryophyllene, which is one of the volatile organic compounds proven to lower the susceptibility of fruits to *B. cinerea* (Cappellin et al., 2013). Identification of VOCs present in raspberry fruits have been recently extensively reviewed (Aprea et al., 2015). In addition to VOCs previously described, we observed the presence of tetradecanal, 3-heptene isomers and 1-octyl acetate. The latter was found to be emitted in a significant higher amount in basil plants treated with NaCl (Landi et al., 2020). In our work, this compound was significantly more abundant in organic raspberries which might suggest that these plants underwent and seek to react to a more stressful condition with respect to IPM ones. Overall, these results might be explained by the lack of use of pesticides and fertilizers in the organic management. Indeed, these conditions might stimulate plant self-protection mechanism and promote the synthesis of secondary metabolites in plants and fruits (Brandt et al., 2011).

Bacteria produce VOCs that act as direct plant-protectants against pathogens, induce plant defenses or promote plant growth and nutrition (Cellini et al., 2021). Here, we observe a difference in the microbiome and volatilome of raspberry fruits originating from different cultivation methods. Although demonstrating the bacterial origin of a particular volatile compound requires dedicated research, it is interesting to note that emission of acids by raspberry fruits is highly correlated to the presence of *Gluconobacter* bacteria (Figure 6.7). In our work, among the volatile compounds belonging to the acids, part of the acetic acid emitted by fruits might be of bacterial origin. Indeed, *Gluconobacter* spp. is known to colonize sugar-rich niches (such as fruit skin) and to efficiently convert sugars to acetic acid (Amaresan et al., 2020). *Rosenbergiella* is a distinctive and abundant colonizer of IPM grown raspberries, here correlated with ketones and monoterpenes emissions. The draft genome of *R. nectarea* suggests that this species may affect fruit quality by releasing terpenoids and by degrading pectins (Laviad-Shitrit et al., 2020), thus confirming a possible role in volatile emission diversity. *Methylobacterium*, *Brevibacillus* and *Sphingomonas* are more abundant in IPM grown fruits and positively correlate with aldehydes emission. Among these bacterial genera, *Methylobacterium* (in particular *M. extorquens*) has been thoroughly studied for its ability to enhance strawberry flavour (Verginer et al., 2010; Nasopoilou et al., 2014). In fact, *M. extorquens* possesses alcohol dehydrogenase enzymes capable of oxidizing a wide range of alcohols to aldehydes and ketones.
Volatiles produced by bacterial isolates on raspberry juice were used for the assembly of in silico fruit volatilome of organic and IPM fruits. Although authors are aware of the limitations imposed by such an artificial system, a clear separation among VOC typical of microbiomes of differently grown berries was observed (Figure 6.8). Fragments of m/z 59.049, 87.08, 97.0274 and 107.0349 were identified as belonging to the aldehyde or ketone class, and were typical of the IPM in silico volatilome. Interestingly, this result is in accordance with volatile emissions of the real fruits and, together with correlations between microbiome and VOC emissions, pave the way for further studies about the role of bacteria in shaping raspberry aroma.

6.5 Conclusions

This work provides evidence of the effects of organic and IPM cultivation methods on quality and aroma of raspberry fruits, and relates them with the cultivation-mediated

changes in fruit-associated bacterial microbiome, supporting the hypothesis of a contribution of the fruit-associated microflora on raspberry quality traits. Organic farming practices were associated to lower bacterial biodiversity of fruit epiphytic microbiome, and resulted in smaller fruits with higher anthocyanin content and lower titratable acidity with respect to IPM fruits. Additionally, IPM fruits were characterized by a higher emission of sweet and fruity odours. Although the manipulation of fruit microbiome composition by cultivation methods requires further research, a higher microbial diversity plausibly determines a more complex, and overall more pleasant fruit aroma, and its enhancement may positively impact on raspberry quality. The involvement of bacteria in fruit development and quality was corroborated by known hormonal and metabolic activities of the microbial species isolated or recognized in the genera identified by NGS analysis. Some bacterial genera, including *Gluconobacter*, *Sphingomonas*, *Rosenbergiella*, *Brevibacillus* and *Methylobacterium* are suggested to contribute to fruit quality, and to enable the control of certain aspects of raspberry.

6.6 Supplementary Materials

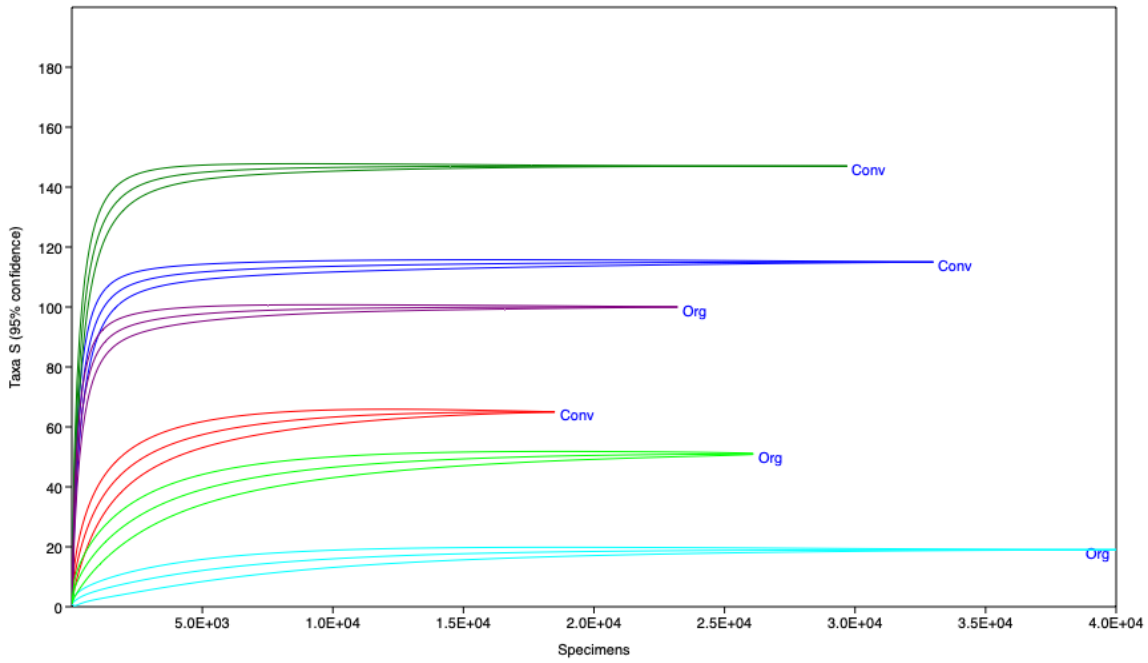

Figure S6.1. Rarefaction curves of community richness estimates of IPM (Conv) and (Organic) raspberry samples.

Table S6.1. Diversity indexes for IPM and organic raspberry samples.

