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DISCLOSING THE ROLE OF ROOT EXUDATES IN MODULATING THE INTERACTION BETWEEN PLANT AND XENOBIOTIC DEGRADING BACTERIA

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Abstract

Positive interactions between plants and root-associated bacteria are fundamental to maintain the homeostasis of the holobiont, especially upon environmental stresses. This crosstalk is mediated by root exudates (REs), including plant secondary metabolites, that are released by the plant as a 'cry-for-help' to recruit beneficial bacteria. Plant-bacteria interactions are particularly relevant for rhizoremediation, a sustainable strategy for the clean-up of recalcitrant xenobiotics like polychlorinated biphenyls (PCBs), contaminants that induce severe phytotoxic effects, as well as posing serious threats to ecosystems and human health. The aim of this PhD thesis was to disclose the role of root exudates differentially released under PCB stress, in influencing bacterial rhizocompetence traits, necessary to guarantee efficient root colonization and induce PCB degradation.

Among root exudates, flavonoids are well described as signaling molecules and were reported as inducers of the PCB degradation pathway. The first objective of this thesis was to test this capability using the PCB degrader *Paraburkholderia xenovorans* LB400, by assaying pure flavonoids and the complex blend of REs released by *Arabidopsis thaliana*. LB400-*Arabidopsis* interaction was monitored in presence and absence of PCB-18 using a fluorescence-tagged LB400 strain, to observe root colonization and plant-growth-promotion potential upon stress. Our findings indicated that flavonoids stimulated LB400 proliferation, induced PCB degradation potential and increased biofilm formation, swimming motility and chemoattraction, features overall implicated in the early root colonization. However, other non-flavonoids molecules seemed to be involved in later stages of colonization and supported the plant growth promotion effect induced by LB400 under phytotoxic growth conditions. The REs alteration caused by PCBs was therefore explored to identify the main players in the contaminant-driven 'cry-for-help'. A metabolomic analysis was performed on the REs of *Arabidopsis* grown in control conditions and in presence of a phytotoxic concentration of PCB-18, to observe the differential abundancies of secreted compounds and assess the role of five identified metabolites on the metabolism of PCB-degrading bacteria. PCB-18 dramatically affected plant physiology and altered *Arabidopsis* exudation pattern. Remarkably, the secretion of scopoletin, a plant secondary metabolite with known antimicrobial characteristics, was inhibited in presence of PCB stress, thus putatively remodeling the rhizosphere ecological niche to provide more suitable conditions for PCB-degrading bacteria. Instead, over-exuded metabolites were utilized as carbon and nitrogen sources and affected different rhizocompetence features. Finally, a bacterial biosensor was developed to clarify the role of root exudation by using a more realistic plant-bacteria interaction experimental set-up. The use of *Pseudomonas* JAB1 biosensor strain allowed the prompt visualization of root colonization and of the spatial distribution of cells in which PCB degradation was triggered by the REs. Our findings substantiated the conclusions about a putative ‘cry-for-help’ of the plant exposed to PCB-18 and about the centrality of flavonoids in sustaining this beneficial interaction.

To conclude, the results of this thesis unveiled the functioning of plant-bacteria associations upon PCB stress and confirmed the relevance of REs in assembling a rhizosphere microbiota that provides the plant with useful services to improve contaminant degradation and support the holobiont health. These findings will be crucial to develop improved rhizoremediation interventions in PCB contaminated soils.

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INTRODUCTION

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Rationale and aim of the work

Environmental contamination by persistent xenobiotics like polychlorinated biphenyls (PCBs) is a worldwide concern and poses serious risks for human health and ecosystem wellness. PCBs constitute a class of 209 synthetic compounds that have been extensively produced and utilized for many industrial applications during the last century. Given the arising concerns about their toxic and carcinogenic nature, PCBs were classified as persistent organic pollutants (Stockholm Convention on POPs, 2015; IARC, 2015) and their production banned about 40 years ago. However, the high stability and recalcitrance to biodegradation of these pollutants led to their massive spread and accumulation in the environment, particularly in the sites of former industrial production and in the surrounding agricultural soils and groundwater due to improper disposal or leakage from the industrial plants (Passatore et al., 2014). In northern Italy, the contaminated site Brescia-Caffaro extends over more than 100 ha of agricultural land and presents a PCB concentration largely exceeding the legal thresholds for residential areas, therefore impeding any cultivation for food and feed purposes on the area (Di Guardo et al., 2017). Being lipophilic molecules, PCBs can accumulate in animal tissues and biomagnify in the food chain, thus threatening human health (Jha et al., 2015). Indeed, the consumption of contaminated food, especially when rich in lipids like dairy products, meat and fish, emerged as the main source of assimilation of PCBs for humans (Reddy et al., 2019). Soil decontamination is therefore urgent both for public health reasons and to possibly return the contaminated fields to agricultural use.

Conventional remediation techniques are not suitable for the decontamination of large areas due to the high costs and energetic consumption involved. They often require soil digging from the contaminated site for *ex situ* thermal and chemical treatments and subsequent refilling of the cleaned matrix (Jing et al., 2018). Therefore, rhizoremediation has emerged as a promising *in situ* strategy for soil decontamination by PCBs being less disruptive, more sustainable and allowing the preservation of soil function and fertility (Vergani et al., 2017). This technique relies on the complex frame of natural interactions occurring between plants and their root-associated microbiota that together constitute the plant holobiont. This crosstalk enables the establishment of a win-win relationship essential for maintaining the holobiont fitness in polluted environments. Indeed, experimental evidence in the last decades highlighted the importance of the ecological services provided by the plant-associated microbial community to improve the host resistance to stresses and to promote its adaptation to environmental changes. Plants, on the other hand, exploit the process of root exudation, consisting in the release of a complex blend of primary and secondary metabolites, to nurture and influence the functionality of the associated microbiota (Berlanga-Clavero et al., 2020; Trivedi et al., 2020). The modification of root exudation chemistry of the plant as a stress response is crucial to shape the composition of the root-associated microbiota in order to improve beneficial features that are essential to cope with stress. This strategy is defined as 'cry-for-help' and has been documented only recently to be activated by the presence of biotic stresses like the attack of phytopathogens as a defense strategy (Rolfe et al., 2019) as well as under abiotic stresses like drought, high salinity or nutrient deprivation (Xiong et al., 2020; Rizaludin et al., 2021). In this work, we inquire whether such response could be triggered by PCB stress in plants. Indeed, PCBs are phytotoxic compounds that cannot be degraded nor detoxified completely by plant metabolism and often the modifications induced by the plant detoxification routes lead to the formation of by-products like OH- and MeO-modified PCBs that are even more deleterious for plants than the parental compounds (Subramanian et al., 2018). Therefore, plants can survive in PCB-contaminated soils only by establishing beneficial associations with microorganisms endowed with the enzymatic degradation machinery to catabolize PCBs. In **Chapter I** of the thesis introduction, research works related to plant-microbe interactions in polluted soils are reviewed, highlighting how the findings and knowledge collected support the hypothesis of a 'cry-for-help' in these environments. According to the proposed vision, when exposed to phytotoxic compounds like PCBs, plants resort to this specific survival strategy to recruit PCB-degrading bacteria possessing the catabolic genes encoded by the *bph* operon that are necessary for PCB degradation and transformation into less phytotoxic compounds.

As previously stated, plant competence to select and attract a degradative bacterial community and the enzymatic versatility of root-associated microorganisms to catabolize persistent pollutants are fundamental assumptions for rhizoremediation. Therefore, understanding the crucial mechanisms involved in the dialogue between plant and PCB-degrading bacteria mediated by root-exuded secondary metabolites is of great importance to improve and fine-tune PCB rhizoremediation strategies. Being among the most abundant secondary metabolites synthesized by plants, flavonoids play a key role in mediating interactions with beneficial bacteria. These phenolics have been studied in detail for their role as signal molecules in initiating legume-rhizobia symbioses, as mediators of the interaction with arbuscular mycorrhizal fungi, as quorum sensing regulators and as allelochemicals against parasitic plants (Shaw et al., 2006; Hassan and Mathesius, 2012). Besides this, flavonoids have been extensively described in scientific literature for their diverse roles as signal and defense molecules to maintain the homeostasis of the holobiont especially when subjected to biotic and abiotic stresses (Shah and Smith, 2020). For this reason, flavonoids can be considered molecules that regulate inter-kingdom communications: a comprehensive literature review of the main findings in this regard is provided by **Chapter II**.

However, evidence about flavonoid role in coordinating plant relationships with non-rhizobial bacterial species, particularly in the context of xenobiotic contamination stress, is still limited. In this framework, the aim of this PhD work was to improve the knowledge about the mechanisms on which rhizoremediation relies, like the stimulation of traits involved in the bacterial ability to colonize and persist on plant roots (rhizocompetence) and the maintenance and biostimulation of the activity of the degrading microbiota through root exudation. A further objective was to clarify root-exudation alterations induced by PCB stress and test the putative 'cry-for-help' hypothesis by analyzing plant-bacteria interactions in presence of PCBs. The findings from this thesis potentially have applicative implications by contributing to improve the effectiveness of soil rhizoremediation strategies for PCB transformation and for the clean-up of polluted soils through sustainable and green technologies.

Previous research highlighted the role of root-exuded secondary metabolites, particularly flavonoids, as co-metabolites or inducers of PCB degradation. Most of these studies analyzed the enhancement of PCB depletion rates via resting cell assays in presence of root exudates or the level of expression of the *bphA* gene encoding for the α subunit of the dioxygenase that initiates biphenyl aerobic degradation pathway (Pham et al., 2015; Vergani et al., 2019). In **Chapter III**, the model PCB-degrading strain *Paraburkholderia xenovorans* LB400 was employed with the aim to investigate in detail the impact of flavonoids on functional features related to rhizocompetence. The study also examined the role of flavonoids in inducing the expression of the enzymatic catabolic machinery involved in PCB degradation in strain LB400, thus potentially alleviating plant stress. By using both a selection of representative root-exuded flavonoids, known from previous studies as signaling molecules (Tadra-Sfeir et al., 2015; He et al., 2022) and biostimulators of PCB degradation (Toussaint et al., 2012; Pino et al., 2016; Zubrova et al., 2021), and the more complex mixture of root exudates naturally released by *Arabidopsis thaliana* mutant lines differentially affected in the biosynthesis and exudation of flavonoids (Narasimhan et al., 2003), it was possible to analyze the function of these secondary metabolites in improving the rhizocompetence of the strain and its PCB degrading activity. The analysis of root colonization efficiency and plant growth promotion by strain LB400 on *Arabidopsis* plants subjected to PCB stress was possible though an *in vitro* plant-bacteria interaction assay developed in this work that effectively simulates the phytotoxic effects caused by the contaminant. Finally, strain LB400 colonization pattern was observed via microscopy analysis by generating and employing a novel fluorescence-tagged version of the strain. There is still a lack of studies that analyze in detail the mechanisms underlying plant-bacteria interactions and root colonization mediated by flavonoids under PCB stress, in particular using the model PCB degrader *Paraburkholderia xenovorans* LB400, or that explore the potential of this strain as a plant growth promoter under contamination stress. Our results highlighted that the presence of flavonoids is crucial for early root colonization and for the recruitment of PCB-degrading strains and that, in parallel, also other metabolites might be relevant in root exudation chemistry changes and adaptations induced by PCB stress.

Consequently, it was important to gain a deeper understanding about the specific root exudates involved in recruiting and maintaining a degrading bacterial community in the rhizosphere.

Considering the lack of broader analyses on plant root exudation when exposed to PCB stress, to support the hypothesis of a specific contaminant-driven ‘cry-for-help’ response, the study reported in **Chapter IV** was developed. A metabolomic analysis was performed on the model plant *Arabidopsis thaliana* Col-0 to characterize root exudation in control conditions and when exposed to PCB stress. The analysis allowed the identification of 65 differentially exuded compounds and five of these, including primary and secondary metabolites which exudation was either up-regulated or down-regulated upon PCB exposure, were further characterized. As described in the previous chapters, primary and secondary metabolites may act as carbon sources or signaling molecules that stimulate metabolic traits of PCB-degrading bacteria. The exudation of these molecules upon stress facilitates the recruitment of beneficial strains that can provide beneficial services to the plant holobiont and restore its fitness. Conversely, other specific secondary metabolites, such as coumarins, may have a repulsive role for specific rhizospheric bacteria as, among others, these molecules showed selective antimicrobial properties (Stringlis et al., 2018; Voges et al., 2019). Therefore, our hypothesis was that challenged plants that are subjected to abiotic stress might down-regulate the exudation of these potential antimicrobials to avoid hindering the survival of beneficial strains upon stress. The beneficial or deleterious properties of the identified molecules were tested on three PCB-degrading bacterial strains, namely *Acinetobacter calcoaceticus* P320, *Pseudomonas alcaliphila* JAB1 and *Paraburkholderia xenovorans* LB400.

Finally, a bacterial biosensor was designed, with the aim of gaining a deeper insight into the complex interactions occurring in the holobiont subjected to PCB stress and about the spatial dynamics of recruitment of beneficial bacteria through root exudation. This could be a simple and immediate approach to monitor the activation of the PCB catabolic pathway *in vitro* and to observe root adhesion of PCB degraders and their colonization efficacy in order to validate plants useful for rhizoremediation strategies (Van Der Meer and Belkin, 2010). **Chapter V** focuses on the development of the biosensor strain using the PCB degrader *Pseudomonas alcaliphila* JAB1. The strain was genomically tagged with a fluorescent mScarlet protein expressed constitutively, to monitor root system colonization, and with an eGFP protein regulated by the *bph*-operon promoter, to observe the induction of the PCB catabolic pathway eventually triggered by root exudates. The JAB1 biosensor strain was validated by testing its sensitivity with different concentrations of biphenyl as main inducer of the degradative pathway. Subsequently, the JAB1 biosensor strain was applied *in planta* for an interaction assay with *Arabidopsis thaliana* and the mutant lines for flavonoid biosynthesis and exudation under stress caused by PCBs as described in Chapter III. Through the novel engineered strain, it was possible to observe the root exudation-mediated ‘cry-for-help’ through microscopy analysis as well as identify the main root portions involved in triggering the PCB degradation response in strain JAB1.

To summarize, **general conclusions and future perspectives** arising from this PhD work are reported in the last chapter of the thesis.

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CHAPTER I

‘Cry-for-help’ in contaminated soil: a dialogue among plants and soil microbiome to survive in hostile conditions

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Summary

An open question in environmental ecology regards the mechanisms triggered by root chemistry to drive the assembly and functionality of a beneficial microbiome to rapidly adapt to stress conditions. This phenomenon, originally described in plant defence against pathogens and predators, is encompassed in the 'cry-for-help' hypothesis. Evidence suggests that this mechanism may be part of the adaptation strategy to ensure the holobiont fitness in polluted environments. Polychlorinated biphenyls (PCBs) were considered as model pollutants due to their toxicity, recalcitrance and poor phyto-extraction potential, which lead to a plethora of phytotoxic effects and rise environmental safety concerns. Plants have inefficient detoxification processes to catabolize PCBs, even leading to by-products with a higher toxicity. We propose that the 'cry-for-help' mechanism could drive the exudation-mediated recruitment and sustainment of the microbial services for PCBs removal, exerted by an array of anaerobic and aerobic microbial degrading populations working in a complex metabolic network. Through this synergistic interaction, the holobiont copes with the soil contamination, releasing the plant from the pollutant stress by the ecological services provided by the boosted metabolism of PCBs microbial degraders. Improving knowledge of root chemistry under PCBs stress is, therefore, advocated to design rhizoremediation strategies based on plant microbiome engineering.

1. The 'cry-for-help' hypothesis is poorly investigated in polluted environments

An impressive body of evidence proves that the root chemistry, that includes the metabolites exudated by the plant, their breakdown compounds and the soil microbe-degraded sub-products, plays a crucial role in shaping the structure and functionality of the root-associated microbiome (Olanrewaju et al., 2019). The extent and nature of exudation varies with the age of the plant (Pausch and Kuzyakov, 2018), with the plant genetics (Cordovez et al., 2021), pathogen or symbiotic interaction or exposure to abiotic stresses, such as drought, high salinity, flooding, extreme temperatures or nutrient starvation (Vives-Peris et al., 2020). The root chemistry drives the wellness of the holobiont, the meta-organism resulting from the proficient interactions between the plant and the associated microbiome (Vandenkoornhuyse et al., 2015). In this win–win relationship, primary and secondary metabolites, volatile organic compounds, cells debris and by-products of senescent root cells, accounting for ~10% of the total photosynthetically fixed carbon (Pausch and Kuzyakov, 2018), are allocated in the flux of root exudation to recruit, feed and influence the metabolism of specific microbial taxa (Liu et al., 2020). Recruited soil microbes establish in the rhizo/endosphere and assist the plant homeostasis by encoding for functionalities that extend the plant genome (Berendsen et al., 2012).

Despite the accumulating evidence, the molecular mechanisms that rule over the causal connection between (i) the plant phenotypes relevant for fitness, like development, growth, and health and (ii) the microbiome structure and functionality are still unknown.

In recent years, an experimental framework related mainly to pests- (Cotton et al., 2019; Liu et al., 2021) and herbivore-attacked plants (Hu et al., 2018) has been validated to describe the proficient interplay between plant and microbiomes and that is encompassed in the 'cry-for-help' hypothesis (Rolfe et al., 2019). According to this theory, under phytopathogen or herbivore-induced stress, the plant exudation pattern is shifted to release specific chemicals that favour the recruitment of beneficial microbes or of antagonists able to hinder the growth of pathogens (Rasmann and Turlings, 2016; Carrión et al., 2019; Rolfe et al., 2019). For example, *Fusarium oxysporum*-infected cucumber roots were showed to increase tryptophan and decrease raffinose exudation, enhancing the colonization ability of the beneficial strain *Bacillus amyloliquefaciens* and counteracting the pathogen proliferation. On the other side, *B. amyloliquefaciens* releases tryptophan-dependent auxin, in a positive feedback loop to support phytohormone homeostasis (Liu et al., 2017). The 'cry-for-help' hypothesis also explains the development of disease-suppressive properties in infested soils (Wilkinson et al., 2019) and the maintenance of this legacy to subsequent plant generations growing in the same soil (Kong et al., 2019). For instance, through a metatranscriptomic approach targeting the sugar beet rhizosphere communities growing in a *Rhizoctonia*-suppressive soil, bacteria affiliated to various families, (including *Oxalobacteraceae*, *Sphingobacteriaceae*, *Burkholderiaceae*, *Alcaligenaceae*, *Cystobacteraceae*, *Sphingomonadaceae*, *Cytophagaceae*, *Comamonadaceae* and *Verrucomicrobia*) were demonstrated to upregulate stress-related genes to enhance survival strategies to cope with the pathogen (Chapelle et al., 2016; Expósito et al., 2017). Similarly, entomopathogenic nematodes are attracted by (E)-ß-caryophyllene, exudated by maize roots to hinder the damages caused by the insect *Diabrotica vergifera* (Rasmann et al., 2005).

In response to pathogen and pest attack, the plant microbiome is often enriched of bacteria that potentially can enhance plant defences or directly counteract the pathogen proliferation. Members of the genera *Chitinophaga*, *Chryseobacterium*, *Flavobacterium*, *Microbacterium*, *Pseudomonas*, *Sphingomonas*, *Stenotrophomonas* and *Xanthomonas* have been recently identified among the beneficial bacteria (Liu et al., 2020).

Beyond the 'adapt or migrate' strategy, plants could employ the 'cry-for-help' approach to benefit from microbial associations in contaminated environments, in case the pollutants are highly recalcitrant and toxic, poorly biodegradable and phyto-extractable. Often plants fail to achieve full metabolism of persistent organic compounds, resulting in slow removal and incomplete degradation because they lack the catabolic enzymes necessary for their complete mineralization (Eapen et al., 2007; Schwitzguébel, 2017). As a consequence, xenobiotics commonly induce molecular injuries that disrupt biochemical, physiological and signaling processes and unbalance plant growth and survival (Ramel et al., 2012). The impact of xenobiotics exposure on rhizodeposition is underrated and the knowledge is sparse, limited to heavy metals like cadmium (Bali et al., 2020) and aluminum (Saha et al., 2020). The root system plunging in polluted soil, by changing the rhizodeposit fingerprint, resorts to the 'cry-for-help' to recruit microbes for their plant-growth promoting activities, with the potential to release the plant from stress (Vergani et al., 2017a). Furthermore, the eventual enrichment of bacteria possessing the enzymatic machinery able to degrade the pollutant can decrease its local concentration, enhancing the detoxification in the root/rhizosphere compartments. This beneficial association is the keystone for rhizoremediation strategies, consisting in pollutant removal by microorganisms in the root zone (Balloi et al., 2010; Vergani et al., 2017b).