	Organic	*IPM*
Taxa (S)	147	269
Individuals (n)	29955	27216
Simpson (1-D)	0.7272	0.9146
Shannon (H)	2.008	3.626
Equitability (J)	0.4024	0.6481
Chao-1	154.5	282

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7. Investigating the contribution of fruit microbiome to raspberry volatile organic compounds emission

Abstract: Raspberry fruit (*Rubus idaeus* L.) is highly appreciated by consumers for its quality characteristics and aromatic profile, determined by nearly 300 volatile organic compounds (VOCs). Although several microbes produce VOCs, their direct involvement in fruit aroma determination has been largely overlooked. In this study, the contribution of fruit-associated microbiota to fruit volatile emissions was evaluated by performing an untargeted GC-MS analysis of VOCs occurring in naturally colonized (C), sterile (S) and artificially reinoculated berries (R). C and R bacterial fruit microbiomes were characterized by next generation sequencing (NGS). The treatments significantly affected the fruit volatilomes, thus confirming the role of bacteria in fruit aroma construction. In particular, aldehydes, monoterpenes, norisoprenoids, and other aroma-active compounds were significantly lower in S raspberries, and recolonization could only partially restore the emission of terpenoid compounds. Significant correlations were found among NGS data and volatile emissions, including a positive correlation between *Lactobacillus* and *Paenibacillus* spp. and norisoprenoids, and a negative correlation between Enterobacteriaceae and monoterpenes. Several VOC-emitting bacterial taxa (including *Bacillus*, *Lactobacillus, Methylobacterium, Paenibacillus*, *Pseudomonas* spp.) are recurrently found in the raspberry-associated microbiome, suggesting that future applications aimed at the control of microbial colonization may enhance fruit aroma.

7.1 Introduction

Small berry fruit include several species characterized by remarkable nutraceutical and aromatic properties, harvested at physiological maturity and consumed within a few days. Among these fruit, raspberry (*Rubus idaeus* L.) is widely cultivated in America and Europe. Besides being appreciated for its high content in health-beneficial compounds, such as anthocyanins, flavonols, catechins, ascorbic and ellagic acid derivatives (Schultz & Chim, 2019), raspberries are well recognized by consumers for their characteristic flavor and aromatic profile. Nearly 300 volatile organic compounds (VOCs) have been reported to contribute to raspberry sensory quality (Aprea et al., 2015). Abundance and composition of volatile compounds in fruit is

highly dependent on several factors, such as cultivar, maturity, pre- and postharvest fruit handling (El-Hadi et al., 2013).

Plants are naturally colonized by diverse bacterial and fungal communities, referred to as microbiota (Bulgarelli et al., 2013), which may vary according to organ, genotype (Morella et al., 2020) and environmental conditions. The microbiota can influence the host plant phenotype, and convey resistance to stress and biotic factors (Berlec, 2012; Finkel et al., 2017; Van Wees et al., 2008; Purahong et al., 2018). Long-known effects of plant-associated microbes on their host plants include nutritional enhancement, growth promotion and induction of resistance/tolerance to stress (Bailly and Weisskopf, 2017; Sharifi and Ryu, 2018), and induction or the control of postharvest diseases in fruit (Mari et al., 2016; Zhang et al., 2020). More recently, microbial communities have been held responsible for the production of VOCs with physiological and ecological roles, for instance, in inducing plant resistance responses (Cellini et al, 2018) or modulating floral emissions (Peñuelas et al., 2014; Cellini et al, 2019, Ponzoni et al., 2008). In contrast, the direct involvement of the fruit-associated microbiota in the determination of fruit quality has been largely overlooked.

Raspberry has been the subject of microbiome studies (i.e., the genetic characterization of the microbiota) on soil (Oszust and Frac, 2021) and fruit (Perpetuini et al, 2019), highlighting the functions expressed by the plant-associated microflora. Although genotype-specific bacterial communities have been found on the surface of raspberry fruit (Perpetuini et al., 2019), their contribution to VOC emission has not been investigated. The aim of this study, therefore, was to investigate the actual contribution of fruit-associated microbiota to fruit aromatic properties by analyzing volatilomes of naturally colonized and artificially reinoculated berries.

7.2 Material and methods

7.2.1 Sample origin and treatment

Raspberry plants of cultivar 'Enrosadira' were grown at Ponte di Pietra (FC, Italy) during summer 2019 following standard agriculture practices. Harvest was performed by hand-picking berries at full maturity stage. Harvested berries were put in cold boxes and immediately brought to the laboratory.

The experimental design included three replicates, made up of six berries each, for the three different treatments (Control, C; Sterile; S; Recolonized, R). For the C samples, berries were dipped for 10 s in 0.01 M $MgSO_4$ supplemented with cycloheximide (0.05

g L^{-1}), to eliminate epiphytic fungi but at the same time avoiding the washing out of the bacterial microbiome. Another batch of fruit was washed 5 min under agitation (70 rpm) in 0.01 M $MgSO_4$ plus cycloheximide, and the wash was collected. Berries were then surface-sterilized by sequential rinsing with 1.5 % NaClO, 70 % ethanol, and three times with sterile deionized water; S samples were taken from this fruit batch. The wash collected before sterilization was concentrated by centrifugation for 15 min at 5,000 × g, resuspended in 0.002 L, and used to reinoculate previously sterilized berries (R samples) with 0.0001 L of concentrated suspension.

They were then let dry in sterility, put in a glass pot and closed with a paper lid, to allow aeration. After 24 h, three berries for each replicate and treatment were collected, the pot was closed with an air-tight pierceable lid, maintained 24 h at room temperature and subsequently stored at -80°C. The remaining berries were washed 15 min at 100 rpm in $MgSO_4$ solution in order to resuspend the epiphytic microbial population. C and R washings were stored at -80 °C for subsequent DNA extraction, whereas S washings were plated on Luria-Bertani agar medium (Sigma Aldrich, St. Louis, MO, USA) to check sterility.

7.2.2 Fruit volatile analysis

Each replicate (n=3) consisted of a pool of three berries. Raspberry fruit volatiles were analysed by solid phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS) as described in Sangiorgio et al. (2021). Briefly, samples were taken out from -80 °C and put at -20 °C for 20 min before equilibration. 1-octanol 0.05 % (v/v in H2O) was used as internal standard and equilibration was carried out at 40 °C for 20 min. Samples were then exposed to a DVB Carboxen PDMS Stable Flex SPME fibre (Supelco, Bellefonte, PA, USA) for 40 min, which was desorbed in the injector of a Shimadzu GC-MS-QP2010 Plus (Shimadzu, Tokyo, Japan) at 250 °C for 10 min in the split mode. The chromatographic separation of VOCs was performed on an RTX-WAX fused-silica capillary column (Restek, Bellefonte, PA, USA). GC-MS heating program and conditions were the same as those reported by Sangiorgio et al. (2021). VOCs were identified based on their mass spectra and linear retention indices, which were compared with the NIST/EPA/NIH Mass Spectral Database (NIST 08, National Institute of Standards and Technology, Gaithersburg, MD, USA) and the ChemSpider information resource (http://www.chemspider.com).

7.2.3 DNA extraction and next generation sequencing for microbiome analysis

To perform metagenomic analysis, DNA was extracted from washing suspensions according to the CTAB protocol (Maguire et al., 1994), using the pellet obtained from centrifuging the washing solutions at 13000 × g for 10 min. DNA quality and quantity were measured by spectrophotometric quantification with a NanoDrop ND-8000 V1.1.1 spectrophotometer (Thermo Fisher Scientific, Dreieich, Germany). Bacterial V3-V4 regions were amplified with 16S Amplicon PCR Forward= 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and
Reverse=5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGG GTATCTAATCC primers according to Illumina protocols and subjected to automated sequencing by BioFab research (Rome, Italy).

7.2.4 Bioinformatic analysis

BBMap version 38.79 was used to remove low-quality reads using a quality-trim left and right ends before mapping with an average phred score ≥25 (Bushnell, 2014). Sequences shorter than 170 bases were discarded. The sequences were analysed using the Qiime2 v2020.2.0 (Bolyen et al., 2019). Qiime2 dada2 plugin was used to length trimming, denoising, chimera and PhiX removal. SILVA 16S rRNA sequences database, release 312 (Quast et al., 2012) with a 97 % identity criterion was used to assign taxonomy to features. Bacterial genera were considered to be part of the core microbiome if they were present in at least three out of four cultivars as long as, their family was present in the excluded genotype.

7.2.5 Data analysis

Past software (Version 4.0) (Hammer et al., 2001) was used for basic statistical functions. One-way ANOVA was computed to investigate whether single VOCs and VOCs classes were significantly different among treatments. The significance level of all analysis was p=0.05. R (Version 1.1.463), together with the external package "mixOmics" (Rohart et al., 2017), was used for PCA analysis and visualization employed in this work. Multiple Factor Analysis was performed with the external package "FactoMineR" (Le et al., 2008). Pearsons' and Spearmans' correlations were analysed with "Hmisc" (Harrel, 2004) and predicted-R2 with "olsrr" package

(Hebbali, 2020). R-squared and Predicted R-squared were computed for VOC/NGS pairs when both Pearsons' and Spearmans' R were significant. Pearsons' R value between NGS and VOC data was calculated using raw NGS data, as well as transformed NGS data calculated as follows: Transformed OTU abundance = -1/(1+OTU abundance). When the difference between R-squared and predicted R-squared was lower than 0.25, the association model between VOC and NGS was considered acceptable for prediction. VOC and NGS cores of raspberry cultivars 'Enrosadira', 'Anne', 'Imara', 'Regina' were assembled by merging data included in this and in previous work (Perpetuini et al., 2019; Sangiorgio et al., 2021).

7.3 Results

7.3.1 Effects of surface sterilization and recolonization on the fruit volatilome

Fruit samples of the cultivar 'Enrosadira' were washed, surface-sterilized, and re-inoculated with the wash suspension to recolonize the fruit with its original microbiota. VOC emission from control (C), sterilized (S) and recolonized (R) samples was investigated by gas chromatography-mass spectrometry (GC-MS). PCA shows a clear separation of sample classes based on their VOC profiles (Figure 7.1 a). 2-pentanone, *o*-cymene, limonene, β-myrcene and β-pinene contributed to the differentiation of sterile and recolonized berries from the control ones. Surface-sterilized berries evidenced a generalized reduction of VOC emissions. In particular, aldehydes, monoterpenes and norisoprenoids significantly decreased with sterilization treatment, and recolonization could partly restore the emission of terpenoid compounds (Figure 7.1b). Specifically, hexanal, octanal, 1-nonanol and β-pinene emissions significantly decreased in S and R treatments (the latter two compounds being detectable only in the control). Limonene, *o*-cymene and α-ionone were reduced in S, but R emissions were similar to C. In R berries, β-phellandrene could not be detected (Table 7.1).