Classified within the most deleterious persistent organic compounds (POPs) for human, animal and ecosystem health (Stockholm Convention on POPs, 2015; Hens and Hens, 2017), polychlorinated biphenyls (PCBs) are a wide class of 209 congeners containing biphenyl with one up to 10 chlorine atoms. Although their production was banned worldwide in 1979 (Passatore et al., 2014), PCBs still represent a threat for their teratogenic (Berghuis and Roze, 2019), carcinogenic (Magoni et al., 2019; Guo et al., 2020), mutagenic potential (Murati et al., 2020), together with their recalcitrance in the environment (Simhadri et al., 2020), persistence and biomagnification in the food web (Amutova et al., 2021). For land reclamation, PCB-polluted soils should be excavated for off-site treatment by solvent extraction, thermal alkaline dechlorination, incineration or landfilling (Campanella et al., 2002). While these techniques are expensive and, in many cases, almost infeasible due to the general large extension of the contamination (Van Aken et al., 2010), rhizoremediation offers a sustainable, potentially efficient and cost-effective technology for PCBs removal from soil (Vergani et al., 2017b).

In this review, the role of root exudation as plant driver of the 'cry-for-help' strategy in PCBs contaminated soil is discussed, showing its central role in recruiting and sustaining the microbial services to reduce the contaminant load, thus restoring the holobiont health.

2. Plant fitness falters in PCBs contaminated soil

The routes of PCBs uptake and translocation in plant tissues are largely unknown: as foreign molecules not naturally present in the environment, they presumably enter plant tissues by hijacking the pathways required for the uptake of soil nutrients (Greenwood et al., 2011).

PCBs mainly absorb on root epidermal surfaces and then diffuse in less amount within the root tissues (Fig. 1), as observed in alfalfa plants exposed to PCB-28 (Teng et al., 2017). The root capacity to absorb extremely hydrophobic compounds like PCBs depends on the thickness of waxes on the root epidermis (Zhang et al., 2009). Pectins, as well, may mediate PCBs accumulation in the root tissues: the *Arabidopsis* mutant *quasimodo1*, affected in a galacturonosyltransferase enzyme, showed a reduced amount of PCB 18 in the root system (Bao et al., 2013). Although pectins are well documented in chelating heavy metals (Shao et al., 2021), their role in PCBs phyto-extraction remains elusive.

Several lines of evidence suggest that lower chlorinated PCBs are preferentially uptaken and accumulated by the roots than higher chlorinated PCBs (Asai et al., 2002; Luo et al., 2020; Fig. 1). Once collected in the root tissue, PCBs can be then translocated at some extent in the epigeal compartment (stem and leaves), as observed in pumpkin plants cultivated in a soil polluted by the commercial PCBs mixture Aroclor (Whitfield Åslund et al., 2008). Similar indications were observed in poplar plants grown in a hydroponic medium artificially contaminated by PCBs where the higher chlorinated compounds were compartmentalized in the root and the lower chlorinated ones were mobilized in the stem (Liu and Schnoor, 2009). The translocation seems to occur through the xylem sup, as documented in pumpkin plants (Greenwood et al., 2011) (Fig. 1). PCBs concentration decreases in the sup along the shoot as the distance from the roots increases, with low chlorinated compounds that are more easily mobilized toward the plant aerial compartments (Whitfield Åslund et al., 2007; Greenwood et al., 2011). PCBs translocation seems to be influenced not only by the PCB congener and the chlorine substitution pattern, but also by the plant species (Asai et al., 2002; Iwabuchi et al., 2020).

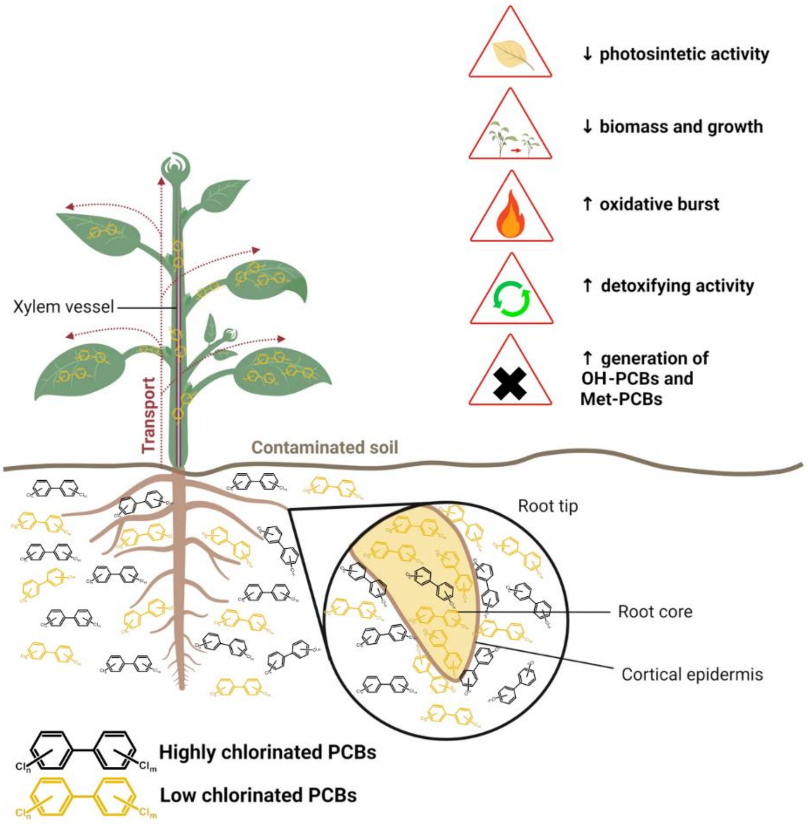

Fig. 1 Polychlorinated biphenyls (PCBs) distribution in plant tissues and phytotoxic effects. Several studies reported a differential distribution of low and highly chlorinated PCBs in plant tissues. The external cortical epidermis of the root can interact and be enwrapped by PCBs with a different degree of chlorination, but low chlorinated PCBs are favoured in entrance in the root internal core and then can be translocated through the xylem sup in the plant aerial compartments, both stem, shoot and leaves. Plants are unable to fully mineralize PCBs and the exposition to these toxic pollutants result in a plethora of phytotoxic effects, affecting plant growth and health.

Controversial conclusions were drawn for PCBs-induced phytotoxicity, which has been estimated on a wide array of heterogeneous experimental settings, including in vitro assay or field trial with different plant species at various developmental stages and different PCB congener's mixtures (Zeeb et al., 2006; Wang et al., 2017). Thus, variable plant responses to PCBs exposition were recorded. Some studies reported a phytotoxic effect given by PCBs on plant growth only at high concentrations (Jin et al., 2011; Wang et al., 2017), and low to moderate negative effect for lower levels of PCBs contamination (Zeeb et al., 2006; Deavers et al., 2010; Subramanian et al., 2018; Urbaniak et al., 2020). On the contrary, in some cases PCB were described to induce plant growth stimulation (Subramanian et al., 2018; Urbaniak et al., 2020), claiming for a hormesis effect (Calabrese, 2014).

A possible explanation to the observed variability in PCBs toxic effect is linked to the above-mentioned factors affecting PCB uptake by the roots and translocation, such as the plant species and PCB hydrophobicity and bioavailability, strongly dependent on the congener and substitution patterns (Van Aken et al., 2010).

Xenobiotics responses in higher plants are still cryptic, although it is ascertained that POPs could disrupt molecular and biochemical processes, thus affecting signaling circuits (Ramel et al., 2012). Transcriptomics study of *Arabidopsis* plants exposed to 2,5-dichlorobiphenyl (Jin et al., 2011) and tetrachlorobiphenyl (Subramanian et al., 2018) showed an enrichment in gene functions related to the metabolism of toxic substances and to response to oxidative stress, oomycetes and bacteria, confirming, as observed for other stresses, the convergence of biotic and abiotic signaling responses (Fujita et al., 2006).

Phenotypically, PCBs exposure showed to cause fall in germination rate (Meier et al., 1997; Subramanian et al., 2018), reduction of the plant biomass (Kučerová et al., 2000), decrease in the chlorophyll content with leaf bleaching effect (Bao et al., 2013) and burst of oxidative stress (Ahammed et al., 2013) (Fig. 1).

To cope with xenobiotics, plants adopt a three-steps detoxifying strategy, summarized in the green liver model (Sandermann, 1994), that includes: (i) activation of POPs by oxidation to produce hydroxylated molecules with a higher solubility; (ii) conjugation to generate adducts with glutathione to decrease the POPs toxicity and (iii) sequestration of the conjugates in the vacuole or incorporation into plant cell wall (Coleman et al., 1997).

Unfortunately, this detoxification mechanism is inefficient and has a limited impact on plant release from PCBs-induced stress (Schäffner et al., 2002). Indeed, much evidence suggest that plant catabolism of PCBs is limited to tetrachlorinated and lower congeners (Kučerová et al., 2000; Van Aken et al., 2010), and, moreover, the substitution patterns of these molecules can dramatically affect their recalcitrance (Rezek et al., 2007; Van Aken et al., 2010).

Furthermore, the initial metabolism of PCBs in plants involves the activity of monooxygenases of the cytochrome P-450 family and peroxidases that generate hydroxylated (OH-) and methoxylated (MeO-) PCBs as observed in rice, maize, wheat and *Arabidopsis*, having an enhanced toxicity than the parental molecule (Sun et al., 2016; Subramanian et al., 2018; Sun et al., 2018; Lin et al., 2020), posing a further stress for plant physiology and an environmental risk for human and ecosystem health (Wu et al., 2014). Exposure to OH- and MeO-PCBs in rice triggers an enhanced energy demand, promoting the catabolism of pyruvate, the TCA cycle (tricarboxylic acid), the transfer of acetyl groups into mitochondria and finally resulting in a higher biomass reduction than the parental 2,3,4,5-tetrachlorobiphenyl treatment (Lin et al., 2020). The plant displays the most efficient detoxification effect, glutathione-mediated, only after exposure to the parental congener, while a vast array of less effective antioxidant mechanisms is activated by OH- and MeO-PCBs, explaining, therefore, their higher phytotoxicity (Lin et al., 2020).

Recently, in the soil of a historically polluted site in Italy (Di Guardo et al., 2017), sulfonated- and hydroxy-sulfonated-PCBs were detected (Bagnati et al., 2019). The synthesis of these sulfonated compounds, maybe through the action of the glutathione S-transferase enzyme (Blanchette and Singh, 2003), has been hypothesized to be part of the detoxifying strategy to enhance the PCBs mobility and bioaccessibility (Bagnati et al., 2019) as already demonstrated for other molecules potentially harmful for the plant (Chen et al., 2015). Plant and environmental fate of such modified PCBs remains still to be elucidated.

Knowledge about plant metabolism of xenobiotics is still fragmentary, although it was described that plants contribute to de-adsorb PCBs from soil particles and mobilize them, up to 62% of the initial PCB contamination (Chekol et al., 2004). During growth and development, plants influence in the soil surrounding root the abundance and composition of dissolved and particulate organic carbon (DOC and POC respectively) that are the main drivers for the chemical movement of hydrophobic compounds in soil (Terzaghi et al., 2020). The increased PCB bioavailability could further compromise plant growth. Therefore, the selection of plant species effective for PCB phytoremediation like *Medicago sativa* (alfalfa), *Lespedeza cuneate* (Chinese bushclover), *Lathyrus sylvestris* (everlasting pea), *Phalaris arundinacea* (reed canary grass), *Cucurbitaceae* (cucurbits), *Sparganium* (bur-reed), *Salix alaxensis* (Alaska willow) and *Picea glauca* (white spruce; Chekol et al., 2004; Ficko et al., 2010; Slater et al., 2012; Terzaghi et al., 2020), have been suggested as crucial for land reclamation of PCB-polluted areas. The increase of PCB bioavailability in the rhizosphere makes these highly recalcitrant molecules more accessible also for microbes featuring the enzymatic arsenal to degrade them (Vergani et al., 2017b), generating propitiatory conditions for the ‘cry-for-help’ strategy. Although it can be supposed that PCB- induced effect on plant physiology and stress response could affect rhizodeposition, the changes in the root chemistry have still to be mechanistically demonstrated.

3. PCBs-degradative potential of soil microbiomes

Despite PCBs have been introduced in the environment by human activity in relatively recent times, bacterial communities inhabiting polluted soils are well known for their potential to degrade this class of molecules, via both dechlorination and cleavage of the biphenyl ring. These capacities are probably derived from the cycling of natural organohalogens (Atashgahi et al., 2018; Temme et al., 2019) and the degradation of plant-derived aromatic organic compounds (Fuchs et al., 2011) as a consequence of structural similarity.

PCB dechlorination has been confirmed only as anaerobic reductive activity among members of the phylum Chloroflexi, including *Dehalococcoides*, *Dehalogenimonas* and *Dehalobium*, either by organohalide-respiration or by co-metabolism (Xiang et al., 2020). There is evidence that also bacteria classified as *Clostridia* (Firmicutes) and *Geobacteraceae* (δ-Proteobacteria) are involved in this activity (Wang and He, 2013; Praveckova et al., 2016).

The most studied PCB-dechlorinating bacteria are strains belonging to the only described species of the genus *Dehalococcoides*, *D. mccartyi*. This species is an obligate organohalide-respiring bacteria (OHRB), strictly anaerobic, with a small-sized genome yet encoding for several reductive dehalogenase enzymes (RDases) active towards a broad range of chlorinated compounds that represent its sole source of energy (Taş et al., 2010; Bedard, 2014). *Dehalococcoides* presence and activity was reported in different environments such as polluted soils, sediments and groundwater (Taş et al., 2010), however, RDases responsible for PCB dechlorination were described for the first time in 2014 (Wang et al., 2014). Combining cultural techniques with metagenomic and metatranscriptomics, the authors identified in three new isolates of *D. mccartyi* different reductive dechlorinases, PcbA1, PcbA4 and PcbA5, catalysing the meta and para-dechlorination of several PCB congeners of the mixture Aroclor 1260 (Bedard, 2014).

The role of biotic and abiotic factors influencing the activity of OHRB in soils is still poorly understood; however, the interactions with other bacteria populations, the soil type and water content seem to be crucial. A pivotal role is likely played by other bacteria providing electron donors, carbon sources and the essential corrinoid cofactors for RDases (Men et al., 2012). It has been proposed that *D. mccartyi* forms a core community with *Methanosarcina* and *Desulfovibrio* populations, which have been observed to support PCB dechlorination mediating acetate and hydrogen sources (Wang et al., 2019). The structure and activity of organohalide-degrading communities are also connected to soil edaphic factors and the presence of anaerobic niches allowing the metabolism of strictly anaerobic OHRB. In a recent study, the soil water content was identified as the main parameter positively related to PCB dechlorination, increasing the cell-to-contaminant availability (Shen et al., 2021). Both the presence of oxygen-depleted niches, due to root respiration activity, and the increase in water retention due to the release of exopolysaccharides, indicates the plant rhizosphere as a potential hot spot for PCB reductive dechlorination.

As most of the knowledge about PCB dechlorination deals with OHRB, far less is known about the possible role of other bacteria and associated non-reductive enzymes. According to recent metagenomic studies, both respiratory and non-respiratory processes seem to be widespread in terrestrial ecosystems and involved in chlorinated-natural organic matter (Cl-NOM) cycling (Weigold et al., 2016; Temme et al., 2019). RDases and hydrolytic or oxidative dehalogenase enzymes were retrieved in many environments, including sediments from urban lakes and in enrichment cultures from a PCB-polluted soil, and the abundance of these enzymes increased in the presence of Cl-NOM (Temme et al., 2019). These findings suggest that both respiring and non-respiring dehalogenating microbial communities may be important for the dechlorination of PCB in contaminated soils, though this hypothesis requires to be further investigated.

The possibility of aerobic degradation of PCB is tightly related to the number and position of chlorine substitutions. Indeed, highly chlorinated congeners are not accessible to aromatic ring-degrading enzymes and therefore their metabolism is dependent on the removal of chlorine by dehalogenating microorganisms (Borja et al., 2005). PCB aerobic catabolism by soil bacteria has been extensively reviewed (Abraham et al., 2002; Field and Sierra-Alvarez, 2008; Furukawa and Fujihara, 2008). The process is known to occur mainly through the enzymes of the biphenyl pathway, encoded by the *bph* operon that is shared among different bacterial phyla, probably as a result of the ancient evolution of the degradation of biphenyl-related molecules of plant origin such as the lignin complex (Fuchs et al., 2011). These genes are often found on mobile genetic elements and therefore may be spread through horizontal gene transfer. Recent studies showed that *bph* clusters of β- and γ-proteobacteria were located as integrative conjugative elements both on plasmids or on the chromosome and could be transferred among bacterial cells (Suenaga et al., 2017; Hirose et al., 2019).

The aerobic biphenyl degradation pathways potentially lead to the conversion of the biphenyl ring into benzoate (upper pathway) and to further degradation into the TCA cycle (lower pathway). The key enzyme responsible for initiating PCB degradation is biphenyl dioxygenase (BphA), a multi-component Rieske non-heme iron oxygenase encoded by *bphA1A2A3A4*. Differences in *bphA1* sequence, encoding for BphA α-subunit, are determinant for substrate specificity and consequently biodegradation capability of different PCB congeners, as found among the most studied PCB-degrading strains such as *Pseudomonas furukawaii* KF707, *Paraburkholderia xenovorans* LB400 and *Rhodococcus jostii* RHA1 (Vergani et al., 2017a). Other *bph* operons of bacteria belonging to different species have been sequenced, presenting some differences in their genetic organization and regulation, as well as different enzymes specificity towards ortho-, meta- or para- substituted congeners. However, the characterization of the degradation pathways confirmed that their function is highly conserved (Gómez-Gil et al., 2007; Ridl et al., 2018; Garrido-Sanz et al., 2020).

Although *bph* pathways associated to diverse strains are incomplete, causing the accumulation of intermediates that can be toxic for other bacteria and organisms, it is important to note that the biodegradation processes by soil bacterial communities likely occur through a complex metabolic network, rather than a linear series of reactions, and involve the interaction of different populations of degraders active at different catabolic steps (Leewis et al., 2016; Duarte et al., 2017). A few studies coupling stable isotope probing (SIP) with 16S rRNA gene sequencing showed that biodegradation potential in PCBs polluted soils is widespread among different species, with a prevalence of Actinobacteria and Proteobacteria, suggesting that active populations vary depending on site and environmental conditions (Uhlik et al., 2013; Leewis et al., 2016; Jiang et al., 2018). Also, aromatic ring-hydroxylating dioxygenases metagenomic studies highlighted a high degree of sequence diversity, indicating that the catabolic potential hosted by microbial communities inhabiting contaminated soils remains widely unexplored (Aguirre De Cárcer et al., 2007; Iwai et al., 2010; Standfuß-Gabisch et al., 2012). In a recent study, the metabolic pathways involved in biphenyl biodegradation by a bacterial consortium isolated from PCBs polluted soil were identified via metagenomic analysis, revealing three different pathways converting biphenyl into benzoate and five pathways degrading benzoate to the TCA cycle intermediates (Garrido-Sanz et al., 2018).

This approach also allowed to reveal which bacterial populations carried out specific reactions in the network, showing that the *bphABCD* operons of *Rhodococcus* strains were responsible of all the three upper pathways. The lower pathways were mainly initiated by *Pseudomonas* and *Bordetella* via benzoate 1,2-dioxygenase (*benABC*), by *Variovorax* and *Achromobacter* that were involved in benzoate ligation with acetyl-CoA (benzoate CoA-ligase, *blcA*), while 4-hydroxybenzoate 3-monooxygenase (*PobA*) were associated to *Pseudomonas*, *Bordetella*, *Achromobacter*, *Ralstonia* and *Rhodococcus*. A more complex bacterial community was then involved in the subsequent catabolism of intermediates.