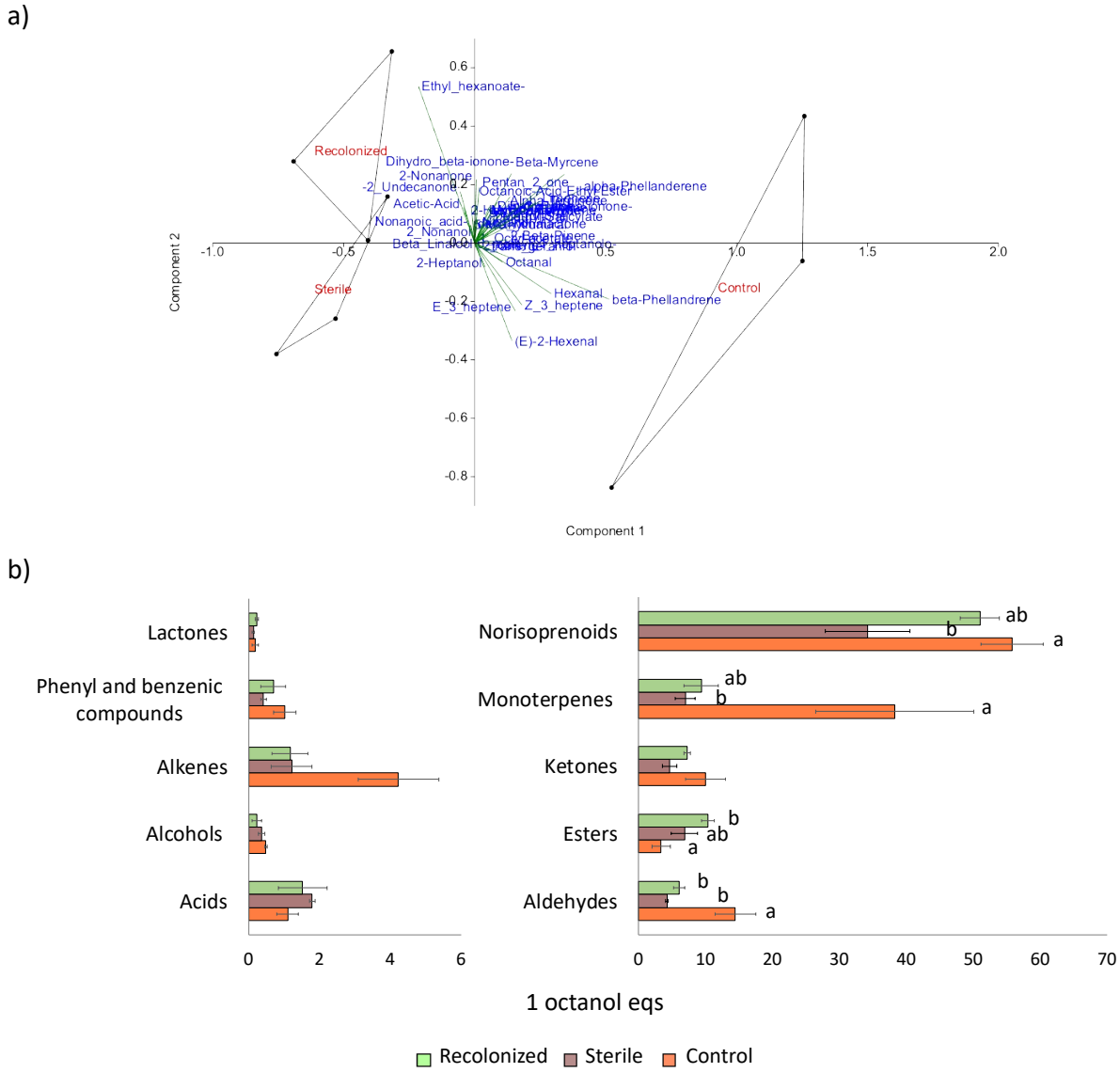

Figure 7.1. (a) PCA of VOCs emitted by untreated (Control), sterile and recolonized raspberries ('Enrosadira') analyzed by GC-MS. (b) VOC emissions (divided in chemical classes) from differently treated raspberries. Data are expressed as mean ± SE. One-way ANOVA was performed. Different letters indicate significant differences between treatments ($p \leq 0.05$) for each VOC class, according to Tukey's post-hoc test.

Table 7.1. VOCs detected by GC-MS in control, sterile and recolonized raspberries ('Enrosadira'). Data are expressed as mean ± SE of 1-octanol equivalents; n.d. = not detected. One-way ANOVA was performed. Different letters indicate significant differences between treatments ($p \leq 0.05$) for each VOC, according to Tukey's post-hoc test.

		Control	Sterile	Recolonized
Acids	Acetic acid	1.02 ± 0.22	1.73 ± 0.05	1.3 ± 0.66
	Nonanoic acid	0.08 ± 0.08	0.07 ± 0.04	0.23 ± 0.07
Alcohols	2-Heptanol	0.43 ± 0.08	0.31 ± 0.07	0.16 ± 0.11
	2-Nonanol	0.05 ± 0.05	0.06 ± 0.03	0.08 ± 0.04
	Nonanol	0.18 ± 0.09	n.d.	n.d.
	6-methyl 1-heptanol	0.03 ± 0.03	n.d.	n.d.
Aldehydes	Hexanal	5.65 ± 0.75 **a**	0.82 ± 0.16 **b**	1.57 ± 0.31 **b**
	(E)-2-hexenal	6.82 ± 2.35	2.51 ± 0.17	2.9 ± 0.78
	Octanal	0.76 ± 0.11 **a**	0.15 ± 0.08 **b**	0.16 ± 0.08 **b**
	5-methyl furfural	0.81 ± 0.03	0.63 ± 0.06	1.17 ± 0.19
	Tetradecanal	0.44 ± 0.22	0.15 ± 0.04	0.29 ± 0.1
Alkenes	(E)-3-heptene	1.89 ± 0.56	0.54 ± 0.17	0.49 ± 0.23
	(Z)-3-heptene	2.34 ± 0.58	0.67 ± 0.4	0.69 ± 0.28
Esters	Ethyl hexanoate	2.26 ± 1.13 **a**	5.96 ± 1.88 **ab**	9.14 ± 0.81 **b**
	1-Octyl acetate	0.75 ± 0.15	0.73 ± 0.08	0.65 ± 0.02
	Ethyl octanoate	0.35 ± 0.18	0.17 ± 0.1	0.61 ± 0.15
Ketones	2-pentanone	7.35 ± 2.35	1.41 ± 0.3	2.83 ± 0.15
	2-heptanone	0.45 ± 0.16	0.55 ± 0.12	0.73 ± 0.08
	2-nonanone	1.08 ± 0.27	0.92 ± 0.25	1.63 ± 0.31
	2-undecanone	1.15 ± 0.24	1.77 ± 0.47	2.05 ± 0.05
Monoterpenes	β-pinene	0.54 ± 0.16	n.d.	n.d.
	α-phellandrene	15.33 ± 4.72	2.93 ± 0.63	4.7 ± 1.78
	β-myrcene	4.91 ± 1.9	0.81 ± 0.28	1.21 ± 0.59
	α-terpinene	0.86 ± 0.43	0.1 ± 0.06	0.28 ± 0.14
	Limonene	2.54 ± 0.79 **a**	0.4 ± 0.09 **b**	0.68 ± 0.26 **ab**
	β-phellandrene	9.65 ± 2.96	1.8 ± 0.39	n.d.
	γ-terpinene	0.67 ± 0.37	0.06 ± 0.06	0.82 ± 0.32
	o-cymene	2.69 ± 0.9 **a**	0.4 ± 0.06 **b**	0.96 ± 0.03 **ab**
	2-carene	0.38 ± 0.24	0.12 ± 0.07	0.31 ± 0.05
	(E)- geraniol	0.67 ± 0.09	0.35 ± 0.03	0.39 ± 0.03
Norisoprenoids	Dihydro-*α*-ionone	0.96 ± 0.24	0.4 ± 0.14	0.68 ± 0.17
	Dihydro-β-ionone	6.53 ± 1.33	3.17 ± 0.91	5.45 ± 0.8

	α-ionone	13.35 ± 0.8 **a**	6.65 ± 1.32 **b**	11.16 ± 1.34 **ab**
	β-ionone	35 ± 2.49	24.01 ± 4.18	33.7 ± 1.04
Phenyl and benzenic compounds	Methyl salicylate	1.02 ± 0.31	0.42 ± 0.09	0.7 ± 0.35
Lactones	δ-nonalactone	0.18 ± 0.09	0.14 ± 0.03	0.24 ± 0.05

7.3.2 Bacterial microbiome of differently treated raspberries

Next Generation Sequencing (NGS) analysis revealed that control raspberries were colonized by a wide variety of bacterial taxa, while R berries mainly hosted taxa belonging to Enterobacteriaceae family (Figure 7.2). Indeed, C berries showed a higher biodiversity (Shannon's diversity index $H = 2.18$) with respect to R ones ($H = 0.61$). C berries were mainly colonized by *Brevibacillus* (31 %), *Rosenbergiella* (27 %) and *Methylobacterium* (13 %) genera. After sterilization, S berries were washed and a wash aliquot was plated on LB agar. The absence of bacterial growth confirmed the efficacy of the sterilization treatment.