Besides bacteria, PCB degradation has been focused mainly on wood-decay fungi, which have been studied due to their ligninolytic activity. In fact, as xylophagous organisms, they produce extracellular non-specific laccases and peroxidases enzymes that efficiently attack the biphenyl ring of several PCBs congeners. Intracellular enzymes such as cytochrome P-450 and dehydrogenases are also involved in the detoxification process (Čvančarová et al., 2012; Stella et al., 2017). Instead, the degrading activity of soil-dwelling fungi is poorly studied. Some species of filamentous fungi belonging to the genus *Penicillium*, *Aspergillus*, *Scedosporium*, *Doratomyces*, *Myceliophthora*, *Phoma* and *Thermoascus* were isolated from PCBs polluted soils, and their degradation potential was characterized in a few studies (Tigini et al., 2009; Mouhamadou et al., 2013; Germain et al., 2021). PCB depletion is thought to occur mainly through the activity of laccases, however the biodegradation pathways of these microorganisms need further investigation.

4. Plant–microbes dialogue mediated by the root exudates: sources of carbon, co-metabolites or signal molecules for PCB degrading bacteria

Prolonged exposure to xenobiotics in historically polluted areas promotes a Darwinian selection of holobionts that adapt as a whole to thrive in presence of the contaminant (Osmanovic et al., 2018). The ‘cry-for-help’ contributes to the structure of the holobiont, driving the ecosystem services provided by the microbial functionalities involved in organic pollutants degradation (Uhlik et al., 2013) (Fig. 2). Furthermore, plants adopt a variety of strategies to facilitate the microbial viability and metabolism in an adverse environment, challenged by the high organic contaminant concentration (Di Guardo et al., 2017). Such adverse conditions could lead to the entry of bacterial cells in a dormant phase, like for example the so-called ‘viable but non culturable’ (VBNC) state, from which they can resuscitate under proper environmental and nutritional stimuli (Murugan and Vasudevan, 2018). PCBs pose a threat to bacterial cells viability, as they may accumulate in the cytoplasmatic membrane and disrupt its functionality (Chávez et al., 2006). Notably, PCB toxicity, as for plants, even for bacteria is mostly degradation-dependent, with catabolic products such as dihydrodiols, dihydroxybiphenyls and catechols affecting cell growth and viability much more than the parental compounds due to their hydrophilic substituents, which increase the impact on the cell membrane structure and function (Cámara et al., 2004). To cope with these effects, the strong degrader *Paraburkholderia xenovorans* LB400 has been shown to activate an efficient detoxification response that is induced only upon PCB degradation (Parnell et al., 2006). Besides the activation of such detoxification pathways, bacterial tolerance and stress response to PCBs and related metabolites is variable and includes also structural adaptive changes of the cell membrane and the expression of nonspecific stress shock proteins (Kim and Masunaga, 2005; Chávez et al., 2006).

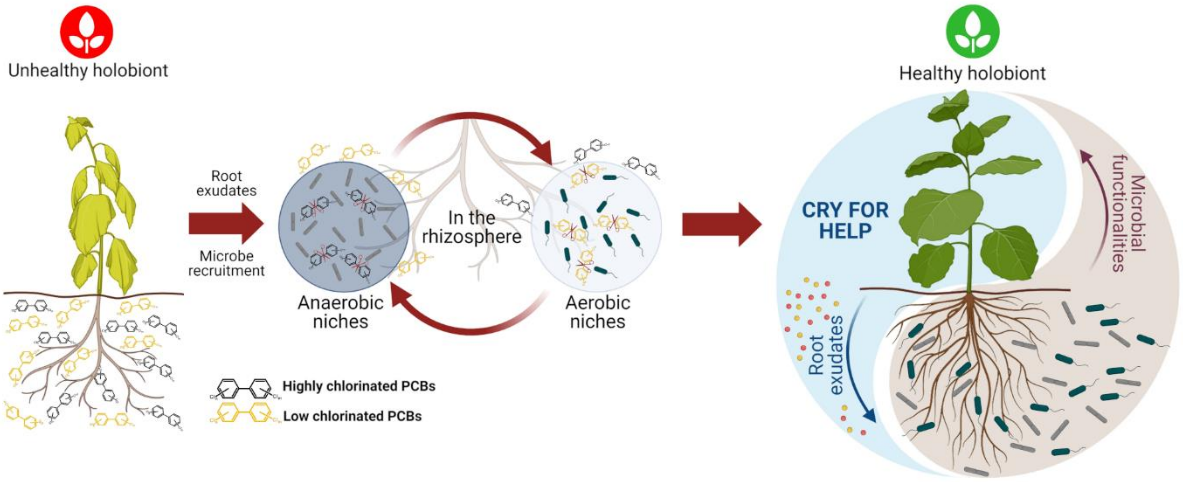

Fig. 2 The complex network interaction between the plant ‘cry-for-help’ strategy and the ecological services provided by the degrader microbiome sustain the holobiont fitness in polychlorinated biphenyls (PCBs) contaminated soils. The PCB-induced phytotoxic effects negatively impact the growth and health of plants growing in polluted soils, which modify their root chemistry in a ‘cry-for-help’ strategy to recruit, feed and sustain PCB-degrading microbes in the rhizosphere. Plant primary and secondary metabolites are valuable candidates to support the microbial aerobic co-metabolism and anaerobic dechlorination of PCBs. Different arrays of microbial populations are involved in PCBs degradation, acting in a metabolic network and in different aerobic and anaerobic micro-niches that establish in the rhizosphere also as consequence of microbial growth and PCB metabolism, favouring further clean-up of the pollutant. The ‘cry-for-help’ approach and the microbial functionalities encoded by the recruited microbiome contribute to foster this healthy system, restoring the holobiont fitness.

Among the vast array of mechanisms that plants adopt to set off the ‘cry-for-help’, the release of primary metabolites like sugars, amino acids and organic acids can support the growth of specific microbial taxa (Macek et al., 2000). The release of a specific profile of root exudates had been considered responsible of the decline in PCBs concentration in *Salix caprea* and *Armoracia rusticana* vegetated soil correlated positively with the population size of culturable PCB degraders enumerated in the rhizosphere of the two plant species (Ionescu et al., 2009). Moreover, the root-associated soil was enriched with bacteria showing a broad PCB congeners catabolism, belonging to the *Pseudomonas*, *Burkholderia* and *Sphingobacterium* genera (Ionescu et al., 2009).

Besides being used as nutrients, plant primary metabolites can be deployed as electron donors to support aerobic co-metabolism or anaerobic dechlorination of PCBs (Leigh et al., 2006). As a side effect, the enhanced aerobic metabolism consumes oxygen, favouring the formation of anaerobic niches for dehalogenation of high-chlorinated PCBs (Chaudhry et al., 2005). This will presumably generate a positive feedback mechanism. The decrease of PCB concentration in the rhizosphere would remove the inhibitory effect of these molecules on plant growth, favouring root development and penetration in soil. As a consequence, oxygenation of soil particles would increase, driving the aerobic oxygenases-mediated degradation of PCBs and generating a cascade effect of oxygen consumption with important implications for the further anaerobic depletion processes (Fig. 2).

Besides primary metabolites, plants can contribute in accelerating microbial PCB degradation by releasing secondary metabolites that act as inducers or co-metabolites and trigger the expression of the microbial enzymatic degradation machinery (Musilova et al., 2016). These root exudate compounds include phenolic molecules like flavonoids, terpenoids, steroids and alkaloids that often share a high structure similarity with PCBs (Jha et al., 2015). Flavonoids, in particular, encompass a huge variety of molecules that influence plant development, response to stress and interaction between plants, microbes and animals (Mathesius, 2018) and have been implicated in the plant-mediated induction of PCB-degrader mechanisms in soil microorganisms (Toussaint et al., 2012; Pham et al., 2015; Subramanian et al., 2018). An altered exudation profile of flavonoids in *Arabidopsis* mutants affected the colonization and consequently the PCB degradation ability of *Pseudomonas putida* PLM2 (Narasimhan et al., 2003). Indeed, flavone and isoflavone were demonstrated to act as inducers of the biphenyl degradative pathway in *Rhodococcus erythropolis*, providing the energy necessary for the contaminant metabolism (Toussaint et al., 2012; Pham et al., 2015).

Terpenes, composed of two or more isoprene units, are responsible for the fragrances of fruits and essential oils and act as chemoattractants for soil microbes (Huang and Osbourn, 2019). The PCB degrader *Pseudomonas stutzeri*, exposed to the terpenes limonene and carvone, showed differential degradation ability towards the PCB mixture Delor 103, that was dependent on the concentration of the inducer (Tandlich et al., 2001).

The role of primary and secondary metabolites can also overlap by acting as attractants to stimulate chemotaxis toward the rhizosphere, as demonstrated for the strain *Rhodococcus erythropolis* U23A that showed motility toward phenolic compounds exudated by *Arabidopsis* roots (Toussaint et al., 2012). Furthermore, variation in the concentration profile of exudated nutrients would affect the residency in the rhizosphere of microbial taxa characterized by diverse life-history strategies (i.e. copiotrophs vs. oligotrophs). This effect was recently documented by a metabolomic approach on the exudates released by *Avena barbata*. During plant development, besides the fast-growing taxa that rapidly consumed the labile exudates, there were a high proportion of slow-growing strains adapted to specialized metabolic niches (Zhalnina et al., 2018).

Rhizodeposition can also occur through decomposition of deposited litter and lysis of sloughed-off root cells (Dennis et al., 2010), presumably containing a diverse array of metabolites. Past (Hernandez et al., 1997; Musilova et al., 2016) and recent studies (Terzaghi et al., 2019; Terzaghi et al., 2021) pinpoint for the effectiveness of the combination of compost supply and plant exudation in increasing cell viability and the microbial activity of PCB-degrading microbiome, while decreasing the load of higher and lower-chlorinated PCBs (Hayat et al., 2019; Di Lenola et al., 2020).

Through the mixture of organic compounds released by roots, litter decomposition and sludged root cells, plants could contribute to resuscitate microbes from the dormant VBNC state, in which they can enter as an adaptive response limiting their growth and division (Murugan and Vasudevan, 2018). Plants contribute, moreover, to increase PCB mobility and bioavailability for microbial degradation through the release of molecules that can act as surfactants (Campanella et al., 2002) or affecting the composition of particulate organic carbon in soil (Terzaghi et al., 2020).

Recent evidence suggests that the ‘cry-for-help’ is not a merely unidirectional strategy employed by the plant to maximize its fitness through the services provided by beneficial microbes. It implies multiple interactions and a molecular dialogue of the holobiont components, the plant and its microbiome, aimed to preserve its functionality under adverse environmental conditions (Fig. 2). Once established in the rhizosphere, after recruitment and feeding through root exudation, microbes can manipulate rhizodeposition to ensure and consolidate their metabolic niche (Korenblum et al., 2020). For example, the release of coumarins, like scopoletin, by *Arabidopsis* roots under iron starvation, showed to improve the colonization by the beneficial strain *P. simiae* that, in turn, stimulated the further exudation of scopoletin to counteract the growth of fungal and bacterial competitors that are sensitive to the antimicrobial activity of coumarins (Stringlis et al., 2018).

The chemistry and mechanisms of communication between the plant host and PCB-degrading soil microorganisms open new challenges for ecological investigation.

5. Concluding remarks

Today, an arsenal of new methodologies is available to unveil the complex crosstalk between plants and the associated microbiome (Park and Ryu, 2021). The use of *Arabidopsis* mutant lines (Huang and Osbourn, 2019; Voges et al., 2019) and the -omics approaches, including culturomics for setting up microbial synthetic communities (Ziegler et al., 2013; de Souza et al., 2020) and metabolomics to identify the plant exudate compounds (Jaini et al., 2017; Pétriacq et al., 2017; Kawasaki et al., 2018; Dietz et al., 2020) potentially will allow to decipher the messages involved in the ‘cry-for-help’ dialogue adopted by plants as response strategy to cope with different environmental stresses (Liu et al., 2020). As recently highlighted by Stringlis and colleagues (2018), who investigated the plant response to iron deficit condition, the microbiome is not merely a receiving component of this dual system. On the contrary, the ability of certain microbes to promote the synthesis and root release of specific compounds ultimately influences the composition of the microbiome itself. The bacteria-mediated induction of specific plant genes recalls the fine talk taking place in the establishment of the legume-

Rhizobium symbiotic relationship, where the bacterium, after rhizoplane colonization, causes morphological changes in the root epidermis related to the expression of the plant early-noduling genes and, from the other side, in response to the flavonoids exudated by roots initiate the synthesis of the Nod Factors which in turn act as transcriptional factors for the plant (Geurts and Bisseling, 2002).

A co-adaptative strategy is established between recruited microorganisms and plants exposed to a particular stress, as observed for drought (Williams and de Vries, 2020), metal toxicity (Timm et al., 2018), plant predation (Adaikpoh et al., 2020) and nutrient limited growth conditions (Ham et al., 2018) to support plant growth under these specific abiotic stressors (Liu et al., 2020). The coumarin scopoletin breaks this paradigm, showing that a molecule exudated under iron starvation is also involved in counteracting pathogen proliferation and favouring the recruitment of beneficial microbes (Stringlis et al., 2019). Identifying similarities and peculiarities in the root exudation profile responding to different abiotic stresses will contribute to decipher the mechanistic aspects of microbial communities assembly upon the plant 'cry-for-help' strategy, and the study of root exudation in contaminated soils will highlight the plant-microorganisms interplay in pollutant degradation.

Improving the knowledge on plant secondary metabolites and root exudates effect in response to PCBs polluted soil paves the way for microbiome manipulation to gain ecological services like rhizoremediation. Though most of the 'cry-for-help' pioneering studies have been realized using the model plant *Arabidopsis thaliana*, future research could be addressed on plant species that demonstrated to induce a decrease in PCBs concentration by biostimulating the microbiome of historically and highly polluted soils (e.g., *Festuca arundinacea*, *Medicago sativa*, *Cucurbita pepo ssp. pepo*, Terzaghi et al., 2019). A fine metabolomic characterization of the exudation pattern of these plant species challenged in PCBs contaminated soil, coupled with metagenomic studies aimed at identifying the soil microbiome response in terms of functional traits related to biodegradation, will thus represent a milestone to steer the recruitment of PCB-degrading microbial populations and design effective rhizoremediation strategy based on microbiome engineering.

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CHAPTER II

Flavonoids are intra- and inter-kingdom modulator signals

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Abstract

Flavonoids are a broad class of secondary metabolites with multifaceted functionalities for plant homeostasis and are involved in facing both biotic and abiotic stresses to sustain plant growth and health. Furthermore, they were discovered as mediators of plant networking with the surrounding environment, showing a surprising ability to perform as signaling compounds for a multitrophic inter-kingdom level of communication that influences the plant host at the phytobiome scale. Flavonoids orchestrate plant-neighboring plant allelopathic interactions, recruit beneficial bacteria and mycorrhizal fungi, counteract pathogen outbreak, influence soil microbiome and affect plant physiology to improve its resilience to fluctuating environmental conditions. This review focuses on the diversified spectrum of flavonoid functions in plants under a variety of stresses in the modulation of plant morphogenesis in response to environmental clues, as well as their role as inter-kingdom signaling molecules with micro- and macroorganisms. Regarding the latter, the review addresses flavonoids as key phytochemicals in the human diet, considering their abundance in fruits and edible plants. Recent evidence highlights their role as nutraceuticals, probiotics and as promising new drugs for the treatment of several pathologies.

1. Flavonoids: plant secondary metabolites with a broad and diversified spectrum of activities across all life kingdoms

A diverse set of environmental cues triggers plant production of specialized metabolites in a highly regulated spatial and temporal dynamic [1] aiming to face unfavorable growth conditions [2]. Such phytochemicals are key players in plant defense, protection, adaptation to abiotic and biotic stress and communication with the other members of the phytobiome [3].

Flavonoids form one of the broadest groups of specialized metabolites and include over 9000 derivatives [4]. Beyond the canonical C_6-C_3-C_6 core structure of polyphenolics, the flavonoid family encompasses in sensu lato chalcones, flavones, flavonols, anthocyanins and proanthocyanidins [5]. This multiplicity in chemical structure is mainly due to secondary modifications by glycosylation, prenylation, methylation and acylation that affect the bioactive role, transport and accumulation of the resulting compounds [6], a complex set of molecules that enables plants to interact dynamically with the environment. The wide distribution of biosynthetic genes in the plant kingdom, already observed in microalgae, suggests that plants were equipped early during evolution with the ability to produce flavonoids [7]. This ancestral origin may be related to flavonoids’ protective role against biotic threats such as pathogenic fungi and bacteria and herbivore predation, and abiotic ones such as ultraviolet (UV) light [8]. It has been hypothesized that the evolutionary success of embryophytes in the Paleozoic as land-colonizers was achieved by the antioxidant activity of phenolic compounds, able to screen for UV radiation [9].

So far, flavonoids are documented to exert a wide spectrum of biological activities in plant physiology [10] and, being differentially produced and regulated in different tissues, organs and cell-types, contribute to a plastic response to environmental stimuli [2]. These phytochemicals have a role even in plant reproduction strategies: they encompass pigmented molecules, such as anthocyanins, that show from blue to red colors, and accumulate in flowers and fruits to attract pollinators and animal dispersers to favor the dispersal of seed [11].

Flavonoids are involved in many plant developmental processes [12,13] and, more precisely, they contribute to stress-induced morphogenic responses under a variety of abiotic conditions [14] including salinity [15], drought [16], heat [17], freezing [18], metal toxicity [19], presence of xenobiotics [20], and UV exposure [21].

Flavonoid aglycones and glycosides are secreted in root exudates [22], acting as communication signals that rule, for instance, plant-to-plant interactions in allelopathic interference between different species [23] and that mediate root association with below-ground soil microbiomes [24,25]. In the soil microbiome framework, the role played by flavonoids in the legume-rhizobia symbiosis has been extensively investigated [26–28]. In response to nitrogen deficiency, legumes release species-specific flavonoids as chemical impulses to attract nitrogen-fixing rhizobia and to activate the transcription of bacterial genes coding for Nod factors [29,30]. This, in turn, affects the plant root morphogenetic development, culminating in root infections and nodule organogenesis [31]. Besides bacteria, flavonoids are signaling compounds also for arbuscular mycorrhizal fungi, influencing spore germination, hyphal growth and root colonization efficiency [32], in turn contributing to the plant’s nutritional balance for micronutrients such as phosphorus and iron [33]. Evidence is accumulating that flavonoids could act as a more universal signaling system for soil microbes [34] as suggested by the huge proportion they represent in root chemistry: in *Arabidopsis*, for instance, they constitute 37% of the total secondary metabolites released into the rhizosphere [35]. Flavonoids can be metabolized by microorganisms as nutrients [36], can affect microbial traits involved in rhizocompetence such as motility and chemotaxis [37,38] or operate as co-metabolites to induce highly specialized pathways in bacteria, such as the expression of the *bph* operon involved in the bioremediation of recalcitrant xenobiotics like polychlorinated biphenyls [39,40].

Flavonoids are essential components of food and edible plants and, once introduced through the diet, they are transformed by the human microbiome in the intestine [41]. Parental and digested flavonoids exert their action locally, by sustaining the intestine functionality in terms of detoxification from toxins and inhibition of pathogens, increasing nutrients up-take and maintaining the intestinal barrier integrity [42]. Part of the flavonoid molecules are released in the blood flow where they act systemically. Numerous medical studies highlighted flavonoid therapeutic potential in the treatment of diverse pathologies, paving the way for the development of new drugs or combinatory treatments for human health [43,44].

This review aims to shed light on (i) the multifaceted roles played by flavonoids in extremely diverse biological systems, (ii) their ability, as signal molecules, to facilitate communications among organisms belonging to different life kingdoms, (iii) their plant-beneficial effect in improving resilience to adverse environmental conditions and (iv) their cross-reaction with animal signaling pathways, thus exerting therapeutic effect for the medical treatment of several human acute illnesses.

The source analyses for this review encompassed the scientific literature deposited in online databases such as the Web of Science Core Collection, PubMed, Scopus and Google Scholar, using keywords that describe flavonoids and their involvement in biotic and abiotic stresses (including oxidative stresses, nutrient scarcity, soil pollution and attack by phytopathogens), allelopathy, microbiome assembly and function, root colonization, chemotaxis, biofilm formation, rhizoremediation, insect, oviposition, feeding, quercetin, naringenin, nutraceuticals, dietary phytochemicals, human health and disease. The data critically discussed in the present review were retrieved from studies published in the years from 2002 to 2022 (accounting for 98% of the cited literature).

2. Flavonoids Tune Plant Physiology Response under Biotic and Abiotic Stress Conditions

Due to their structural heterogeneity, flavonoids play multiple roles in mediating plant physiology and in responding to different abiotic stresses (Figure 1).