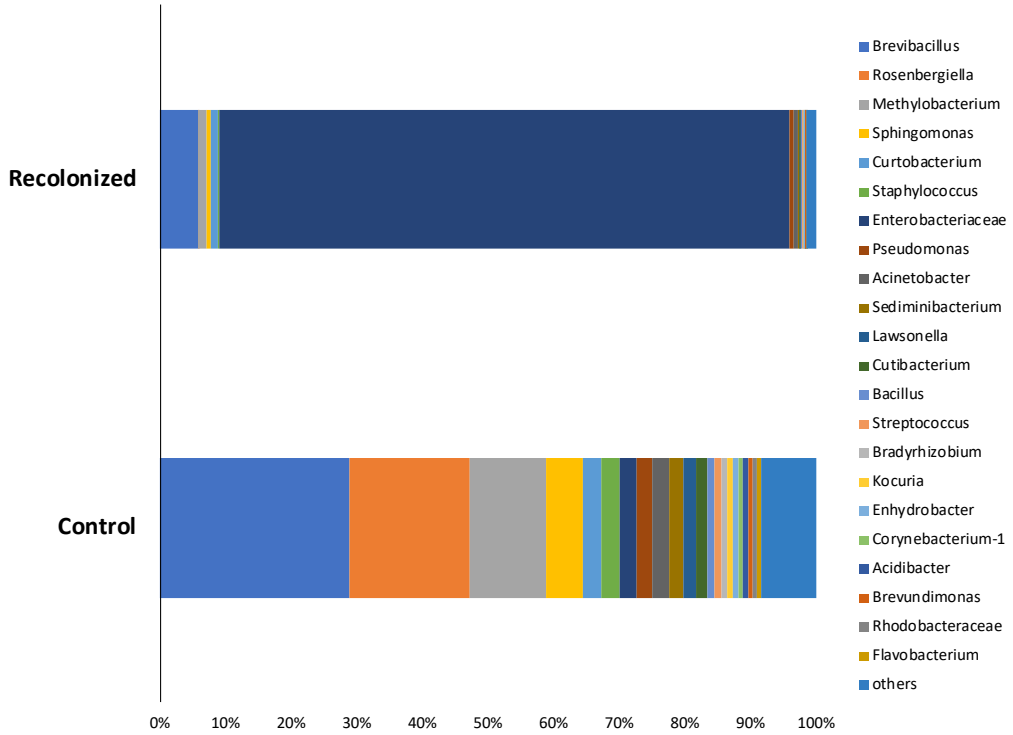

Figure 7.2. Barplot showing (a) Control and (b) Recolonized fruit microbiome composition.

7.3.3 Determination of raspberry core microbiome and volatilome

Volatiles of raspberry cultivars 'Imara', 'Regina', 'Anne' and 'Enrosadira' were combined to construct a core volatilome (i.e. VOCs present in all genotypes analyzed), which consisted of seventeen VOCs (Figure 7.3a). It included the main classes found in fruit from different genotypes with the exception of alkenes, phenyl and benzenic compounds and lactones which were not present in all berries. Similarly, microbiome data of the four raspberry cultivars were combined to construct a core microbiome (Figure 7.3b), which included 2 genera of Alphaproteobacteria (*Methylobacterium*, *Sphingomonas*), 1 of Betaproteobacteria (*Burkholderia*) and 3 of Gammaproteobacteria (*Acinetobacter*, *Pantoea*, *Pseudomonas*). Moreover, it contained 4 genera of Bacilli (*Bacillus*, *Lactobacillus*, *Paenibacillus* and *Vagococcus*) (Figure 7.3b). All compounds of the core volatilome have been found to be produced by at least one bacterium belonging to the core microbiome, except for γ-terpinene, o-cymene, β-ionone, dihydro-α-ionone, dihydro-β-ionone (Lemfack et al., 2018; Sangiorgio et al., 2021).

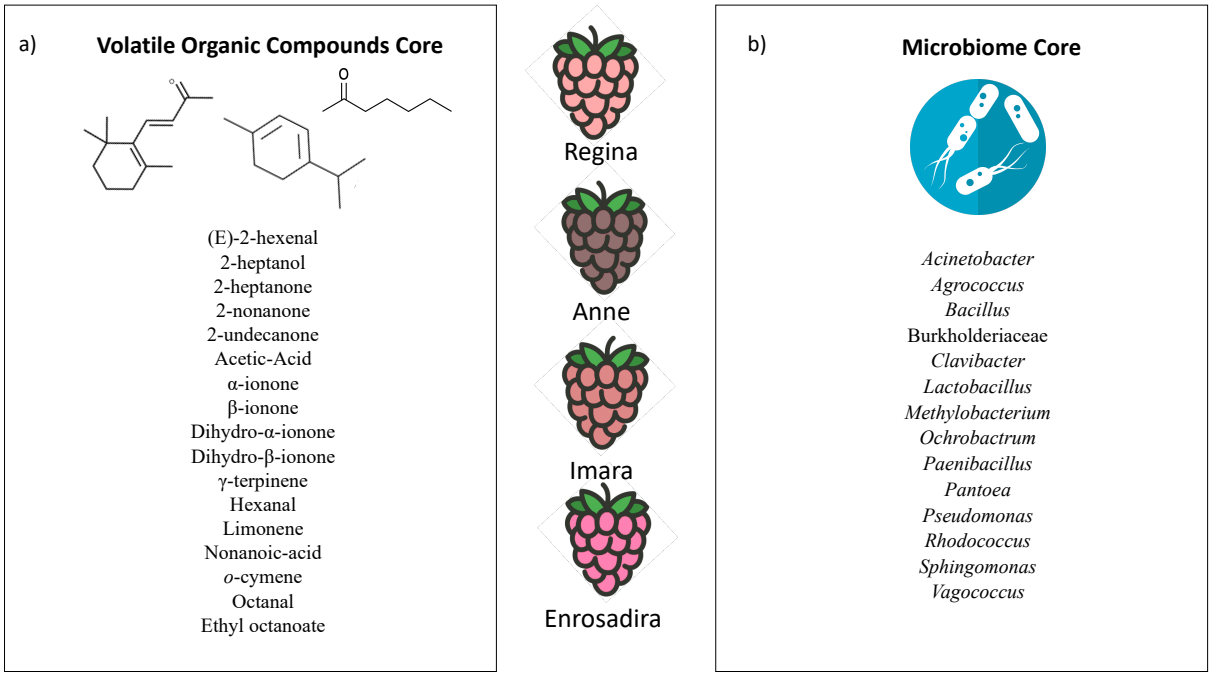

Figure 7.3. Core volatilome (a) and microbiome (b) of raspberries ('Imara', 'Regina', 'Anne' and 'Enrosadira').

7.3.4 Fruit volatiles correlation with bacterial microbiome

Multiple Factor Analysis (MFA) was performed to assess the correlation between microbiome composition (at genus level) (NGS) and VOCs emission by control and recolonized berries (Figure 7.4). MFA model explained a variance of 42.2 % on dimension 1 and of 30 % on dimension 2. RV coefficient of MFA evaluates the relationship between two sets of variables analyzed for the same samples. In this work, NGS and VOCs data were associated with a RV score of 0.78 ($p = 0.02$).

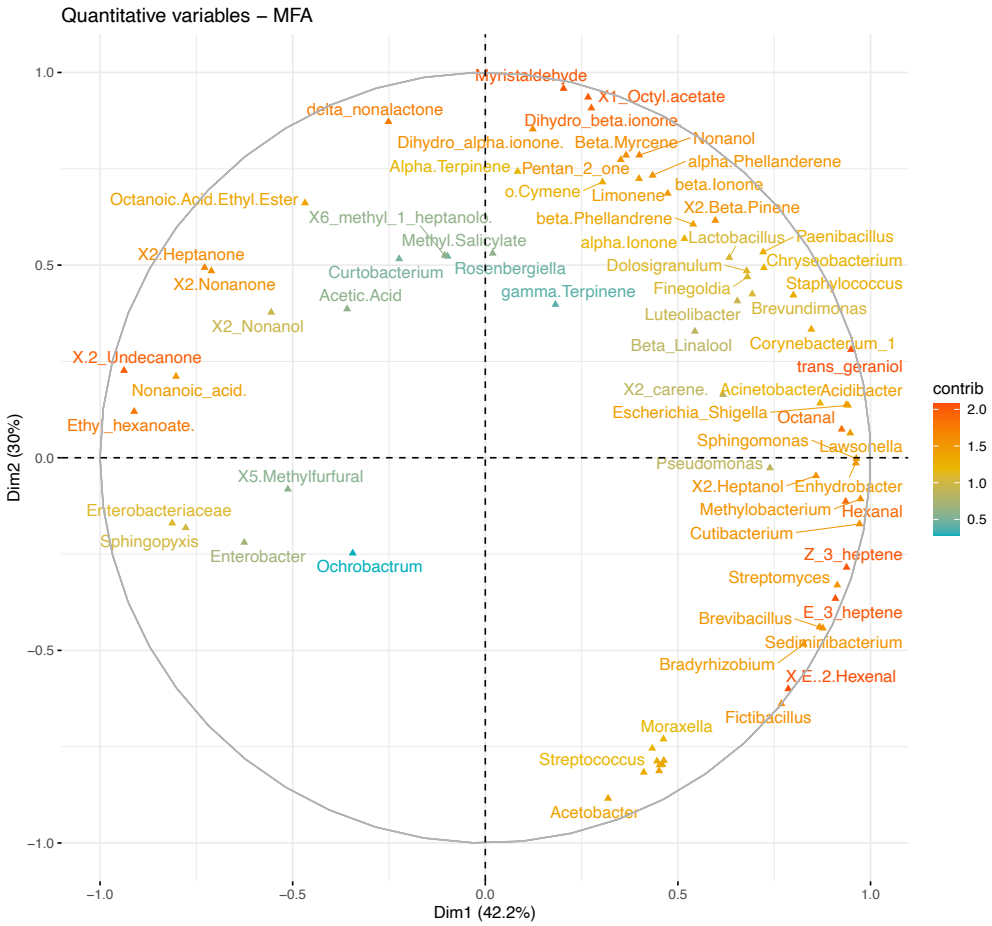

Figure 7.4. Multiple Factor Analysis of VOCs and bacterial genera identified by NGS analysis of raspberries. Diagram plotting quantitative VOC and NGS variables in a bidimensional space. Variables are coloured according to their contribution to Dimension 1.

To better understand specific interactions between volatiles and fruit-associated microbiome, a correlation analysis between bacterial genera and volatile emission was performed. Spearman's rank correlation matrix was computed and results were further screened considering only significant ($p < 0.05$) correlations (Table 7.2). 42

positive and 52 negative correlations were identified between VOCs and bacterial genera.

Table 7.2. Significant correlations between VOCs and NGS, as determined by Spearman's test ($p < 0.05$) and Pearsons' correlation. For the latter, R value was calculated, either using original or transformed (Transformed OTU abundance = -1/(1+OTU abundance) NGS data. The highest values are reported if significant. R-squared and predicted R-squared are shown; * symbol indicates that R-squared and predicted R-squared were calculated with transformed NGS data.

VOC class	VOC	Bacterial Genus	Spearman's R value	Pearsons' R value	R-squared	Predicted R-squared
Acids	Nonanoic-acid	*Bradyrhizobium*	-0.88			
		Corynebacterium	-0.99	0.93	0.86	0.64*
		Enhydrobacter	-0.81			
		Escherichia_Shigella	-0.82	0.83	0.68	0.437*
		Lawsonella	-0.99	-0.87	0.75	-1.832*
		Methylobacterium	-0.81	-0.81	0.66	0.36
		Sediminibacterium	-0.99	-0.84	0.70	0.27
		Sphingomonas	-0.93	-0.84	0.70	0.22
		Streptomyces	-0.97	-0.90	0.81	0.63
Alcohools	2-nonanol	*Acidibacter*	-0.89	-0.82	0.68	0.414*
		Brevibacillus	-0.83			
		Curtobacterium	0.94			
	Nonanol	*Enterobacter*	-0.82	-0.98	0.96	0.863*
	2-heptanol	*Corynebacterium*	0.83			
		Escherichia_Shigella	0.85			
		Lawsonella	0.83			
		Pseudomonas	0.89	0.85	0.73	0.43
Aldehydes	(E)-2-hexenal	*Fictibacillus*	0.90	0.99	0.98	0.97
	Hexanal	*Staphylococcus*	0.89			
	Octanal	*Cutibacterium*	0.94	0.96	0.92	0.865*
		Escherichia_Shigella	0.85	0.88	0.77	0.586*
		Sphingopyxis	-0.94	-0.94	0.88	0.74
		Staphylococcus	0.94			
	5-methylfurfural	Enterobacteriaceae	0.94			
		Sphingopyxis	0.88			
		Staphylococcus	-0.83	-0.97	0.94	0.875*
	Tetradecanal	*Acetobacter*	-0.85	-0.87	0.75	0.525*
		Kocuria	-0.82			
		Paenibacillus	0.89			
		Pedobacter	-0.85	-0.87	0.76	0.557*
Alkenes	(E)-3-heptene	*Fictibacillus*	0.81	0.93	0.87	0.68

		Sediminibacterium	0.83	0.96	0.91	0.84
	(Z)-3-heptene	*Fictibacillus*	0.81	0.90	0.82	0.44
		Sediminibacterium	0.83	0.93	0.87	0.73
Esters	1-octyl-acetate	*Blastomonas*	-0.85			
		Rothia	-0.85			
	Ethyl octanoate	*Acetobacter*	-0.85	-0.91	0.83	0.71
		Pedobacter	-0.85	-0.86	0.75	0.346*
Ketones	2-pentanone	*Enterobacter*	-0.87	-0.96	0.93	0.718*
		Ochrobactrum	-0.85			
		Rosenbergiella	0.83	0.81	0.66	-886.79
	2-heptanone	*Acidibacter*	-0.84			
		Brevibacillus	-0.94	-0.95	0.91	0.78
		Curtobacterium	0.81	0.85	0.72	0.171*
		Cutibacterium	-0.94	-0.90	0.81	0.423*
		Sphingopyxis	0.82			
		Staphylococcus	-0.83			
	2-nonanone	*Acinetobacter*	-0.89	-0.69	0.48	-0.70
		Fictibacillus	-0.99	-0.86	0.75	0.29
	2-undecanone	*Staphylococcus*	-0.89			
Monoterpenes	β-pinene	*Enterobacter*	-0.92	-0.98	0.95	0.892*
		Enterobacteriaceae	-0.88	0.87	0.76	0.45
		Sphingopyxis	-0.87	-0.86	0.74	0.42*
	α-phellanderene	*Enterobacter*	-0.93	-0.96	0.91	0.767*
	β-myrcene	*Enterobacter*	-0.93	-0.95	0.91	0.718*
	α-terpinene	*Kocuria*	-0.83			
	β-phellandrene	*Enterobacter*	-0.92	-0.96	0.92	0.776*
		Enterobacteriaceae	-0.88			
		Rosenbergiella	0.88			
		Sphingopyxis	-0.87	-0.85	0.72	0.381*
	Limonene	*Enterobacter*	-0.93	-0.94	0.89	0.653*
		Ochrobactrum	-0.85			
		Rosenbergiella	0.94			
		Sphingopyxis	-0.88			
	2-carene	*Acidibacter*	0.81	0.85	0.72	0.12
		Dolosigranulum	0.94	0.91	0.83	-0.53
		Finegoldia	0.85	0.90	0.81	0.57
	o-cymene	Enterobacteriaceae	-0.93			
		Sphingopyxis	-0.83			
		Staphylococcus	0.81	0.45	0.20	-0.61
	(E)-geraniol	*Enterobacter*	-0.84	-0.75	0.57	-0.33
		Escherichia_Shigella	0.85	0.93	0.86	0.77
		Sphingomonas	0.83	0.90	0.81	0.54