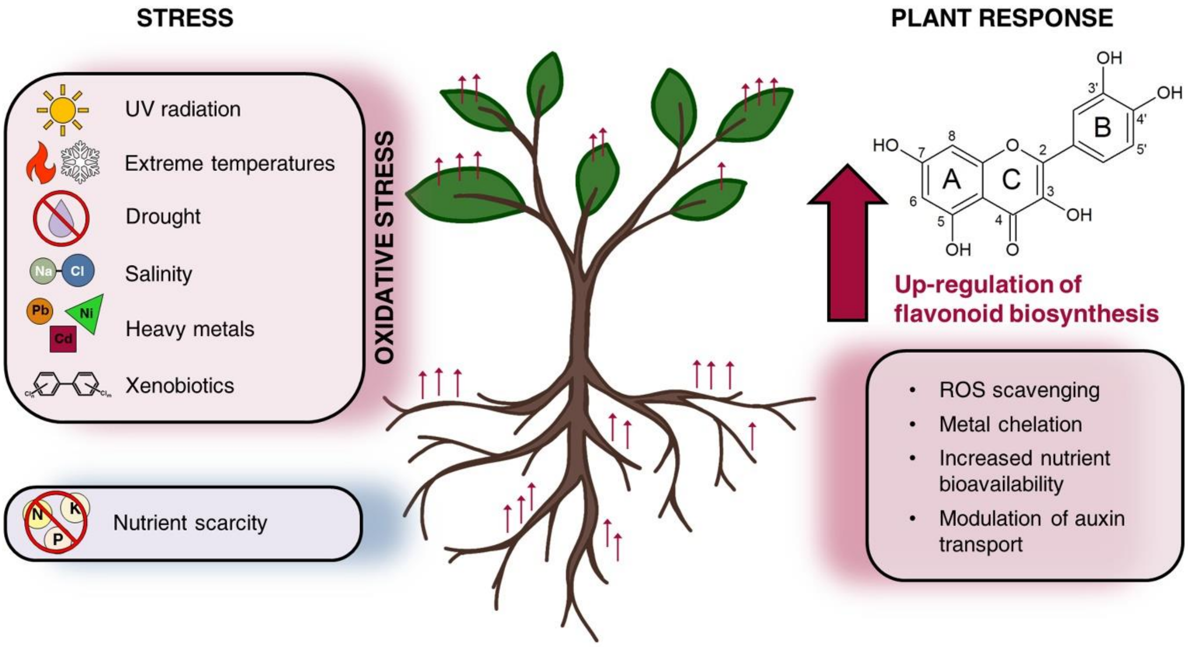

Figure 1. Flavonoid role in plant physiology under abiotic stress. To cope with poor nutrient availability and a variety of abiotic stresses featured by oxidative stress, plants trigger an enhanced synthesis of flavonoids that contributes to tune plant homeostasis and development. A schematic representation of the main structural components and substituent positions in the flavonoid backbone is reported in the image.

The same flavonoid can have multiple functions and be involved in plant responses to high salinity [45], drought stress [46], UV radiation [47], extreme temperatures [48], the presence of heavy metals in soils [49] and nutrient scarcity [10,50]. Therefore, despite the energetically highly-consuming process of their synthesis, flavonoids' versatility contributes to plant terrestrialization [51] and represents an advantageous molecular resource to maintain plant homeostasis, especially under stress conditions [52,53]. Acting as reactive oxygen species (ROS) scavengers is one of the main functions of these molecules, an essential feature to mitigate the damages caused by environmental conditions that trigger oxidative stress [54–56]. ROS-scavenging flavonoids are an essential defense to counteract oxidative damage, such as DNA and protein oxidation and lipid peroxidation [57], supporting the activity of plant antioxidant enzymes [58,59]. The antioxidant activity of flavonoids is related to their polyphenolic structure and to the presence of a wide variety of attached substituents, such as catechol groups on the B ring and hydroxyl groups [60] (Figure 1). For these reasons, flavonoids such as quercetin and myricetin showed a stronger antioxidant capacity than kaempferol [61]. This type of structure allows the efficient quenching of free radicals and singlet oxygen, the chelation of metal ions such as $Fe^{2+/3+}$ and Cu^{2+}, essential to prevent ROS generation [62] and the repression of the activity of ROS-producing enzymes such as oxygenases and oxidases [63]. Quercetin derivatives, in particular, are involved in detoxification under different oxidative stresses caused by intense light and drought, because they possess the ideal structural requirements as dihydroxy-B-ring-substituted compounds. Moreover, their biosynthesis is crucial for plant fitness [64].

In the context of food quality, growth in a condition of oxidative stress is sometimes sought in edible plants to increase their content in beneficial antioxidant metabolites such as flavonoids: the leafy vegetable *Amaranthus tricolor*, naturally endowed with antioxidant pigments and metabolites, enhanced its total flavonoid content and antioxidant capacity when subjected to salinity stress by irrigation with 50–100 mM NaCl water solution [45]. Similarly, the application of short-term high light irradiation with LED lights on hydroponic lettuce improved its nutritional quality and induced a higher accumulation of antioxidant flavonoids and anthocyanins [65].

Exposure to excessive UV radiation is indeed one of the main triggering conditions for oxidative stress in plants, leading to DNA breaks, cell damage, and impairment of the photosynthetic process [14,50]. To counteract UV stress, increased biosynthesis of specific types of flavonoids is induced to limit excessive light radiation damage, acting in synergy with antioxidant enzymes, the plant main defense strategy against ROS [66]. This is the case of UV light-induced quercetin 3-O- and luteolin 7-O-glycosides that were observed to act as O_2^- scavengers in *Ligustrum vulgare* exposed to UV-B radiation [67]. Also, apigenin biosynthesis was enhanced under UV-B stress in transgenic *Arabidopsis* plants expressing maize flavone synthases, leading to reduced membrane damage explained by the role of flavonoids in the reduction of lipid peroxidation [68]. Besides showing higher accumulation of flavonoids, stressed plants exhibit a marked up-regulation of genes involved in their biosynthesis: in UV-B-exposed hairy roots of *Isatis tinctoria* L. the level of gene transcription for chalcone synthase was 405-fold higher than the control, together with a 16.5-fold increase in flavonoid levels [69]. In the mutant line *tt4* of *Arabidopsis thaliana*, the knock-out of the chalcone synthase gene leads to the inability to synthesize kaempferol, therefore making the plant more sensitive to UV-light exposure [14].

Regarding their location in plant tissues, light-induced flavonoids such as flavonols and anthocyanins are not present exclusively in leaf epidermal cells or external appendages such as trichomes, where they can prevent ROS formation through a photoprotective action as UV-shelter molecules [68,70]. Since their beneficial function is mostly related to ROS detoxification, flavonoids are also translocated toward more internal tissues such as, for instance, the mesophyll [71]. Here, flavonoids exert their role inside or near the target cellular compartments where ROS are generated after severe light exposure, such as the nucleus and the chloroplast [72]. However, flavonoids translocation mechanism to the target organelles is still unknown [64]. In *Phillyrea latifolia*, for instance, *in vivo* observations showed that flavonoids protected the plant against singlet oxygen generated by excessive UV light stress in mesophyll chloroplasts [73]; they can also be transported to the vacuole, where their coupled activity with peroxidases was shown to increase H_2O_2 scavenging [74].

Besides radiations, even high temperatures, often combined with drought stress [75], can cause ROS accumulation by affecting evapotranspiration, stomatal closure and by decreasing water content in leaves [76,77]. Flavonoid biosynthesis is an essential trait for plant tolerance and adaptation to extreme temperatures and climatic variations. Plants were shown to activate chemical defense mechanisms by inducing the expression of phenylpropanoid pathway genes (e.g., chalcone isomerase, flavone and flavonol synthase, anthocyanidin synthase), leading to increased concentrations of flavonoids in leaves and photosynthetic tissues [78,79]. The up-regulation of flavonoid biosynthetic genes was seen in wheat and rice, suggesting a role for flavonoids in defense from drought and high temperatures [80,81]. Similarly, in the citrus *Cleopatra mandarin*, which is a highly susceptible species to heat and water stress, a strong antioxidant response was marked by the accumulation of apigenin and polymethoxylated flavones [82]. Furthermore, the response to high temperatures was seen to be stress-specific in tomato (*S. lycopersicum* cv. Boludo); heat stress alone preferentially led to the accumulation of flavonols, and their biosynthesis was reduced under salt stress or under a combination of heat and salt stress, which instead revealed higher concentrations of hydroxycinnamic acids [72].

Excessive soil salinization is often correlated with heat and drought and can cause osmotic stress and toxic ion accumulation leading to ROS formation [22]. Increased concentrations of flavonol glycosides, especially quercetin, isorhamnetin, and kaempferol were indeed observed in *Ginkgo biloba* and in *Casuarina glauca* leaves after treatment with low concentrations of NaCl and helped to improve plant tolerance to the stress [83,84].

Low temperatures during winter can be a source of oxidative stress in plants and therefore cause seasonal fluctuations of flavonoid levels [85]. Sorghum, for instance, is susceptible to frost conditions and responds by accumulating antioxidant molecules such as 3-deoxyanthocyanidin luteolinidin in the roots [86]. To mitigate desiccation stress caused by frost, also the resurrection plant *Haberlea rhodopensis* was seen to accumulate flavonoids and anthocyanins [87]. These observations are consistent with previous studies carried out in *A. thaliana* where increased flavonol content, in particular quercetin and anthocyanin compounds in the over-expressing *pap1-D* line, and the expression of genes involved in flavonoid biosynthesis were proven to be related with cold acclimation, supposedly due to their role as protectors for freezing damages of membranes and proteins. Indeed, most of the flavonoid knock-out mutants showed impaired freezing tolerance or the accumulation of known or unknown flavonoids with potential compensatory effects, highlighting a functional role for these molecules under cold stress [18].

Due to their aromatic structure and the presence of the cathecol group, some flavonoids act as metal ion chelators, therefore avoiding the generation of oxygen radicals and lipid peroxidation induced by phytotoxic concentrations of heavy metals in soil [66,88]. Indeed, flavonoids were seen to form transition complexes with metals in lettuce leaves irrigated with industrial wastewaters polluted by heavy metals such as Pb, Co, Ni and Cd [89], preventing heavy metal ions' participation in ROS production [54]. An analogous effect was observed in *Erica andevalensis*, a heavy metal-resistant plant that grows in mine soils, showing higher tolerance to Cd due to the defense mechanisms prompted by the flavonoid rutin, among other phenolics [90]. Moreover, the treatment with increasing concentration of copper generated a boost of flavonoid concentration (reaching the order of magnitude of milligrams) in *Belamcanda chinensis* calli, probably to mitigate the impaired expression of the antioxidant enzyme guaiacol peroxidase due to the elevated stress level [91].

The role of flavonoids in plant physiology and homeostasis is not only related to the prevention of oxidative damage but also to other fundamental aspects for plant fitness such as the modulation of auxin transport and the influence on bioavailability of soil nutrients [92,93].

Flavonoids regulate plant morphogenic response to stresses through the modification of the transport of auxins, important phytohormones that coordinate root formation and elongation, phototropic and gravitropic responses [76,94,95]. Numerous studies highlighted that, depending on the substitution groups, flavonoids could exert a negative role in polar auxin transport (PAT), such as kaempferol and quercetin [63,96], or the opposite effect of promoting PAT and root elongation, such as scutellarin [97]. The role of flavonoid in auxin-mediated morphogenic responses was also observed in halotropism, where the accumulation of light-induced flavonoids generated root bending in the perennial grass *Poa trivialis* L. [98], and in cotton axillary buds where decreased flavonoid content generated enhanced auxin efflux and the outgrowth of new shoot branches [99]. Flavonoids appear to directly modulate membrane trafficking of *PIN* proteins, the auxin efflux facilitators: exogenous supply of 1 nM naringenin restored *PIN1* localization in the root tip in the *Arabidopsis tt4* mutant, lacking flavonoid biosynthesis and showing altered auxin transport [100].

The extreme versatility of flavonoids in plant physiology extends to the enhancement of nutrient acquisition in plants due to their involvement in metal chelation and complexation and to their role as reducing agents. Often, essential macro- and micro-nutrients, such as N, P, Fe and Mn, are poorly bioavailable for the plant that, among other strategies, secretes flavonoids to enhance their mobilization and solubility [101]. In fact, aside from their well-known role as chemoattractors and *nod* genes inducers in *Rhizobium*-legume symbiosis [102,103], flavonoid biosynthesis was reported to be upregulated under nitrogen deficiency in tea and tomato plants as a stress response [104,105]. Flavonoid exudation also increased in apple trees subjected to phosphorus depletion, in order to cope with the nutritional stress by prompting either Fe reduction or chelation and consequently P solubilization [106].

3. Flavonoids Mediate Plant-Plant Interactions

Allelopathy is generally used to indicate a phytotoxic interference of a plant on the growth of another plant belonging to the same species (autotoxicity) or to a different one (allelopathy *in sensu stricto*), in the same soil niche [107] (Figure 2).

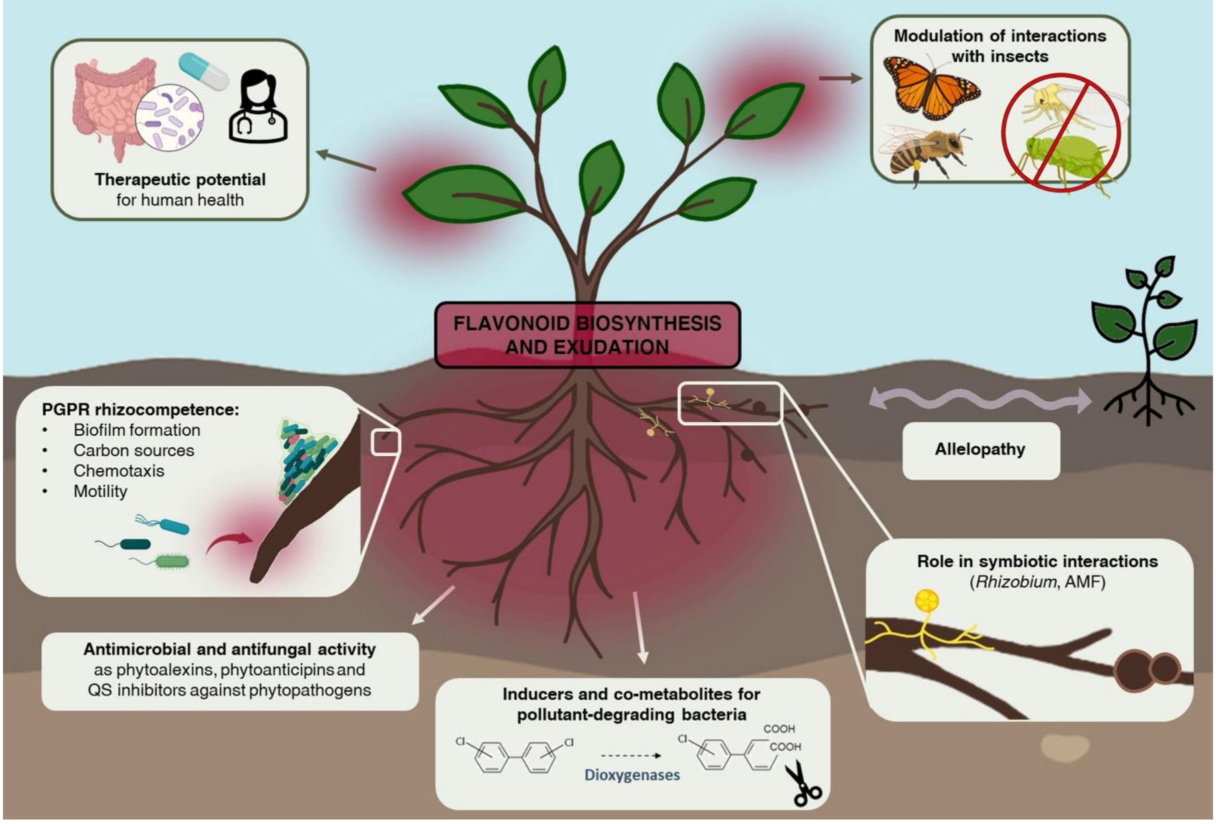

Figure 2. Plant intra- and inter-kingdom interactions mediated by flavonoids. The main aboveground and underground multitrophic interactions, that are orchestrated by flavonoids, are illustrated in the figure. The release of these phytochemicals in the soil modulates allelopathic interactions with neighbor plants and the symbiotic relationship with rhizobia and arbuscular mycorrhizal fungi (AMF). It also mediates rhizocompetence traits of plant growth promoting rhizobacteria (PGPR), exerts antimicrobial and antifungal functions against phytopatogens and stimulates the bacterial catabolism for xenobiotics degradation. Furthermore, flavonoids can tune interactions with beneficial and opportunistic insects. Recently, flavonoids have been proposed as nutraceutical and prebiotics for human health. The image was partially realized using graphic contents from Bio-render (https://biorender.com/ (accessed on 2 October 2022)).

The process is mediated by a complex network of biochemical signals, due to the release of root or leaf exudates by the allelopathic plant, and volatile molecules or leachate from plant litter decomposition. In soil, these allelochemicals range from 10^{-5} to 10^{-6} M [108] and, despite these low concentrations, they lead to the delay and/or suppression of germination, growth and development of neighboring sensitive plants [23]. As an example, the invasive tree black cherry (*Prunus serotina*) releases seasons-variable phytotoxic compounds from the leaves.

Among them, the monoterpene linalool did not show a germination inhibitory effect but affected the root length and the seedling growth of pine plantlets in European forests [109]. Several reports indicated that young seedlings are more prone to the phytotoxic effect of allelochemicals rather than germinating seeds, as observed also for radish and maize plantlets exposed in soil to ground leaves of *Fallopia japonica* and *bohemia*, one of the most invasive species in Europe [110].

Perennial legumes, like clover and alfalfa, showed both autotoxic and allelopathic features [111] that were speculated as arising from their evolutionary establishment in extremely dry and nutrient-poor Mediterranean soils. Under these adverse conditions, plants adopted allelopathic strategies to improve their survival chances by preventing the growth of young seedlings near mature plants [112]. The alfalfa saponin medicagenic acid, assayed at 500 ppm, exerted strong phytotoxicity on germination and seedling growth. The knowledge of this autotoxicity has an influence on agronomical decisions on alfalfa replanting in fields in which alfalfa allelochemicals of previous plantation could persist [113]. Furthermore, allelopathy explains weeds fitness in invasive ranges, enhancing the competitive ability against native plant species, competing for resources, light, space, and nutrients [114,115]. It is estimated that weeds contribute to 28% reduction of crop yield [116], with severe economic losses [117].

The role of flavonoids as allelopathic molecules has been largely acknowledged [118–120]. Often, unique compounds are specifically synthesized, as in the case of the barley-produced saponarin, a newly identified flavonoid, to suppress *Bromus diandricus* spread [121]. According to the “novel weapon hypothesis” flavonoids accumulation may drive the establishment success of invasive plants and, in particular, allelopathic actions exerted specifically by molecules belonging to the flavonols family may represent a signature of tree-invasive species [122].

Recently, allelopathy was exploited to develop agronomic practices for weed integrated management by planting allelopathic crops, thus reducing the use of agrochemicals [123,124], or by using identified flavonoid allelochemicals, such as coumarin, and bioherbicides [113]. Coumarin supplements (10 µM) can significantly alter *Arabidopsis* root architecture, by reducing up to 50% primary root length and significantly increasing the number of lateral roots [125]. Modifications in root anatomy are a well-described mechanism in allelopathic responses: exposition to allelochemicals can trigger toxic responses mediated by proteolytic activities and eventually culminate in organ and tissue cell death, thus affecting plant growth [107].

To develop sustainable bio-herbicides, allelopathic plants may be used as a source of phytochemicals to cope with weed invasiveness. Methanolic extracts (20%) of *Artemisia santolinifolia*, for instance, were shown to cause severe oxidative stress and permanent wilting in major weeds, and this effect seemed to be due to a higher content of phenols and flavonoids (rutin and quercetin) in plant extracts [126].

In Africa, smallholder farmers cultivate *Desmodium uncinatum* as intercropping plant to prevent the attachment and germination of the parasitic weed *Striga* [127], an effect that was found to be mediated by several (iso)flavonoid glycosides released in the legume root exudates [128]. In this case, flavonoids orchestrated a complex network of biological events that culminated in *Striga* suicidal germination, whereas uncinanone B promoted weed seed germination, uncinanone C and several di-C-glycosylflavones prevented the propagule attachment to host roots [129].