		Staphylococcus	0.83	0.88	0.78	-17.41
Norisoprenoids	α-ionone	*Paenibacillus*	0.94	0.98	0.97	0.933*
	β-ionone	*Blastomonas*	-0.85			
		Chryseobacterium	0.85	0.92	0.84	0.467*
		Lactobacillus	0.85	0.96	0.93	0.73
		Paenibacillus	0.83	0.92	0.95	0.27
		Rothia	-0.85			
	Dihydro-α-ionone	*Paenibacillus*	0.83			
	Dihydro-β-ionone	*Acetobacter*	-0.85	-0.86	0.73	0.526*
		Kocuria	-0.82			
		Paenibacillus	0.89			
		Pedobacter	-0.85	-0.86	0.73	0.522*
Lactones	δ-nonalactone	*Acetobacter*	-0.86	-0.98	0.97	0.95
		Chryseobacterium	0.82			
		Dolosigranulum	0.83			
		Lactobacillus	0.82			
		Paenibacillus	0.87			
		Pedobacter	-0.86	-0.92	0.84	0.429*

To validate the correlations found among VOCs and NGS data, predicted R-squared was calculated for those correlations that were significant both by applying Pearson's and Spearman's method (Table 7.2). Pearson's R value was calculated both using original and transformed NGS data (Transformed OTU abundance = -1/(1+OTU abundance) and the highest value was reported. Data transformation was included to account for asymptotic correlations. Twelve predicted R-squared, calculated with original NGS data, appeared to be coherent with their respective R-squared value: *Streptomyces*-nonanoic acid, *Fictibacillus*-(E)-2-hexenal, *Sphyngopyxis*-octanal, *Fictibacillus*-(E)-3-heptene, *Sediminibacterium*-€-3-heptene, *Sediminibacterium*-(Z)-3-heptene, *Acetobacter*-ethyl octanoate, *Brevibacillus*-2-heptanone, *Finegoldia*-2-carene, *Escherichia-Shigella*-(E)-geraniol, *Lactobacillus*-β-ionone and *Acetobacter*-δ-nonalactone. Additionally, 10 cases were identified correlating VOC emissions with transformed NGS data: *Corynebacterium*-nonanoic acid, *Cutibacterium*-octanal, *Staphylococcys*-5-methylfurfural, and *Enterobacter* with five monoterpene compounds (β-pinene, β-myrcene, α-phellandrene, β-phellanderene and limonene), nonanol and 2-pentanone.

7.4 Discussion

7.4.1 Sterilization and recolonization treatments modify fruit volatile emissions

While microbial communities have traditionally received more attention for their roles in the soil and rhizosphere, their involvement in fruit quality has raised interest only recently (Leff and Fierer, 2013). With regard to fruit aroma, for instance, the contribution of *Methylobacterium* spp. to the VOC profile of strawberries has been suggested in different studies (Verginer et al., 2010; Nasopoulou et al., 2014). The ability of *Bacillus* spp. to produce 4-(4-hydroxyphenyl)-butan-2-one (also referred to as raspberry ketone or frambinone) has also been pointed out (Feron et al., 2007). Nonetheless, all these researches focused on single species (e.g. *Methylobacterium* or *Bacillus*) and targeted specific volatiles (e.g. furanones). Moreover, they were performed by experimentally enriching the microbial community with selected bacteria.

In this work, the contribution of fruit-associated microbiota to fruit volatile emissions was evaluated by performing an untargeted analysis of VOCs in naturally colonized (C), sterile (S) and artificially reinoculated raspberries (R). Sterilization and recolonization significantly affected the volatilome profile of fruit, thus confirming the role of bacteria in fruit aroma construction. To further dissect the influence of different bacterial genera on fruit aroma, the whole microbiome was characterised by NGS. Aldehydes, monoterpenes, norisoprenoids, and other aroma-active compounds (octanal, nonanol) were significantly lower in sterile berries, and recolonization partially restored the emission of terpenoid compounds (Figure 7.1b). Only for one compound (ethyl hexanoate, characterised by overripe fruit aroma) the emission from recolonized fruit was higher than from control, as a possible result of plant tissue degradation. The absence of β-pinene and nonanol in sterile and recolonized berries, suggests a crucial influence of the microbiome in their emissions. In this view, the alteration of the microbiome structure observed in recolonized fruit may have not allowed to fully restore their emission. However, an effect of the sterilization treatment on the emission of these two compounds cannot be fully ruled out, despite the fact that compounds with similar chemical properties and concentrations, such as 2-carene and 2-nonanol, were not significantly affected by the sterilization treatment. Interestingly, β-pinene has been observed to be produced by several bacterial genera found on raspberry, such as *Paenibacillus* spp., which is part of the core microbiome, *Pedobacter* spp. and *Streptococcus* spp. (Lemfack et al., 2018). Similarly, nonanol has been found to be produced by several bacteria isolated from raspberry,

such as *Pseudomonas* (Ercolini et al., 2009), *Streptococcus* (Hertel et al.,2015) and *Bacillus* (Guo et al., 2020; Reese et al., 2020). The strong reduction of these genera in recolonized berries might partly explain the absence of β-pinene and nonanol when fruit microbiome was manipulated. VOCs belonging to the class of furfurals have not yet been reported to be produced by plants, whereas several studies highlight both production and degradation of these compounds by microorganisms, such as *Pseudomonas* spp. and *Cupriavidus* spp., respectively (Koopman et al., 2010; Crigler et al., 2020). 5-methyl furfural has been found to be produced by several bacterial species isolated from raspberry fruit and grown on its juice (Sangiorgio et al. 2021). This might justify its emission increase observed in artificially recolonized berries.

In addition to hedonic properties, several compounds reported in this work may play a role in defense against pathogens. Hexanal, for instance, is an inducer of systemic resistance in plants and an inhibitor of bacterial quorum-sensing (Anusha et al., 2021; Zhang et al., 2018), and has been reported to extend fruit shelf-life (Song et al., 1996). Methyl salicylate is also one of the main regulators of plant defenses. Terpenes are natural antimicrobial compounds, also effective on postharvest fruit preservation (Sivakumar and Bautista-Baños, 2014).

Taken together, the data presented here suggest that the fruit-associated microbiota probably contributes to a more intense and complex raspberry aroma, and its perturbation during fruit handling and storage might affect final fruit quality and shelf-life duration.

7.4.2 Commonalities of VOCs and fruit-associated bacteria in different raspberry cultivars

To evaluate the robustness of the findings of this work, both the microbiota and the VOC emissions of four raspberry cultivars were compared. Twenty-one VOCs were present in all the cultivars, including monoterpenes, norisoprenoids, and medium-length ketones and fatty acids. Microbiome data show the occurrence of genera potentially contributing to fruit aroma, such as *Paenibacillus*, Enterobacteriaceae, *Lactobacillus*, *Bacillus* (for which the emission of C5-C9 alcohols, aldehydes, ketones and acids was reported; Lemfack et al., 2018, Sangiorgio et al., 2021), and *Methylobacterium*, known for producing strawberry flavour components such as 2,5-dimethyl-4-hydroxy-2*H*-furan-3-one (Verginer et al., 2010; Nasopoulou et al, 2014).

Moreover, *Bacillus*, *Lactobacillus* and *Sphingomonas* spp. include specialised plant symbionts, modulating processes such as fruit quality and defense from pests and

pathogens (Sansinenea, 2019; Daranas et al., 2019; Asaf et al., 2020). The presence of microbes conserved among cultivars, highly integrated with plant metabolism, and potentially coevolved with their host, suggests that these species may have a significant role in determining the fruit phenotype.

7.4.3 Investigating association between volatiles and bacterial microbiome

To further dissect the influence of different bacterial genera on fruit aroma, after characterising 'Enrosadira' volatilome on the same fruit, microbiome was characterised by NGS. This analysis revealed that remarkable differences in the proportion of OTUs emerged after the recolonization process (Figure 7.2). The qualitative differences in the microbiota were reflected by the change in aroma profiles, as shown by the significant MFA association. However, the association of microbiome data resulting from NGS (characterized by high dimensionality, discrete values and sparsity) with other -omics data is not straightforward (Xia et al., 2018, 2020). Thus, Spearman's correlation test seemed the most adequate tool in absence of direct biological information on VOC relation to bacteria (Table 7.2).

For some compounds, such as nonanoic acid, monoterpene family, medium-chain fatty acid esters, the previously described antimicrobial properties can explain their negative correlations with a variety of OTUs (Sahin et al., 2006; Nakayama et al. 2015; Álvarez-Martínez et al., 2021). On the other hand, correlations between OTUs and VOCs may indicate direct production or utilization by bacteria, enhanced release by the plant tissues, selective effects of VOCs on specific bacterial taxa, or even indirect, non-causal relations. Thus, the overall aroma profile probably results from the complex interaction of all these effects and from the proportion of bacterial populations at their ecologic equilibrium.

7.4.4 OTU-specific correlations with VOCs

The possibility to gain further insight in the role of specific OTUs was explored by assessing the predictive power of their linear or asymptotic correlation with VOCs. Asymptotic correlation may fit several biological processes like substrate limitation, volatilization from liquid phase, or microbial interspecies interactions.

Some bacterial taxa (*Fictibacillus*, *Lactobacillus*, *Paenibacillus*, *Sediminibacterium*, but also potential or opportunistic human pathogens such as *Escherichia* and *Cutibacterium*)

show a significant positive correlation with VOCs, such as (E)-2-hexenal, octanal, heptene isomers and norisoprenoids. The latter compounds are key components of raspberry aroma, and the observed correlation with *Lactobacillus* and *Paenibacillus* might indicate a positive effect of these genera on fruit quality. *Lactobacillus* spp. have been associated to an increased production of norisoprenoids from carotenoid-rich media (Mapelli-Brahm et al., 2020). However, norisoprenoid synthesis by *Lactobacillus* and *Paenibacillus* spp. has not been reported, and the contribution of these microbes to raspberry aroma requires further investigation.

The most striking effect of recolonization of sterilized fruit was the enrichment of Enterobacteriaceae populations. This group, and most prominently the genus *Enterobacter*, showed a negative correlation with several monoterpenes (limonene, β-pinene, α- and β-phellandrene, β-myrcene). An explanation may be the monoterpene subtraction by the increased bacterial population (Park et al., 2003; Yang et al., 2007; Harder et al., 2000). Alternatively, the observation of similar monoterpene emission levels from sterile (Enterobacteriaceae-free) and recolonized (Enterobacteriaceae-enriched) raspberries would rather suggest that monoterpenes, emitted by the whole microbiota, are highly effective in antagonising Enterobacteriaceae colonization. Since this group includes potential human pathogens, this aspect of VOCs relations with the microbiota has a relevance for fruit safety.

7.5 Conclusions and future perspectives

The untargeted approach used in this research shed a new light on the contribution of the bacterial microbiota to raspberry aroma. Correlation analysis highlighted several significant associations between bacterial genera and volatile emissions, which might be better explained analysing bacterial metabolism and the complexity of interactions occurring at fruit-niche level. This research provides the first characterization of raspberry core microbiome and, based on our results, bacteria naturally resident on raspberry fruit could be selected to improve fruit quality, aroma, shelf-life and safety in an ecological and sustainable way. Future work will aim at clarifying the mechanisms of interaction with the fruit, as well as the optimal conditions for the enhancement of raspberry aroma, safety and overall fruit quality.

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8. General Conclusions

Altogether, this work provides relevant insights into the role and application of microbes as sustainable strategy to reduce abiotic and biotic stresses, increase productivity and quality of strawberry and raspberry.

In particular, we found that strawberry microbiomes are associated to plants in a genotype- and organ-dependent manner, both considering their taxonomical and functional composition. Additionally, we isolated several Plant Growth Promoting Bacteria from strawberry and proved their ability to protect plants against *Xanthomonas fragariae* infection and from salinity.

We considered raspberry for investigating the effect of the cultivation method on fruit microbiome, quality and aroma determination. Specifically, we found that the cultivation method influenced fruit nutraceutical traits, aroma and epiphytic bacterial biocoenosis. Furthermore, we found that key bacteria might be responsible for aroma emitted by berries produced under different cultural management. Notably, we found that differently treated berries showed significantly different aromatic profile and that these differences correlated with difference in fruit microbiota. This highlight the role of bacteria in fruit aroma development. Our findings open the way to new attractive applications of bacterial-based products ranging from the agricultural field, where microbes might be exploited for increase plant tolerance and resistance to various stresses, to the food industry, where they could represent a valid alternative to the use of chemical additives and aromas.