The allelopathic efficacy seems to rely on the concentration [130] and persistence of allelochemicals in soil water solution, that is affected by environmental factors, soil edaphic properties and plant-related growth stages [131]. White clover stands release at relatively high concentration different aglycones such as formononetin (~4500 µmol/kg plant dry weight), medicarpin (~950 µmol/kg plant dry weight), and kaempferol with phytoinhibitory activity and this latter, among others, persisted for days in field soil (~1200 nmol/kg dry soil) [132]. Similarly, allelopathic rice cultivars accumulated two flavone glycosides in root tissues and, once exudated, they were rapidly converted in aglycone forms, more resistant to microbial degradation and less mobile in paddy soil, thus enhancing their suppressive effect against rice paddies-infesting weeds such as *Echinochloa crus-galli* [133].

Flavonoids' mechanism of action in allelopathy is far from clear [63], although it is believed to be related to the interference with ATP production and to auxin transport and degradation, resulting in dramatic alteration of the root morphogenic program of target plants [134]. Exposition of *Arabidopsis* roots to 4 mg/mL of the invasive *Conyza canadensis* plant extract led a decrease in root tip vitality and a ROS burst, coupled to the induction of stress-responsive genes, pathways and detoxifying machinery [135]. The death of the root system of neighboring plants was observed in knapweed *Centaurea maculosa*-infested fields and this effect was caused by the dramatic wave of ROS induced by the (-)-catechin released by the weed [136], revealing a potential pro-oxidant function of flavonoids in allelopathic interactions [137]. Administration of (-)-catechin triggered programmed cell death that expanded from the root region to the stele, presumably by eliciting a ROS-induced calcium wave that alters cell ionic homeostasis, unbalancing cellular pH [136].

4. Flavonoids as Weapons against Phytopathogenic Attacks

Plants and their associated microorganisms coevolved as a unique meta-organism defined as the plant holobiont [138,139]. The interplay between plant and microorganisms, in particular those colonizing the rhizosphere and the endosphere, is specifically mediated by exuded metabolites and chemical signals exchanged as a form of communication and essential in maintaining the holobiont health status and performance [140]. Flavonoids constitute a conspicuous percentage of root-exuded secondary metabolites and are among the most studied molecules involved in plant-microorganism interactions [33,36] (Figure 2). Flavonoids play a crucial role in orchestrating plant defense upon phytopathogen attacks since they can interfere with the mechanisms that drive bacterial and fungal virulence [141,142]: they are antimicrobial and antifungal agents and act as priming agents of plant defense responses to react rapidly upon infection perception [142,143] (Figure 3).

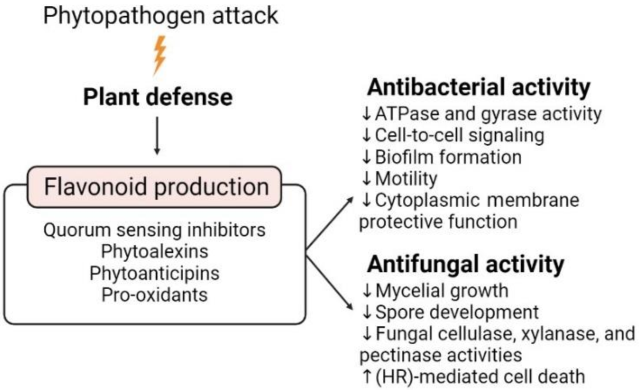

Figure 3. Flavonoid-mediated plant defense strategies against biotic stress caused by phytopathogen attacks. The attack by microbial phytopathogens induces plant defense responses (represented by the thunder symbol), including an enhanced flavonoid production and exudation. Flavonoids play numerous roles in plant defense against biotic stresses: they are quorum-sensing inhibitors, pro-oxidants, and some of them are defined as phytoalexins and phytoanticipins defense compounds. These features confer flavonoids with antibacterial and antifungal activities that are mediated by a diverse array of mechanisms, some of which are reported in the figure. The down-oriented arrow (↓) represents an inhibitory effect, whereas the up-oriented arrow (↑) represents an induction effect.

Indeed, they can act as quorum-sensing inhibitors (QSI) by multiple mechanisms, including the reduction of signal molecules biosynthesis, the inhibition of cell-to-cell signaling and biofilm formation, and the competition for receptor binding sites by mimicking signal molecules [144,145]. Naringenin and quercetin, for instance, interfere with QS by downregulating the expression of genes involved in the production of acyl-homoserine lactones, typical signal molecules during *P. aeruginosa* infections [146–148] and by hindering the correct functioning of QS receptors, specifically due to the presence of two -OH groups on the flavonoid A-ring. This specific molecular structure has an antagonist role toward pathogen autoinducers (AIs), compounds that coordinate the expression of genes for virulence and survival, and prevents DNA binding of the AIs-receptor complexes, hence down-regulating the expression of pathogenic factors [149]. Another example of QS inhibition observed in a phytopathogenic strain was the suppression of the QS-regulated toxoflavin production in *Burkholderia glumae*, causal agent of rice grain rot and wilt, mediated by two apigenin derivatives [150,151]. By using a *B. glumae* biosensor strain, engineered to trigger β-galactosidase activity upon toxoflavin expression [152], it was observed that the inhibitory activity of the apigenin derivatives on the strain occurred at low concentrations, 6.76 and 7.87 µM respectively [150].

Other exemplary flavonoid antimicrobials were found in the root exudates of *Scutellaria baicalensis*, namely wogonin and baicalein. The former was effective *in vivo* in plants infected by the rice fungal pathogen *Magnaporthe oryzae* at the concentration of 500 µg/mL, whereas the latter showed inhibitory effects *in vitro* against phytopathogenic bacteria such as *Acidovorax avenae* and *Ralstonia solanacearum* at MIC 19 µg/mL (MIC, minimum inhibitory concentration) [153]. Furthermore, the enhanced production of phenolics, including quercetin-3-O-glucoside, explained a cultivar-dependent resistance against *Plasmopara viticola* in grapevine compared to a sensitive cultivar [154]. Quercetin-3-galactoside is also able to counteract *in vitro Botrytis cinerea* pathogenic traits by reducing spore germination at the early phase of infection and by inhibiting germ tube elongation, thus potentially reducing the pathogen penetration ability [155].

The role of flavonoids in sustaining plant defense is also expressed by their action as (i) phytoalexins, namely de novo-synthesized and pathogen-elicited defense molecules with antibacterial and antifungal activity, and (ii) as phytoanticipins, constitutive molecules that are produced and accumulated before pathogen exposure, that can be rapidly employed by the plant when needing a swift defense response [156,157]. Isoflavonoids and their derivative pterocarpans, such as medicarpin and pisatin, produced respectively by alfalfa and pea plants, are known phytoalexins, active against fungal pathogens as inhibitors of mycelial growth or inducers of hypersensitive response (HR)-mediated cell death [158–160]. Conversely, flavonols showed a putative phytoanticipin role in mildew-infected blackberry [161]: despite a low concentration in infected plants, they can safeguard protection from the pathogen. This is explained by the fact that, upon pathogen infection, pre-existing phytoanticipin flavonoids can be transformed into active phytoalexins [161,162]. Some exemplary cases are daidzein, a soy phytoanticipin that, once elicited by pathogenic microbes, is transformed by prenylation into glyceollin pterocarpans [163]. Similarly, the active aglycone form of maackiain is obtained by hydrolysis of the conjugated compound and released by infected red clover plants [164].

Flavonoid pro-oxidant activity represents a further weapon against phytopathogens [165]: pretreatment with 250 µg/mL quercetin [166] or 100 µM naringenin [167] induced H_2O_2 production in *Arabidopsis* plants, causing oxidative burst and callose deposition in host cells, magnifying the defense response against *Pseudomonas syringae* pv. tomato DC3000. The addition of catalase or of glutathione caused the reverse effect to that observed with flavonoid pretreatments, making the plant more susceptible and therefore implying the need for H_2O_2 accumulation in plant resistance to *P. syringae* infection [166]. However, the mechanisms through which flavonoid pro-oxidant activity can increase plant resistance to pathogens have not yet been clarified and further investigations are needed to unveil their correlation with plant defense responses.

5. Flavonoid Exudation Affects the Structure and Function of Root-Associated Microbiomes

Flavonoids interactions with rhizosphere/endosphere microbiomes are efficient means exploited by the plant to communicate with soil microorganisms and to recruit beneficial plant growth-promoting (PGP) bacteria [6,168]. These bacteria largely contribute to the holobiont fitness by alleviating nutritional shortages, by producing phytohormones and volatile organic compounds to enhance plant development [169–171] and by repressing attacks from plant pathogens acting as biocontrol agents [38,172]. One of the most studied interactions between plants and beneficial microbes is mycorrhization, where arbuscular mycorrhizal fungi (AMF) colonize roots and improve plant fitness by alleviating biotic and abiotic stress factors [173]. The role of flavonoids in mycorrhizal symbioses is not only associated to the enhancement of hyphal growth and spore germination [32,173]; interestingly, mycorrhiza helper bacteria (MHB), closely associated to specific AMF, can produce Nod factors and induce root exudation of flavonoids that act as signals to attract AMF, thus facilitating root colonization [174,175].

In general, PGP activities are related to the ability of bacteria to colonize and persist on plant roots, which is defined as rhizocompetence [176–178] (Table 1). An important feature in rhizocompetence is possessing the catabolic enzymes to use root exudates like flavonoids as nutritional sources, outcompeting other bacterial species [179,180] (Figure 2). Some beneficial soil bacteria belonging to the genera *Bacillus* and *Pseudomonas* and endophytic fungi like *Paraconiothyrium variabile*, for example, are known to effectively metabolize flavonoids [6,181]. The strain *Pseudomonas putida* PLM2 was seen to degrade quercetin via aerobic dehydroxylation, but it could also use other flavonoids such as naringin, naringenin, daidzein and apigenin as carbon sources [182]. Other flavonoid-influenced rhizocompetence traits are bacterial motility, chemotaxis toward root exudates and biofilm formation for accommodation on root surfaces [183,184]. Naringenin-mediated chemotaxis of *Aeromonas* sp. H1 toward *Arabidopsis* roots, marked also by the over-expression of genes for flagellar biosynthesis, was essential to protect plants from the deleterious effect of drought stress [185]. Similarly, phloretin and apigenin enhanced *Pseudomonas fluorescens* 2P24 growth and swarming motility *in vitro* [37]. Biofilm formation was enhanced by rutin in *Bacillus subtilis* [186] and by apigenin in the N_2-fixing bacterium *Gluconacetobacter diazotrophicus*, both associated to rice plants, thus potentially improving plant growth and defense from pathogens through an indirect mechanism [187].

Table 1. Impact of flavonoids on growth and rhizocompetence traits of plant growth-promoting bacteria (PGPB) through *in vitro* experiments.

Bacteria	Rhizocompetence Traits	Flavonoids	Concentration	Main Effects	Reference
Aeromonas sp. H1	▪ Chemotaxis ▪ Biofilm formation	Naringenin, kaempferol, quercetin	100 µM	▪ Induction of chemotactic movement ▪ Biofilm formation ▪ Up-regulation of flagellar biosynthetic genes	[185]
Gluconacetobacter diazotrophicus	▪ Biofilm formation	Apigenin	20–100 µM	▪ Dose-dependent induction of biofilm formation, without impacting bacterial growth	[187]
Bacillus licheniformis, *Bacillus subtilis*, *Acinetobacter junii* Pb1	▪ Chemotaxis	*Chrysopogon zizanioides* (L.) Roberty-exuded flavonoids	n.d. *	▪ Chemoattraction toward the flavonoids exuded by Pb(II)-stressed plants	[188]
Rhodococcus erythropolis U23A	▪ Chemotaxis	Concentrated *A. thaliana* root exudates containing flavonoids	n.d. * (flavanone approx. 0.5–1 mM)	▪ Chemoattraction toward *Arabidopsis* root exudates	[189]
Pseudomonas fluorescens 2P24	▪ Swarming motility ▪ Production of cellulose and curli fibers	Apigenin, phloretin	100 µM	▪ Increased swarming motility ▪ Up-regulation of flagellar-related genes ▪ Up-regulation of cellulose and curli synthetic genes, crucial in EPS production	[37]
Herbaspirillum seropedicae SmR1	▪ Swimming motility ▪ Maize root colonization	Naringenin	100 µM	▪ Promotion of maize root colonization in early phase (first 36 h) ▪ Down-regulation of flagellar-related genes and reduced motility possibly due to proximity to the root surface	[190]
Bacillus subtilis CIM	▪ Chemotaxis ▪ Motility (swimming, swarming and twitching) ▪ Growth promotion ▪ Biofilm formation	Rutin	1 pM	▪ Chemoattraction ▪ Enhancement of swimming, swarming and twitching motility ▪ Higher bacterial growth (CFUs) on rutin-supplemented growth medium ▪ Induction of biofilm formation	[186]
Azorhizobium caulinodans ORS571, *Herbaspirillum seropedicae*	▪ Root colonization through lateral root cracks in *Arabidopsis thaliana*	Naringenin, daidzein	50 µM	▪ Promotion of endophytic root colonization performed by nitrogen-fixing bacteria through a Nod factors-independent mechanism	[191]
Azorhizobium caulinodans, *Azospirillum brasilense*	▪ Root colonization through lateral root cracks in rice	Naringenin	50 µM	▪ Promotion of endophytic root colonization performed by nitrogen-fixing bacteria through a Nod factors-independent mechanism	[192]
Pseudomonas putida PML2	▪ Use of flavonoids as carbon sources	Naringenin, quercetin	10 mM	▪ Growth on both flavonoids as unique carbon sources ▪ Almost complete depletion of 0.1 mM quercetin added to the medium ▪ Elucidation of quercetin degradation pathway in *P. putida*	[182]
Acinetobacter calcoaceticus MTC 127	▪ Use of flavonoids as carbon sources	(+)-Catechin	3 mM	▪ Growth on catechin as unique carbon source ▪ Elucidation of catechin degradation pathway in *A. calcoaceticus*	[193]

Bacteria	**Rhizocompetence Traits**	**Flavonoids**	**Concentration**	**Main Effects**	**Reference**
Paraburkholderia xenovorans LB400	▪ Use of flavonoids as carbon sources	Morusin, morusinol, kuwanon C	100 µg/mL	▪ Utilization of the tested flavonoids as sole carbon sources	[194]
Rhizobacteria consortium (*Pseudomonas* sp. + *Stenotrophomonas* sp.)	▪ Use of flavonoids as carbon sources	Flavone, flavanone, isoflavone, 7-hydroxyflavanone, 7-hydroxyflavone, 6-hydroxyflavone	200 µM	▪ Utilization of the tested flavonoids as sole carbon sources	[195]

* Not defined: the specific concentration of flavonoids was not specified because a mixture of root exudates was used for the assay.

Concomitantly, plant colonization by PGP bacteria was shown to alter plant flavonoid production, especially under stress conditions [196]. Indeed, a consortium of three PGP microorganisms, including a *Pseudomonas* strain, *Mesorhizobium* sp. and *Trichoderma* enhanced H_2O_2 synthesis in *Cicer arietinum* to counteract *Sclerotium rolfsii* attack. In parallel, the beneficial consortium increased accumulation of the flavonols quercetin, myricetin and kaempferol in chickpea plants to protect plant cell membrane from oxidative modifications [197]. Similar increase in total flavonoids content was reported in maize plants challenged by high salinity stress [198,199] and in drought-exposed pennyroyal [200] treated with beneficial bacteria.

It was hypothesized that, when subjected to biotic and abiotic stress conditions, the plant exudates secondary metabolites to actively recruit beneficial microorganisms in order to respond and minimize the stress [86], exploiting a mechanism known as 'cry-for-help' [201,202] (Figure 4).

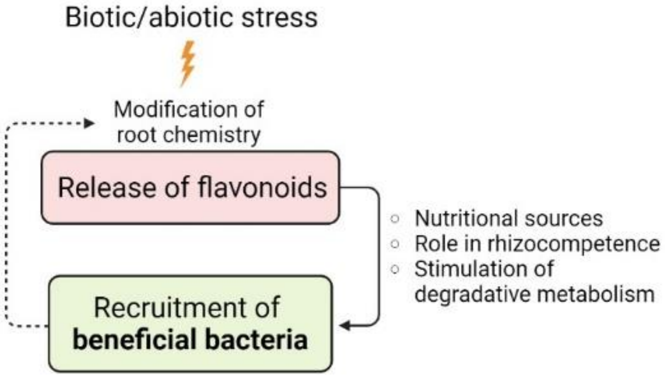

Figure 4. Role of flavonoids in the plant 'cry-for-help' strategy to cope with environmental stresses. Modification of root chemistry is part of the physiological responses that plants adopt to cope with environmental stresses (represented by the thunder symbol). This phenomenon is known as 'cry-for-help' and it induces, among others, the exudation of flavonoids in the rhizosphere. These phytochemicals play multiple roles in the recruitment of soil microorganisms, which can establish beneficial interactions with the host plant and contribute to the holobiont fitness, mitigating the stress deleterious effects. Flavonoids can be exploited as nutrients, induce efficient colonization of the root system, and act as inducers for the expression of catabolic genes for the degradation of xenobiotic compounds. In turn, beneficial microbes colonizing the rhizosphere and the endosphere can affect the root exudation pattern (dashed arrow), favoring the establishment of suitable conditions for their growth in these niches.

This rescue strategy is crucial when plants are exposed to phytotoxic xenobiotics such as, for instance, polychlorinated biphenyls [203]. Under these circumstances, plants tune their exudation profile to favor the recruitment of microbes endowed with the degrading enzymatic machinery to transform recalcitrant substances in more bio-available and less phytotoxic compounds [204,205]. An increasing body of evidence indicates that flavonoids, due to a similarity in the chemical structure, are crucial in stimulating the microbial-degrading metabolism of PCBs (Figure 5) by acting as growth substrates, co-metabolites or inducers of the *bph* operon [206–208], that comprises the catabolic genes for biphenyl aerobic oxidation and encodes for the dioxygenases necessary for its mineralization [209,210].

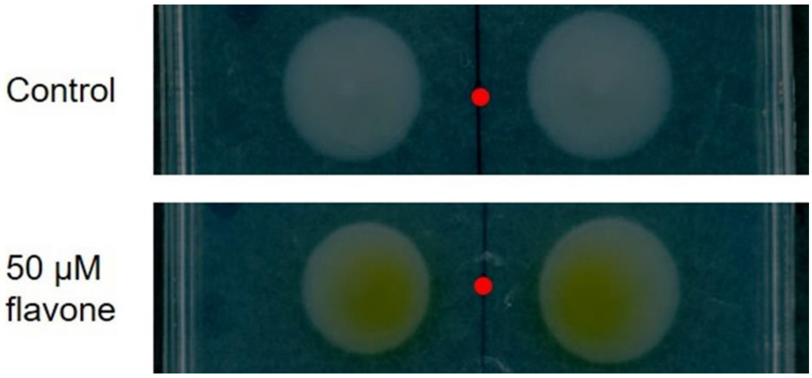

Figure 5. Flavonoids-mediated enhancement of the PCB degrading metabolism. The PCB-degrading strain *Pseudomonas* strain JAB1 was placed in close contact with flavone, which is able to induce its degradative metabolism [211] as visible by the yellow coloration assumed by the grown colonies, possibly due to the presence of a degradation intermediate [212]. The red dots represent the sites in which the solvent (control) or the flavonoid flavone were plated, according to the protocol for chemotaxis described by Reyes-Darias and colleagues [213].

Therefore, the flavonoid-mediated plant-microbe crosstalk paves the way for rhizoremediation approaches for polluted soils clean-up [214,215]. The PCB-degrading bacterium *Rhodococcus erythropolis* U23A, grown co-metabolically with a different combination of carbon sources and flavonoids such as flavanone, flavone and isoflavone, showed the ability to degrade 4-chlorobiphenyl to 4-chlorobenzoate with a higher efficiency than in the presence of biphenyl [189]. This effect was directly dependent on the flavonoid-driven induction of *bph* genes [216], a feature observed also with flavanone and catechin in *Pseudomonas alcaliphila* JAB1 [211]. A microcosm experiment revealed that the addition of naringin to the soil had an impact on bacterial community structure, promoting the growth of *Burkholderia* spp., and led to an enhanced degradation of numerous PCB congeners [217].

Flavonoids exert similar functions on another wide class of pollutants, namely polycyclic aromatic hydrocarbons (PAH): PAH-degrading bacteria isolated from cucurbits used flavonoids like morin as unique carbon sources, suggesting a relation in the metabolic pathways and the putative promotion of PAH degradation induced by flavonoids [218].

A deeper understanding of the mechanisms involved in beneficial plant-bacteria interactions mediated by flavonoids may be crucial in implementing sustainable strategies aimed at increasing plant growth and productivity [36], accelerating phyto-rhizoremediation [219], improving plant protection from pathogens and, more generally, the holobiont resistance to stresses [220].

6. Flavonoids Modulate Behavior and Life History Traits of Insect Herbivores, Natural Enemies and Pollinators

Flavonoids are known to modulate plant interactions with insects, including herbivores, natural enemies (i.e., predators and parasitoids) and pollinators [221,222] (Figure 2). Together with other plant pigments (e.g., betalains and carotenoids), these phytochemicals confer color, fragrance and taste to flowers, fruits and seeds, attracting not only insects, but also birds and mammals, which provide important ecosystem services i.e., pollination and seed/fruit dispersal [223]. For instance, quercetin preference by the honeybee *Apis mellifera* has been verified in free-flight semi-field assays [224]. Mutations in the flavonoid biosynthetic pathway showed a shift of pollinators' preference [225]. Loss-of-function mutations of the transcriptional factor *ANTHOCYANIN2* (*AN2*) in *Petunia axillaris* resulted, indeed, in white-colored and moth-visited flowers, whereas *P. integrifolia* flowers with functional *AN2* alleles harbored anthocyanin-colored petals and were efficiently pollinated by bees and butterflies [225].

Flavonoids have a crucial role in plant protection and defense against phytopathogenic insects by modifying the plant palatability, reducing its digestibility, decreasing its nutritional value, but also attracting useful predators of insect herbivores. They can behave as toxins, binding the insect digestive enzymes, and thus interfere with the host development, reproduction and molting, affecting ultimately the fitness. They can inhibit ecdysone-dependent pathway and acetylcholinesterase, impairing molting, or hinder the activity of glutathione S-transferases enzymes, involved in insecticide detoxification [50,63,226]. High flavonoid levels in tomato reduced the plant attractiveness and the feeding activity of the whitefly *Bemisia tabaci*, which in turn decreased the spread of the whitefly-transmitted virus responsible for an economically important disease (i.e., tomato yellow leaf curl disease, TYLCD) [227]. Feeding tests verified the repellence or feeding deterrent effect of different flavonoids in several in-sect and nematode species [228,229], supporting their use as biopesticides as alternatives to traditional synthetic molecules [230]. Quercetin, for instance, is a repellent and insecticidal compound for different plant-feeder insects like aphids [231]. However, its ingestion activates detoxification enzymes in useful insects like honeybees, extending their longevity and enhancing pesticide tolerance, hence highlighting a beneficial role of quercetin for pollinators [232,233]. In this context, an interesting perspective relies on the use of agricultural wastes as flavonoids' source material under the frame of a circular economy approach, once field experiments and evaluations on features such as environmental fate and target specificity would have ensured their safe use [230]. Flavonoids can have indeed a harmful effect on nontarget beneficial species, as observed on the zoophytophagous generalist predator *Orius sauteri*, a biological control agent of whiteflies, which showed a reduced oviposition, nymphal survival, and development on flavonoids-producing tomatoes [234]. Several studies also reported the phagostimulant or neutral activity of flavonoids in dependence of the insect species considered and of the molecule and dosage selected [229,235]. For instance, quercetin and pinocembrin at low concentration (0.01–1 µg/cm^2) have phagostimulant effects on *S. frugiperda* on treated lettuce foliage [229], whereas at higher concentrations (10, 50, 100 µg/cm^2) the effects of these two molecules were different. Pinocembrin showed an antifeeding effect for all the high concentrations, whereas quercetin had a neutral activity at concentrations of 10 and 50 µg/cm^2, becoming antifeedant at a concentration of 100 µg/cm^2 (although with lower effects than pinocembrin) [229]. A recent review summarizes a literature search on insect-quercetin interactions to suggest the use of this flavanol to reduce herbivory without damaging natural enemies and pollinators: harmful and nonharmful effects were reported on different insect species, considering different application dosages and behavior and life history traits [231]. However, authors highlighted the need for field experiments to substantiate the results [231].

Flavonoids can also regulate insect oviposition [63]. Quercetin glycosides are oviposition cues for the monarch butterflies [236], whereas quercetin, taxifolin, and naringenin (the last one to a lesser extent) can stimulate oviposition in the predatory ladybird beetle *Coleomegilla maculata* [237]. Interestingly, quercetin can also orient *C. maculata* to oviposition sites when conspecifics are present, underlining the possible exploitation of this phytochemical in mass-rearing facilities [238]. Conversely, oviposition can be negatively influenced by the presence of flavonoids [231]. Flavone, rutin, quercetin, myricetin, fisetin, quercitrin, and partially purified flavonoids from *Calotropis procera* (a popular medicinal plant of Africa and Asia tropics), reduced egg laying of the adzuki bean weevil *Callosobruchus chinensis* in a dose-dependent manner [239]. Quercetin also deterred oviposition of the melon fruit fly *Bactrocera cucurbitae* and its presence on test substrates could outcome in a lower number of ovipunctures under multiple-choice conditions, as also exerted by rutin [240]. A decrease in flavonoid content in tomato infested by *B. tabaci* positively modulated the oviposition preference of the insect [241].

Even if many efforts have been performed to elucidate insect-flavonoid interactions, also focusing on specific molecules [221,231], future in-depth work should consider more insect species and different flavonoid compounds, considering a multitrophic level of investigation (i.e., among plant, herbivore, natural enemy, pollinator, and generally nontarget species) and clarifying the molecular mechanisms that underlie these interactions.

7. Flavonoids as Nutraceuticals and Prebiotics for Human Health

Flavonoids and their metabolites can improve human health by acting as phytochemicals [242], nonessential molecules consumed through food or dietary complements, that modulate signaling pathways and decrease inflammation, immune response and oxidative stress processes [43]. This review will treat this topic highlighting flavonoids' role as inter-kingdom signaling molecules also in the context of human pathologies: other works have dedicated valuable efforts to elucidate the medical and nutraceutical potential of these compounds [41,243–247].

Flavonoids were shown to exert a variety of nutraceutical features such as anti-oxidative, anti-diabetic, anti-proliferative, anti-carcinogenic, anti-microbial and immunomodulatory abilities [44]. Due to these abilities, flavonoid supplements have been largely investigated in studies for prevention or drug efficacy evaluation in several pathologies including cancer, atherosclerosis, stroke, neuroinflammatory diseases, age-related neurodegeneration, diabetes, human herpes virus (HHV) infections and recently also COVID-19 respiratory disease [43,60,248,249] (Figure 2). Interestingly, one of the main effects of these illnesses is oxidative stress, which induces harmful damages on membranes, lipids, proteins, lipoproteins and DNA in humans [250], similar to the dangerous effects previously described in plants. The role of flavonoids as conventional hydrogen-donating antioxidants has been extensively reviewed and leads to a paradigm shift in their acknowledged functions in human disorders [43]. According to an emerging scenario, these phytochemicals may exert their beneficial activities in human health mainly by interfering with signaling pathways [243].

Diet is the main source of flavonoids for humans, since they are common constituents of fruits, vegetables and legumes [251]. Berries are known to contain a wide range of antioxidant flavonoids as anthocyanidins, the flavone luteolin and numerous flavonols [252]. Isoflavonoids, like genistein and daidzein, are the main class present in legumes due to their role in nodule formation in the symbioses with rhizobia [253], and flavanones as hesperidin and naringenin can be found in citrus species and grapes [254–256]. Flavonoids are also present in fruit and vegetable food wastes, making them profitable resources to obtain useful biomolecules for the formulation of functional foods and nutraceuticals in a circular economy approach [257]. Fourteen different flavonoids have been identified in tomato seeds extract, including quercetin, kaempferol and isorhamnetin glucosides [258], and rutin and naringenin were found to be the main constituents of total phenolics in tomato peel fiber [259].

Once introduced through the diet, flavonoids are absorbed and digested in the intestine by enzymes expressed by intestinal epithelial cells and by the gut microbiota, giving rise to more absorbable aglycones and a mixture of smaller phenolics with varying degrees of hydroxylation, glucuronidation, sulfation, and methylation [260,261]. Parent flavonoids and their metabolic products can exert their biological function locally in the gastrointestinal tract or can pass to the systemic circulation to reach the peripheral body districts where they perform systemically beneficial actions [262]. At the intestine level flavonoids and their metabolites exert a prebiotic effect for the host's immune system and gut health [245]. Through an unknown mechanism, dietary flavonoids can support the growth of probiotic bacteria such as *Bifidobacteriaceae* and *Lactobacillaceae* and inhibit pathogens such as *E. coli* and *Helicobacter pylori*, thus improving gut health and immune modulation by reducing endotoxin production, sustaining nutrients absorption and intestinal barrier functionality [263,264]. Presumably by inhibiting the gyrase, a topoisomerase involved in bacterial DNA replication [265], 50 µg/mL quercetin was found to inhibit the growth of *Ruminococcus gauvreauii* [266], a gallbladder bile-inhabiting bacterium [267] whose abundance has been associated to altered gut composition in patients affected by coronary artery disease [268]. Depending on their structure and dose, flavonoids can affect the structure of the gut microbiome, by stimulating the abundance of specific genera [42], providing an analogy to the mechanism driving the plant microbiome structuring. Rutin and quercetin supplements at 100 µg/mL stimulated the growth *in vitro* of *Bifidobacterium bifidum*, although at the same concentration quercetin and hesperidin inhibited the growth of *Bifidobacterium adolescentis*, showing contrasting effects depending also on bacterial species and strains [269].

Furthermore, flavonoids can perform other beneficial roles in the gastrointestinal tract. Indeed, diets rich in specific flavonoid families such as anthocyanidins, flavonols, flavones, and isoflavones showed a positive trend for a decreased risk in developing colorectal cancer [270], presumably by inhibiting pro-oncogenic signaling pathways [271].

At a systemic level in the body districts, apigenin, quercetin, chrysin, luteolin, kaempferol and fisetin are flavonoids described for their beneficial role in human health and their mode of action seems to rely on the suppression of inflammatory markers such as interleukins and down-regulation of inflammatory pathways mediators such as NF-kB, that responds early to harmful cellular stimuli, and MAPK cascades, including p38-, JNK-, and ERK-mediated signaling [41]. Myricetin is an isoflavonoid present in different edible parts of the plant and in fruits such as oranges, berries and teas [272]. Several in vitro studies highlighted myricetin as a potential chemotherapeutic agent able to interfere with dysregulated signaling pathways in cancer progression, invasion and metastasis by regulating ER stress, NF-kB, mTOR and telomerase [273]. In human papillary thyroid cancer, 100 µM myricetin treatments induced cytotoxicity and DNA condensation, finally causing cell death of SNU-790 cells by disrupting the mitochondrial membrane potential and regulating caspase cascades [274]. Myricetin-mediated cytotoxicity was observed in triple-negative breast cancer cells due to oxidative stress: autoxidation of 50 µM myricetin in the growth medium led to extracellular H_2O_2 formation and triggered intracellular ROS production, ultimately causing DNA damage [275]. Naringenin, as well, has been demonstrated to be effective against a wide range of tumor types by suppressing cancer progression programs [276]. Genistein, an isoflavonoid present in soy-derived foods, was hypothesized to reduce SARS-CoV-2 infectivity by inhibiting the virus strategy for entrance in human epithelium [277]. Numerous *in vitro* studies also highlighted the role of flavonoids in the treatment of HHVs infections and in preventing the associated neurological diseases [60]. Flavonoids isolated from *Morus alba* L. showed antiviral activities toward the widespread herpes simplex virus type 1 (HSV-1) through different mechanisms that led to inhibited viral proliferation: morusin interfered with the expression of the glycoprotein necessary for cellular binding and infection and reduced the virus-induced ROS content [278], and kuwanon C, T, U, and E hindered HSV-1 replication machinery [279].

Despite being under the spotlight for their wide potentials in therapeutic use, flavonoid administration still lacks *in vivo*, preclinical and clinical studies for their validation in the treatment of human diseases. There is, therefore, a huge need for clinical research to assess flavonoids' efficacy in human health. Furthermore, the elaboration of uniform guidelines in the design and reporting of flavonoid studies is advocated, in order to translate clinical evidence and research findings in valuable recommendations for patients [280]. In a pharmacological perspective, it will be crucial to understand the structure-activity relationship, considering that several flavonoids exert diversified, multiple effects on different pathways. Thus, a precision medicine approach is necessary to identify the mechanisms of action, eventual off-target effects, investigate the most active flavonoid molecules, and use them in combination therapies. This knowledge will be essential to understand the significant inter-individual variability observed in dietary flavonoid benefit supplementations [281].

So far, flavonoids are extracted from natural plant resources through separation techniques [282,283]. Recently, much progress has been made through a synthetic chemistry approach to investigate the structure-activity of flavonoid moieties [284], to promote synthetic units that control a precise interaction site within the biological system [285,286], and to modify the flavonoid backbone to achieve new functionalities [287], paving the way toward the discovery of new drugs [288].

A major drawback in flavonoid medical application is linked to their poor bioavailability and low solubility in oral formulations, which makes it necessary to develop valuable delivery systems to the target cells [273]. Nanocarriers may solve this issue, by protecting encapsulated flavonoids from oxidation or degradation, and increasing their stability, solubility, and bioavailability to the target tissue [44,289]. A diverse set of nanodrug delivery systems (lipidic, polymeric, micelles, nanoemulsions and nanoparticles) has been developed for naringenin encapsulation to overcome its medical application issues due to the molecule instability and low bioavailability [290,291].

8. Concluding remarks

Although their primary ancestral role in evolution seems to be correlated with protection against the excess of radiant energy, flavonoid biosynthesis is triggered by a broader array of abiotic stresses and their main function is to cope with stress-induced ROS oxidative burst. Despite obvious differences between plants and animals in stress responses, ROS trigger very conserved signaling cascades and induce common damages in both systems [292,293], explaining the wide ability of flavonoids to act as ROS scavengers in plants and to modulate inflammation-signaling cascades in animal and human in vitro models [43].

Besides ROS protection, flavonoids showed additional beneficial functions, such as, (i) the influence on phytohormone homeostasis and stress-induced morphogenesis, which contribute to tune the plant plastic response to adverse environmental conditions [294], (ii) the soil detoxification from xenobiotics, and (iii) the microbiome modelling in the 'cry-for-help' mechanism [204]. These metabolites can act also as signaling molecules, endowing plants with the ability to perceive the environment and to communicate with the biotic components belonging to other species, genera, families and kingdoms [36]. These multi-kingdom communication dynamics affect high biodiversity hotspots such as the rhizosphere as well as the human gut, where dietary flavonoids are metabolized [295].

Although plant secondary metabolites have been described for their ability to perform activities across different kingdoms such as, for instance, plant defense against weeds and pathogens [296], so far only flavonoids have shown such a large variety of functionalities and conserved beneficial effects in plant, animal and human systems and the ability to mediate interactions with prokaryotic and eukaryotic cells.

In the vision of phytochemicals application in agriculture to sustain crop growth, phytoprotection and yields, flavonoids are élite molecules. Quercetin, for instance, is a potent flavonoid with a diverse set of functions in plants [226]. Preliminary pot experiments suggest its potential as biofertilizer: spray inoculation of quercetin on wheat leaves increased physiological parameters related to chlorophyll content and fluorescence, and induced the accumulation of phenolics that are correlated with increased resistance to abiotic stress in a dose-dependent manner [297]. Seed priming with quercetin alleviated chromium toxicity in *Trigonella* seedlings, enhancing flavonoids accumulation in plant tissues, potentially quenching ROS induced by the metal contamination [298]. Another promising flavonoid for field application is naringenin, although its involvement in plant defense is poorly elucidated. A preliminary study in tobacco highlights that naringenin is endowed with a low-medium antifungal activity against *Phytophtora nicotianae*, although it induces the expression of salicylic acid responses to counteract the pathogen attack [299].

A main issue for flavonoid applications in agriculture is their stability in soil that depends, among other factors, on the specific molecule and the degradative activity of soil microbiomes. Flavonoids are considered rather stable compounds, although apigenin and kaempferol half-life in soil is estimated within only 4 and 14 days respectively. Nevertheless, these lifetimes of permanence in soil are limiting factors for flavonoid exploitation as biofertilizers or biopesticides because of the requirement for more frequent applications to maintain these phytochemicals at effective concentrations. The adoption of delivery systems based on encapsulation could contribute to increase flavonoid stability and a sustained release in soil. Nanoliposomes were adopted to encapsulate quercetin to efficiently target the phytochemical inside leaf cells through osmosis or endocytosis, overcoming its low solubility which is considered as an important factor limiting the phytopathogen control effect. Nanoliposome-quercetin, indeed, could inhibit the expression of plant hsp70 proteins that are generally induced under stress, and which are host factors hijacked by viruses to assemble their replication complex, thus acting as an outperforming antiviral agent even under field conditions [300]. On the other hand, soil-dissolved organic carbon (DOC) strongly reduces flavonoids' lifetime in soil. It is estimated that ~70% of flavonoid signaling potential is attenuated by sequestration to DOC through a metal-mediated oxidative reaction that leads to dimerization [301]. This phenomenon occurs with a variety of plant sources and mainly implicates Mn^{3+}, a metal that is abundant in litter decomposition [301,302]. Flavonoids are also degraded aerobically by diverse soil microbes through monooxygenase enzymatic activities, as observed in *Herbaspirillum seropedicae* mutated in the *fdeE* gene which lost the capacity to use naringenin as a carbon source [303]. This knowledge could be the picklock to design a holobiont-metabolic network utilizing plant with a specific flavonoid exudation profile [304] coupled to information about the lifespan of these metabolites under different soil conditions. This approach would pave the way to engineer beneficial plant-microbe interactions, reduce phytopathogen outgrowth and propose effective soil amendments [24]. In this vision, further research is needed to increase knowledge about flavonoid bioactivity toward microorganisms, their concentration of use, environmental fate, degradative kinetics and influence on soil microbiota. These data are crucial for an environmentally safe application of flavonoids in agricultural practices. A similar concern arises also for flavonoids utilization in medical treatments. Despite mounting evidence on their protective role in several human pathologies, basic research and clinical trials are needed to assess, respectively, their mechanism of action and their efficacy and safety for drug development.

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CHAPTER III

Flavonoids influence key rhizocompetence traits for early root colonization and PCB degradation potential of *Paraburkholderia xenovorans* LB400

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Abstract

Introduction. Flavonoids are among the main plant root exudation components, and, in addition to their role in symbiosis, they can broadly affect the functionality of plant-associated microbes: in polluted environments, for instance, flavonoids can induce the expression of the enzymatic degradative machinery to clean-up soils from xenobiotics like polychlorinated biphenyls (PCBs). However, their involvement in root community recruitment and assembly involving non-symbiotic beneficial interactions remains understudied and may be crucial to sustain the holobiont fitness under PCB stress. **Methods.** By using a set of model pure flavonoid molecules and a natural blend of root exudates (REs) with altered flavonoid composition produced by *Arabidopsis* mutant lines affected in flavonoid biosynthesis and abundance (null mutant *tt4*, flavonoid aglycones hyperproducer *tt8*, and flavonoid conjugates hyperaccumulator *ttg*), we investigated flavonoid contribution in stimulating rhizocompetence traits and the catabolic potential of the model bacterial strain for PCB degradation *Paraburkholderia xenovorans* LB400. **Results.** Flavonoids influenced the traits involved in bacterial recruitment in the rhizoplane by improving chemotaxis and motility responses, by increasing biofilm formation and by promoting the growth and activation of the PCB-degradative pathway of strain LB400, being thus potentially exploited as carbon sources, stimulating factors and chemoattractant molecules. Indeed, early rhizoplane colonization was favored in plantlets of the *tt8 Arabidopsis* mutant and reduced in the *ttg* line. Bacterial growth was promoted by the REs of mutant lines *tt4* and *tt8* under control conditions and reduced upon PCB-18 stress, showing no significant differences compared with the WT and *ttg*, indicating that unidentified plant metabolites could be involved. PCB stress presumably altered the *Arabidopsis* root exudation profile, although a sudden ‘cry-for-help’ response to recruit strain LB400 was excluded and flavonoids appeared not to be the main determinants. In the *in vitro* plant–microbe interaction assays, plant growth promotion and PCB resistance promoted by strain LB400 seemed to act through flavonoid-independent mechanisms without altering bacterial colonization efficiency and root adhesion pattern. **Discussion.** This study further contributes to elucidate the vast array of functions provided by flavonoids in orchestrating the early events of PCB-degrading strain LB400 recruitment in the rhizosphere and to support the holobiont fitness by stimulating the catabolic machinery involved in xenobiotics decomposition and removal.

1. Introduction

The plant microbiome acts as a reservoir of accessory functionalities that increase the fitness of the holobiont (Marasco et al., 2013; Rolli et al., 2015; Hassani et al., 2018; Trivedi et al., 2020). In particular, these microbial services are crucial when the soil is polluted by recalcitrant and poorly phyto-extractable xenobiotics that hamper plant growth and development (Franchi et al., 2016; Franchi et al., 2017; Correa-Garcıá et al., 2018). Plant competence to recruit a degradative microbiome and the enzymatic versatility of the associated microorganisms to catabolize persistent pollutants are essential features in rhizoremediation processes (Balloi et al., 2010; Vergani et al., 2017b; Simmer and Schnoor, 2022). Due to the advancement of metabolomics (Carper et al., 2022) and exo-metabolomics (Zhalnina et al., 2018), root chemistry is gaining increasing interest for the comprehension of the role of root exudation in tuning the recruitment, colonization pattern, structure, and functionality of the plant-associated microbiota (McLaughlin et al., 2023).

In addition to the exuded carbon-rich primary metabolites, plants secrete a vast array of secondary metabolites (Erb and Kliebenstein, 2020) that are responsible for the major shifts in the structure of soil microbial communities due to the presence of antimicrobial compounds, quorum sensing/quenching molecules, and co-metabolites that affect bacterial physiology (Pang et al., 2021).

Flavonoids constitute a broad class of specialized polyphenols that share the C_6–C_3–C_6 basic structure and undergo a series of secondary modifications (e.g., glycosylation, methylation, acylation) that enable their classification into various classes like flavones, isoflavonoids, flavonols, and anthocyanins (Yonekura-Sakakibara et al., 2019). Flavonoids are among the most abundant compounds released by rhizodeposition (Chaparro et al., 2013; Wang et al., 2022): they represented 37% of total secondary metabolites released by *Arabidopsis thaliana* (Narasimhan et al., 2003) and reached a higher abundance compared with exuded primary metabolites in *Quercus ilex* upon water shortage stress (Singh et al., 2023). A metabolome investigation of the root exudate composition of *Panax notoginseng*, *Zea mays*, *Nicotiana tabacum*, and *Perilla frutescens* showed a high chemical diversity, with flavonoids being among the dominating compounds in terms of abundance and with differential patterns in terms of composition, contributing to the uniqueness of the exudation profile in the different plants (Shi et al., 2023).

Flavonoids are well characterized in legumes for their key role in rhizobial symbioses, facilitating the process of rhizobia recruitment and root architecture remodeling that leads to nodule formation, where the biological fixation of nitrogen takes place (Wang et al., 2022). Despite being acknowledged as inter-kingdom signaling molecules in the interactions between roots and plant-associated microbes (Ghitti et al., 2022), the role of flavonoids in non-symbiotic bacterial species is poorly investigated. Recently, a proteomics approach highlighted that apigenin and phloretin can be perceived by *Pseudomonas fluorescens* 2P24 through the TetR regulator system PhlH and that these flavonoids differently affected bacterial physiological traits involved in their establishment on the root system and in root colonization (Yu et al., 2020). Under nitrogen starvation, maize rhizodeposition was enriched of flavones, that positively selected for a higher abundance of *Oxalobacteraceae* in the rhizosphere microbiota.

This shift in root bacterial communities indirectly supported plant growth and nitrogen uptake by remodeling root architecture with enhanced formation of lateral roots (Yu et al., 2021). Similarly, in the flavonoid hyperaccumulator *Arabidopsis* line *pap1-D* that overexpresses anthocyanins and flavonols, the *Aeromonadaceae* family showed a higher relative abundance in both rhizosphere and endosphere compared with Col-0 plants. Furthermore, flavonoids improved chemotaxis and motility of *Aeromonas* sp. H1 toward the root, enriching the rhizosphere with plant growth-promoting strains, finally able to boost plant dehydration resistance (He et al., 2022). Hence, these findings highlight a similarity in the role played by flavonoids as secondary metabolites involved in bacterial recruitment also in non-rhizobial species.

In bioremediation studies, flavonoids were described to act as inducers or co-metabolites to enhance the expression of the dioxygenases encoded by the *bph* operon for the aerobic degradation of polychlorinated biphenyls (PCBs) (Narasimhan et al., 2003; Toussaint et al., 2012). PCBs constitute a broad class of persistent organic compounds (POPs) that dramatically affect human health and ecosystems and whose removal from the environment, ideally through *in situ* sustainable practices, is of paramount importance and urgency (Di Guardo et al., 2017; Simhadri et al., 2020). Flavonoids are promising inducers of the microbial catabolism of PCBs, supposedly because of their similarity in structure with biphenyl (Pino et al., 2016). It is postulated that the PCB degradation pathway evolved primarily for the catabolism of plant secondary metabolites with a biphenyl-based backbone and that, due to a broad enzymatic specificity, this also allowed the degradation of structurally similar compounds like PCBs (Singer et al., 2004; Musilova et al., 2016; Uhlik et al., 2022). In agreement with this vision, co-metabolism drives the degradation of xenobiotic compounds in presence of plant secondary metabolites endowed with a structural affinity with the contaminants (Singer et al., 2003). Through these low substrate affinity enzymes, emerging pollutants of anthropogenic origin, only recently appearing in natural environments, can be degraded to low-molecular weight intermediates (Uhlik et al., 2022; Singer et al., 2004; Musilova et al., 2016). During co-metabolic growth in the presence of sodium acetate, isoflavone induced the expression of the *bphA* gene in *Rhodococcus erythropolis* U23A, resulting in an even higher degradation efficiency of 4-chlorobiphenyl than in the presence of biphenyl (Pham et al., 2015). Therefore, flavonoids may represent crucial exudates in the plant “cry-for-help” strategy in PCB-contaminated soil (Rolli et al., 2021). According to this ecological hypothesis, plants alter their root exudation pattern under stress aiming to recruit, feed, and sustain a wide variety of beneficial microorganisms, which provide useful functionalities to the plant to alleviate the detrimental injuries caused by biotic and abiotic stresses (Rolfe et al., 2019). Given PCB phytotoxicity and the poor detoxification systems of plants (Van Aken et al., 2010), the ‘cry-for-help’ is hypothesized to be the strategy that plants employ to survive in polluted sites (Vergani et al., 2017a; Mapelli et al., 2022). Indeed, the depletion of flavonoid exudation in the *tt4 Arabidopsis* mutant affected the colonization and consequently the PCB degradation ability of *Pseudomonas putida* PLM2 (Narasimhan et al., 2003). Similarly, the PCB-degrading strain *Pseudomonas alcaliphila* JAB1 was able to metabolize flavone and flavanone through the activity of the biphenyl 2,3-dioxygenase and in parallel used a wide range of secondary metabolites, including flavonoids, as *bph* operon inducers (Zubrova et al., 2021). In wider terms, the identification and characterization of the chemical determinants able to induce PCB degradation would be particularly useful in the frame of tailor-made biostimulation strategies (Uhlik et al., 2013; Jha et al., 2015): providing the contaminated soil with inducer-rich amendments, also through waste biomass (Wang et al., 2023), or selecting plants with specific root exudation profiles would potentially enhance the recruitment of degrading microbial communities and increase the effectiveness of rhizoremediation.

This evidence supports the hypothesis that the role of flavonoids in affecting bacterial crosstalk with the plant host, in particular regarding the interactions with PCB-degrading bacteria, could be broader than supposed and still largely unknown. The aim of this work was, therefore, to go beyond the state of the art and elucidate the contribution of flavonoid molecules in affecting functional traits of rhizocompetence and degradation potential in the versatile PCB degrader strain *Paraburkholderia xenovorans* LB400 (Liang et al., 2014). Our experimental approach was developed along an increasing degree of complexity of flavonoid chemistry: (i) by administering pure compounds (naringin, naringenin, quercetin, flavone, and flavanone) to assess *in vitro* the involvement of flavonoids in stimulating bacterial rhizocompetence traits and in inducing the expression of PCB catabolic genes; (ii) by testing the effect of flavonoid imbalance in a natural complex mixture of root exudates released by the *Arabidopsis* mutant lines *tt4*, *tt8*, and *ttg* affected in flavonoid biosynthesis and exudation; and (iii) in planta in the interaction with the same mutant plants that differ in their root exudation pattern due to either the different genetic background or to the stress induced by growing in the presence of PCBs.

We observed a crucial role for flavonoid pure chemicals in boosting bacterial growth, attracting bacteria to the root system and stimulating the expression of PCB catabolic genes, indicating that flavonoids can play a prominent role during the early events of bacterial colonization. On the other hand, LB400 growth in the presence of the complex blend of root exudates from *Arabidopsis* mutant lines *tt4*, *tt8*, and *ttg*, together with the interaction of the bacterium with *Arabidopsis* plantlets of these backgrounds under control conditions and PCB-18 stress suggests flavonoid-independent mechanisms for the observed phenotypes, prompting that other unknown exudates are involved at later stages of bacterial colonization.

This work contributes to improve knowledge on the interactions between plants and *P. xenovorans* LB400, considered one of the most effective aerobic PCB degraders given its impressive ability to catabolize more than 20 PCB congeners, comprising some highly chlorinated ones (Chain et al., 2006). *Burkholderiaceae* have been described as important plant colonizers, represent a key component of the *Arabidopsis* microbiome, especially the floral one, and comprise beneficial bacteria able to support plant growth and resistance to abiotic stresses (Massoni et al., 2021; Pal et al., 2022). *P. xenovorans* LB400 has been used mainly in degradation studies with slurries or sediments (Payne et al., 2017; Bako et al., 2022): improving the knowledge on its association with plants could lead to more targeted and efficient phyto-rhizoremediation approaches for PCB clean-up in soil.

2. Materials and methods

2.1. Bacterial strain, plant material, culture media, and chemicals

Paraburkholderia xenovorans LB400 (DMSZ, Germany) was grown either in Tryptic Soy Broth (TSB, Merck, Darmstadt, Germany) or Luria-Bertani (LB) broth or in Mineral Medium Brunner (DSMZ, Germany) with a supplement of 30 mM sodium pyruvate as a carbon source. Bacterial cells were kept in glycerol stocks at −80°C and periodically revitalized on LB agar plates. When necessary, antibiotics were added at the following concentrations: 15 µg/mL gentamicin and 100 µg/mL rifampicin. The plant secondary metabolites (PSMs) used in the assays are flavonoids (Merck, Germany) and were solubilized in the respective solvents and prepared in 100× and 1,000× stocks. Flavone (2-phenyl-4H-1-benzopyran-4-one) and flavanone (2,3-dihydroflavone) were solubilized in acetone, naringin, and naringenin in methanol and quercetin in dimethyl sulfoxide (DMSO). The biphenyl crystals (Merck, Germany) were solubilized in acetone in a 0.5 M stock and PCB No. 18 (2,2′ ,5-trichlorobiphenyl, LGC Standards) was solubilized in acetone in a 200 mM stock. PCB-18 was selected to induce phytotoxic effect in *Arabidopsis* based on (i) literature data indicating that low-chlorinated PCBs are preferentially uptaken and assimilated by plant roots than highly chlorinated PCBs (Asai et al., 2002; Luo et al., 2020); (ii) a previous work demonstrating that this congener can indeed affect *Arabidopsis* growth (Bao et al., 2013); (iii) plant–microbe interaction study with PCBs are preferentially performed with single congeners rather than complex mixtures to make the phenotype analysis more straightforward (Subramanian et al., 2017; Wang et al., 2017); (iv) this molecule was retrieved in the historically PCB contaminated site Brescia-Caffaro in Italy, indicating its relevance also from an ecological point of view (Di Guardo et al., 2017). *Arabidopsis thaliana* ecotype L*er* and the mutants for flavonoid biosynthesis *tt4*, *tt8*, and *ttg* (NASC, Nottingham Arabidopsis Stock Centre) were the plants employed for root exudate collection and root colonization assays. Line *tt4* is a null mutant that does not produce flavonoids, whereas *tt8* and *ttg* overaccumulate in the root exudates flavonoid aglycones and flavonoid conjugates, respectively (Narasimhan et al., 2003). Seeds of *Arabidopsis* were surface sterilized under shaking conditions with a 0.05% SDS and 70% ethanol solution for 10 min and washed twice with 95% ethanol. The seeds were cultivated on half-strength Murashige and Skoog (1/2 MS) medium (2.2 g/L MS basal salt mixture, 0.5 g/L MES hydrate, pH 5.4) in square petri dishes.

2.2. Growth on flavonoids as unique carbon sources

Since *bph*-encoded enzymes originally evolved to catabolize plant secondary metabolites either as nutrients or for their detoxification (Singer et al., 2003; Singer et al., 2004), the ability of *P. xenovorans* LB400 to use pure plant flavonoids (flavone, flavanone, naringin, naringenin, and quercetin) as carbon sources was tested in liquid Mineral Medium Brunner (MMB) following the protocol reported by Zubrova et al. (Zubrova et al., 2021). Strain LB400 was cultivated overnight at 30°C on a shaker (150 rpm) in LB medium and cells harvested by centrifugation (10 min, 4,000 rpm). The cells were washed twice in physiological buffer (9 g/L NaCl) and resuspended at a final concentration of 0.025 OD_{600} in MMB containing 3 mM flavonoids in Erlenmeyer flasks. Solvents were used as negative controls by adding the corresponding volumes and 3 mM sodium pyruvate as positive control. Flavonoid solvents were evaporated in a sterile laminar hood for 30 min prior to addition of inoculated MMB. Strain LB400 was incubated for 6 days at 30°C on a rotatory shaker. To evaluate growth, bacterial cultures were plated in serial dilutions using the drop plate count method by spotting 10 µL on LB plates, which were incubated at 30°C for 3 days prior to colony counting. Each condition was tested with two independent replicates each with three technical replicates.

2.3. Bacterial growth assay in presence of flavonoids

To verify flavonoids' ability to act as signaling molecules able to affect growth parameters of the bacterial strain, the protocol illustrated by Huang et al. (Huang et al., 2019) was adopted with some modifications. Briefly, strain LB400 was grown overnight on a shaker (150 rpm) at 30°C in 1/2 TSB liquid medium in ultrapure Milli-Q water up to 0.6 OD_{600}, corresponding to the strain LB400 late-log growth phase. The cultures were centrifuged (5 min, 4,000 rpm), washed in physiological buffer, and diluted 1,000-fold in the various media used for the bioassay, consisting of the 1/10 TSB added with flavone, flavanone, naringin, or naringenin at final concentrations of 10 µM, 20 µM, 50 µM, and 100 µM. For the flavonoid quercetin, a maximum concentration of 70 µM was used to avoid the formation of an insoluble precipitate. The diluted cultures were then aliquoted (200 µL per well) into a transparent 96-well plate (VWR, USA). Cultures diluted in 1/10 TSB with only flavonoid solvents were used as negative controls. Abiotic controls were aliquoted as blanks in order to subtract the absorbance background given by the media. Each condition was tested in three biological replicates with, respectively, three technical replicates. Bacterial growth was monitored by measuring optical density at 600 nm every hour for 24/48 h using a 96-well plate reader (Tecan, Switzerland), keeping the plate incubated at 30°C and shaking for 7 s before each measurement. Bacterial relative growth increments were calculated as specified by Wang et al. (2019) by comparing the growth in the presence of flavonoids and the specific controls, as well as the $\log_2$ fold change at specific time points of the growth curves. Maximum growth rate was calculated as specified by Navarro-Perez et al. (Navarro-Pérez et al., 2022).

2.4. Analysis of bacterial swimming motility

Bacterial motility toward root exudates plays an important role in the colonization of the rhizosphere (Lugtenberg and Kamilova, 2009), and flavonoids were demonstrated to enhance flagellar motility in non-rhizobial strains (He et al., 2022). Following the indications of Kearns (Kearns, 2010) and Bartolini and Grau (Bartolini and Grau, 2019), 1/10 TSB medium with 0.25% (w/v) agar were prepared to evaluate swimming motility. The medium was autoclaved, briefly cooled, and supplemented with the flavonoids at concentrations 50 µM, 70 µM, and 100 µM before being poured into the plate. The appropriate solvent for each flavonoid molecule was added to the plate as negative control. The plates were dried with the lid for 2 h under laminar flow hood. Strain LB400 was grown overnight at 30°C on a shaker (150 rpm) in 1/2 TSB to an OD_{600} of 0.6. The culture was subsequently washed by centrifugation (5 min, 1,670g, 4°C) and diluted in physiological buffer to a concentration of 0.5 OD/mL. After drying the media, 2 µL of the bacterial suspension was spotted at the center of the plate and left to dry for 30 min with the lid, and then 10 min without the lid, under laminar flow hood. After 24 h of incubation at 30°C, the plates were scanned and the colony diameter measured in duplicate for each plate using ImageJ software (https://imagej.nih.gov). Each condition was tested with three biological replicates (two for quercetin) with at least four technical replicates.

2.5. Chemotaxis assay

Chemotaxis enhances bacterial capacity to acquire high-value nutrients (Colin et al., 2021) and is adopted by soil microorganisms to detect root exudates (Feng et al., 2021) that exert a chemoattractant role, as in the case of flavonoids (He et al., 2022). Chemotaxis was tested using the quantitative gradient plate assay proposed by Reyes-Darias et al. (Reyes-Darias et al., 2016). Minimal A gradient plate medium containing 0.25% (w/v) agar was poured in square Petri dishes after autoclaving. The plates were cooled with the lid for 3 h under laminar flow hood. 10 µL of 50 mM–100 mM pure flavonoids were spotted at the central line of the plate as chemoeffectors, whereas the solvents were used as negative controls and 100 mM sodium pyruvate as positive control for chemoattraction. Plates were further dried for 1 h under laminar flow hood and incubated overnight at 4°C to allow gradient formation. Strain LB400 was grown overnight at 30°C in 1/2 TSB and washed with physiological buffer as reported before for swimming motility assay. The bacterial culture was diluted to a concentration of 0.5 OD_{600}, and, after drying the plates for 45 min, 2 µL of bacterial solution was spotted at 2.5 cm distance from the chemoeffector. The plates were dried for 20 min to allow the complete absorption of the bacterial drop and incubated at 30°C for 72 h. Results were collected by scanning the plates and measuring the colony radius toward (D1) and opposite to the chemoeffector (D2) using ImageJ software. The chemotaxis response index (RI) was then calculated as RI = D1/(D1+D2). Each condition was tested in at least three independent biological replicates, each with at least eight technical replicates.

2.6. *In vitro* biofilm formation assay

Biofilms are assemblages of bacterial cells embedded in an exopolysaccharide matrix that contribute to the bacterial adherence to the surface of the root system (Jin et al., 2019). The flavonoid apigenin was reported to stimulate biofilm formation by soil diazotrophic bacteria, promoting bacterial colonization of rice tissues and improving nitrogen fixation (Yan et al., 2022). Biofilm formation in the presence of flavonoids was therefore estimated *in vitro* by quantifying the bacterial cell adhesion to a solid surface (polystyrene 96-well plate, VWR, USA) using crystal violet (CV) staining, following the method applied by Yoshioka et al. (Yoshioka and Newell, 2016) with few adaptations. Briefly, strain LB400 was grown overnight at 30°C on a shaker (150 rpm) in liquid K10T-1 medium and 2 mL of bacterial culture was harvested and washed twice in physiological buffer. The bacterial solution was resuspended in fresh K10T-1, and 10 µL was added to each well, containing 200 µL of K10T-1 supplemented with 1 µM, 10 µM, and 100 µM flavonoids, to obtain a final concentration of 10^6 cells/mL. Solvents were added to the medium as negative controls, and non-inoculated medium was aliquoted as blank. The plate was incubated statically for 2 days at 30°C. After incubation, OD_{600} of the bacterial cultures was measured using a 96-well plate reader (Tecan, Switzerland). The liquid culture was then carefully removed from the plate and the wells washed twice with phosphate-buffered saline (PBS). The cells adhering to the wells were stained for 15 min with 0.5% (weight/volume) crystal violet (CV) solution in 20% ethanol. CV solution was removed and the plate rinsed twice with distilled water and air dried for 15 min. The remaining CV was solubilized for 30 min using 200 µL/well of 97% ethanol, and OD_{600} was measured using a 96-well plate reader (Tecan, Switzerland). The CV optical densities obtained were then normalized using OD_{600} values measured previously for bacterial growth. Each condition was tested with three independent replicates.

2.7. Induction of the biphenyl degradative pathway in the LB400 strain by flavonoids

To assess if flavonoids act as potential inducers of the biphenyl catabolic pathway in strain LB400, an induction assay was performed. Adapting the protocol used by Pham et al. (Pham et al., 2015), LB400 was grown overnight in MMB supplemented with 30 mM sodium pyruvate on a rotatory shaker at 30°C. Aliquots of the bacterial culture (800 µL) were then used to inoculate glass vials containing 40 mL MMB supplemented with 30 mM sodium pyruvate only (as a control) or 30 mM sodium pyruvate plus 40 µL of flavonoids or biphenyl (positive control for *bphA* induction) to obtain a final concentration of 100 µM. The solvents in which flavonoids were dissolved were added in the same amount (40 µL) as negative controls in the absence of the putative inducer. All conditions were tested in triplicates. The vials were incubated at 30°C on a rotatory shaker, and for each condition, 1 mL of culture was sampled in triplicate when the bacterial cultures reached the mid-log phase (OD_{600} = 0.4-0.5), around 8 h after the start of the induction. Bacterial culture aliquots were then pelleted by centrifugation at 4,000 rpm at 4°C for 5 min. The pellet obtained was stored at −20°C for subsequent RNA extraction steps.

2.8. Total RNA extraction and RT-qPCR on the *bphA* gene

The induction of the oxidative biphenyl catabolic pathway for strain LB400 was analyzed by quantification of the expression of the *bphA* gene via reverse transcriptase quantitative PCR (RT-qPCR). Total RNA was extracted using the NucleoSpin RNA kit (Macherey-Nagel, Germany) following the manufacturer’s protocol, and RNA was eluted in 20 µL of RNAse-free water. To digest any residual gDNA, RNA cleanup and concentration protocol was performed using the NucleoSpin RNA XS kit (Macherey-Nagel, Germany) following the manufacturer’s instructions. Concentration and purity ratios of the extracted RNA were measured using a spectrophotometer (BioSpectrometer, Eppendorf, Germany). Reverse transcription (RT) was then carried out on 1 µg of RNA using RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, USA) following the manufacturer’s indications. The thermal protocol requires the incubation of the template RNA with oligo(dT) primer for 5 min at 65°C, followed by incubation on ice for 5 min. After adding the reaction mixture, RT requires an incubation at 42°C for 60 min and 70°C for 5 min. Reactions in the absence of template RNA (NTC) and in the absence of reverse transcriptase (−RTC) were performed as controls. The cDNA obtained from reverse transcription of the total RNA extracted was diluted 1:10 in ultrapure Milli-Q water and subsequently used for qPCR. qPCR was performed using the CFX Connect Real-Time PCR Detection System (Bio-Rad, USA) using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, USA). The reaction volume was 12 µL containing 1 µL template cDNA (5 ng/µL) and 0.25 µM primers for the amplification of LB400 *bphA* gene (F: 5’-AAAAAGGGCTGCTTGATCCA-3’; R: 5’-CGGTTTCAGGCACATGACTCT-3’) as the gene of interest, or the reference 16S rRNA gene (F: 5’- GAATTGACGGGGGCCCGCACAAG-3’; R: 5’-AGGGTTGCGCTCGTTG-3’). Thermal protocol was set up as follows: 95°C (10 min), and then 40 cycles at 95°C (10 s) and 60°C (40 s). A control without the template cDNA was run, and all reactions were performed in triplicates. The relative abundance of *bphA* gene expression was obtained by subtracting the threshold cycle (Ct) of the reference gene to the Ct of *bphA* and obtaining ΔCt. The ΔCt of the *bphA* gene in the presence of the inducer was then further compared with the ΔCt in control conditions in the absence of the inducer. The relative expression value of *bphA* was then calculated as $2^{-\Delta\Delta Ct}$. The baseline *bphA* expression in the absence of inducers is therefore represented by the relative expression value of 1 in the resulting graph.

2.9. *In vitro* growth assay on *Arabidopsis* root exudates collected under PCB-18 stress

The ability of strain LB400 to exploit plant root exudates, released under different conditions (differential abundance of flavonoids, control conditions, PCB-18 stress), was evaluated. For root exudate collection, around 30 surface-sterilized seeds of the *Arabidopsis thaliana* L*er* wild-type (WT) genotype, of the null mutant for flavonoid production *tt4* or of the flavonoid overproducers *tt8* and *ttg*, were cultivated on 1/2 MS liquid medium supplemented with 1% sucrose for 11 days. The medium was subsequently removed, the plants were washed twice with 1/2 MS, and then 10 mL fresh 1/2 MS liquid medium was added, supplemented with either 70 µM PCB-18 to induce the stress or acetone as untreated control. Root exudates from three biological replicates were collected and filtered at day 7 after the induction of the stress and stored at 4°C. Strain LB400 was inoculated in 1/2 TSB medium and incubated overnight at 30°C on a shaker (150 rpm). The next day, cells were collected and washed twice in physiological buffer by centrifugation (5 min, 4,000 rpm). Cells were inoculated in triplicates in a 96-well plate at a final concentration of 10^5 cells/mL, with the previously collected root exudates used as culture media. 1/2 MS media containing only 70 mM PCB-18 or acetone were used as negative controls without plant root exudates. The plate was incubated on a rotatory shaker at 30°C for 3 days, and then the bacteria were re-isolated and quantified by plating serial dilutions using the drop plate count method, obtaining the number of CFUs/mL.

2.10. Generation of a fluorescence-labelled LB400 strain

To allow fluorescence microscopy observations of root colonization, LB400 strain was labeled with a constitutive mScarlet-I fluorescent protein via conjugation, adapting the protocol illustrated by Schlechter and Remus-Emsermann (Schlechter and Remus-Emsermann, 2019) for chromosomic insertion. The two strains used were *E. coli* S17-1 as donor strain, containing the Tn5 transposon delivery plasmid pMRE-Tn5-145 (Addgene, USA) expressing mScarlet-I, and a rifampicin-resistant strain LB400 (RifR LB400) as recipient strain. The strains were grown in LB medium as illustrated by the protocol: *E. coli* was used at a concentration of 0.5 OD_{600}, whereas different growth phases were evaluated for the recipient strain, and 0.2 OD_{600} (early-log phase) was selected for its higher efficacy for strain LB400 conjugation. The bacterial cultures were then mixed at 1:5 (donor:recipient) ratios using a concentration of 10^9 cells/mL of donor strain. Bacterial mating was carried out on nitrocellulose filter on an LB plate incubated a 30°C for 1 h. Trans-conjugants were gently resuspended from the filters using PBS and plated on LB containing 100 µg/mL rifampicin and 15 µg/mL gentamicin as selecting antibiotics. The trans-conjugants were re-streaked and single colonies tested for donor cell contamination via PCR using ITS primers (ITS-F: 5’-GTCGTAACAAGGTAGCCGTA-3’; ITS-R: 5’-

GCAAGGCATCCACC-3’). Tn5 insertion in the recipient cells was then confirmed by multiplex PCR as described by Schlechter and Remus-Emsermann (Schlechter and Remus-Emsermann, 2019) using the primers FWD_Tn5_gt (5’-CTGAGTAGGACAAATCCGCCG-3’), REV_Tn5_gt (5’-GCCTCGGCAGAAACGTTGG-3’), FWD_Tn5/7_gt (5’-ATGGTGAGCAAGGGCGAG-3’) and REV_Tn5/7_gt (5’-CAACAGGAGTCCAAGCTCAG-3’). The phylogenetic identity of the transconjugant LB400 strains was then confirmed via Sanger sequencing of the 16S rRNA gene and the expression of mScarlet-I confirmed by fluorescence microscopy.

2.11*. In vitro Arabidopsis* root colonization assay in the presence of PCB-18

Strain LB400–*Arabidopsis* plantlet interaction was evaluated through *in vitro* assay under control conditions and under PCB-18 stress. Sterilized seeds were sown on 1/2 MS agar plates (9 g/L agar type E, Merck, Germany) containing *P. xenovorans* at a concentration of 2 × 10^5 cells/mL, vernalized for 2 days at 4°C in the dark, and then placed vertically in a growth cabinet for 5 days (22°C, 50% humidity, long day conditions with light intensity of 120 μmol/m–150 μmol/m). Mock-inoculated plates (without the bacterial inoculum) were prepared by adding an equal volume of physiological buffer. Five DAG (days after germination), *Arabidopsis* plantlets were transferred onto fresh 1/2 MS plates containing 20 μM PCB-18 (treated) or an equal volume of acetone (untreated). The plates were incubated for a total of 14 days in vertical position in a growth cabinet. Root systems of the growing plantlets (the number varied depending on root dimension) were collected at DAT (days after transfer) 0, meaning the moment of the transfer to new plates, 7 and 14. The roots were placed in preweighed Eppendorf tubes and their fresh weight measured. The roots were then homogenized with a TissueLyser II (QIAGEN, Germany) using the following protocol: two cycles at 20 Hz frequency for 20 s and, after adding 900 μL of physiological buffer, two cycles at 15 Hz for 1 min. The smashed suspension obtained was used as 10^{-1} solution to prepare serial dilutions for the drop plate count method for cell counting on LB plates. After overnight incubation at 30°C, bacterial colonies were counted and the root colonization efficiency expressed as CFUs/mg root fresh weight. At DAT 7, the plates were scanned to measure root length (RL) and the number of secondary roots (NSR) using ImageJ software, and lateral root density (LRD) calculated as NSR/RL. At DAT 14 plant root, shoot and total fresh weights were measured. All measurements were performed on three independent experiments and on at least 12 plants per condition.

2.12. Fluorescence microscopy analysis

Root colonization analysis was performed by investigating the profile of colonization in different sections of the roots of plantlets colonized by strain LB400 labeled with mScarlet protein, developed as previously described. The analysis was performed on *Arabidopsis* roots 7 days after the transfer on acetone or PCB-18-supplemented plates. The analysis was performed at the microscopy platform Unitech NOLIMITS available at the University of Milan. The fluorescence emitted by the mScarlet-tagged bacteria colonizing *Arabidopsis* plantlets was observed at the stereomicroscope (Stereo Nikon SMZ) by scanning the root system with 15× magnification. For the red signal of mScarlet, the excitation and emission wavelengths used were, respectively, 561 nm and 570 nm–620 nm. For an optimized visualization of the mScarlet-labeled strain on the *Arabidopsis* root system, the maximum brightness of all epifluorescence microscopy images was adjusted to value 100 by using ImageJ software.

2.13. *In vitro* early root colonization assay

This assay was adopted to explore the ability of different patterns of flavonoid exudation to affect early events of root adhesion and colonization by strain LB400. Six-day-old *Arabidopsis* plantlets (L*er* WT, *tt4*, *tt8*, and *ttg*) were transferred individually to occupy a well in a 96-well plate with the root system positioned to ensure submersion in 300 µL of 1/2 MS liquid medium containing 10^7 cells/mL of an overnight-grown bacterial culture of strain LB400. After 1 h of incubation at room temperature, the plantlets were briefly washed by dipping three times in physiological buffer, harvested, and pooled (n = 8) into a preweighed Eppendorf tube with a metallic bead. Root weight was measured, and the root systems were homogenized using a TissueLyser as previously specified. The bacterial colonization ability was estimated as CFUs/mg of root by plating serial dilutions on LB plates.

2.14. *In vitro* screening and quantification of strain LB400 plant growth promotion activities

2.14.1. Indoleacetic acid production

Quantification of auxin produced by strain LB400 was performed as previously described (Bric et al., 1991). Briefly, strain LB400 was cultured on a shaker at 30°C for 72 h in LB medium supplemented with 500 µg/mL tryptophan. Optical density at 600 nm of the bacterial culture was measured, and 1 mL was harvested and centrifuged (10 min, 13,000 rpm) to collect the supernatant. 20 µL of orthophosphoric acid and 2 mL of Salkowski reagent (50 mL of 35% perchloric acid, 1 mL of 0.5 M $FeCl_3$ solution) were added to the supernatant and incubated at room temperature for 20 minutes. The color intensity was measured using a spectrophotometer (OD_{530}), and the quantity of auxin produced was obtained via interpolation with a standard curve obtained with indoleacetic acid (IAA) 10 µg/mL–100 µg/mL and normalized with the bacterial culture OD_{600}.

2.14.2. Production of volatile organic compounds that promote plant growth

Quantification of the plant growth promoting potential of volatile organic compounds (VOCs) released by strain LB400 was tested as in Ryu et al. (Ryu et al., 2003). Five-day-old *A. thaliana* seedlings were transferred on one side of a partition Petri dish containing solid 1/2 MS medium, whereas, on the other side, 20 µL of a bacterial suspension in physiological buffer at a concentration of 10^8 cells/mL was spotted on LB medium. The bacterial suspension was obtained from strain LB400 grown overnight in LB medium and subsequently washed twice in physiological buffer by centrifugation (5 min, 4,000 rpm). 20 µL of physiological buffer without bacterium was used as negative control. Petri dishes were closed with Parafilm and incubated for 14 days in controlled conditions in a growth cabinet. Results of the plant growth promotion activity were registered by weighing the plant shoots (n = 6 from 5 independent plates and bacterial replicates).

2.14.3. Production of siderophores

Bacterial production of siderophores was measured as reported by Cherif et al. (Cherif et al., 2015). An overnight culture of strain LB400 was washed in physiological buffer and resuspended at 0.001 OD_{600}. 10 mL of the suspension was spotted on LB plates and incubated for 72 h at 30°C. Once grown, 12 mL of a CAS-blue agar solution was poured onto the LB plates, forming an overlay for the detection of the siderophore production, and incubated overnight at room temperature. Siderophore production was quantifiable as an orange halo around the bacterial colony, due to the change of color of the CAS-blue. Bacterial colony diameter (C) and siderophore halo diameter (S) were measured, and the siderophore production efficiency (SE) of the strain calculated as SE = (S-C)/C, as reported by He et al. (He et al., 2022).

2.14.4. Formation of extracellular polymeric substances

Congo red (CR) assay for planktonic cells (Soo and Wood, 2013) was used to quantify the production of extracellular polymeric substances (EPS) as the amount of CR bound to the cells. Bound CR was calculated as the difference between the initial OD_{490}, quantifying the CR present in the culture media and the remaining CR present in the supernatant after cell centrifugation after 3 and 6 h. The µg of CR bound to the cells was calculated via interpolation with a standard curve obtained with CR concentrations ranging from 0 µg/mL to 40 µg/mL and normalized with the bacterial culture density (OD_{600}). The results were obtained from the average of five independent replicates.

2.14.5. Measurement of ACC deaminase activity

ACC (1-aminocyclopropane-1-carboxylic acid) deaminase activity was measured as the quantity of α-ketobutyrate (α-KB) produced by the hydrolysis of ACC, which is a precursor of ethylene. The protocol illustrated in detail by Belimov et al. (Belimov et al., 2005) was followed and the amount of α-KB was quantified by measuring the OD_{540} and interpolating the value with a standard curve obtained with α-KB 10-100 µg/mL. α-KB concentrations obtained were normalized with the bacterial culture OD_{600}. The results were obtained from the average of five independent replicates.

2.15. Statistical analyses

Statistical analyses were performed using R. Normal data were tested using one-way analysis of variance (ANOVA), followed by Tukey–Kramer *post-hoc* test for multiple comparisons (confidence interval 95%). For non-normal data, Kruskal–Wallis non-parametric test was adopted, followed by Dunn's *post-hoc* test (confidence interval 95%). To compare distributions with small sample size ($n < 30$), Mann–Whitney non-parametric test was used (confidence interval 95%).

3. Results

3.1. Plant flavonoids promote *Paraburkholderia xenovorans* LB400 growth

To test whether the selected model flavonoids affected *P. xenovorans* LB400 growth, the bacterium was grown in liquid culture (1/10 TSB) amended with increasing concentrations (10 µM to 70 µM/100 µM) of flavonoids as pure compounds. Flavonoids are secondary metabolites, and therefore, this assay had the objective to verify their role as potential signaling molecules that could stimulate or hamper bacterial proliferation in a diluted carbon-rich medium. As indicated in the heatmap in Figure 1A, the assayed flavonoids selectively modulated the growth of strain LB400 in a concentration-dependent manner. In the presence of naringin and quercetin, bacterial growth was promoted at all assayed concentrations. Naringenin improved bacterial growth only at the highest concentrations of 50 and 100 µM, whereas for flavanone the growth promotion effect was observed at 20 µM and 100 µM and an inhibition effect was recorded at 50 µM. Flavone promoted strain LB400 proliferation at 20 µM and 50 µM (Figure 1A and Supplementary Figure 1). The flavonoid-mediated improvement in growth parameters is also mirrored in an increased bacterial biomass reached at the stationary phase, after 24 h/30 h of growth (Figure 1B). The higher relative growth increments compared with the control were recorded for 20 µM flavanone and flavone (+9.2% and +15.3%, respectively), for 50 µM naringin and naringenin (+27.8% and +17.3%, respectively) and for 50 µM quercetin (+40.1%) (Figure 1B and Supplementary Figure 2). The growth curves were elaborated to estimate the effect of flavonoid supplements on bacterial maximum growth rate (Supplementary Table 1). Under all assayed concentrations of naringin, strain LB400 exhibited a higher maximum growth rate compared with the control. An increase in this growth feature was observed for 20 µM flavone and 50 µM naringin, as well.

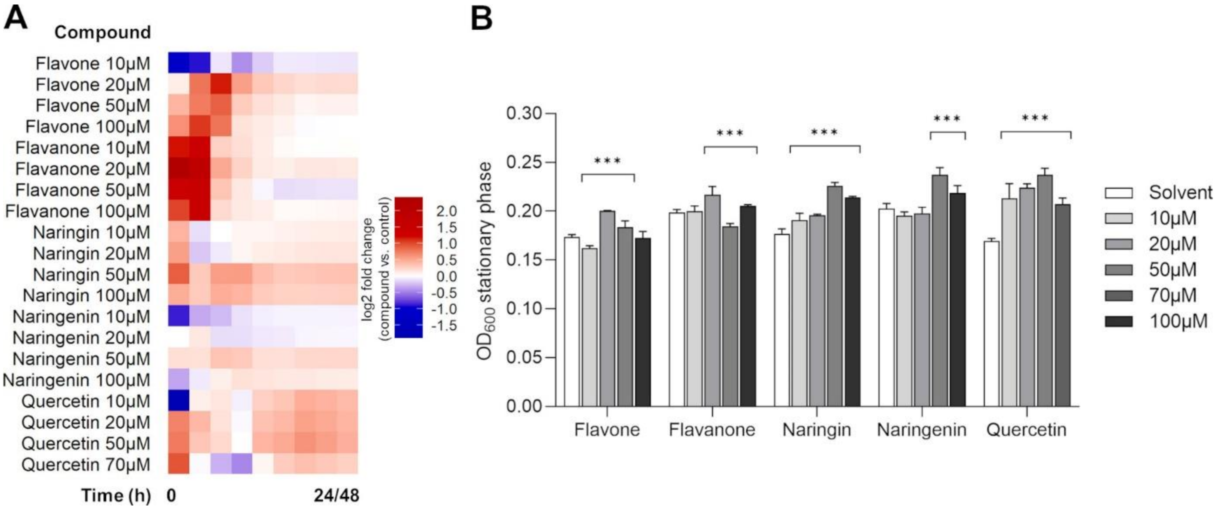

Figure 1. Effects of flavonoid compounds on the growth of *Paraburkholderia xenovorans* LB400. **(A)** Growth modulation activity of flavonoids supplemented as pure chemicals to 1/10 TSB liquid medium. The heatmap shows $\log_2$ fold change of strain LB400 treated with different concentrations (10 μM to 70 μM/100 μM) of the assayed flavonoids versus the respective solvent control at various time points over 24 h (48 h for quercetin only). The corresponding graphical growth curves are depicted in Supplementary Figure 1. **(B)** Bacterial biomass reached the stationary phase at 24 h (30 h for quercetin only) is expressed as OD_{600}. The bars represent the average ± standard deviation of three independent replicates. Statistical analysis was performed using the Mann–Whitney test by comparing the flavonoid concentrations with the respective solvent control (***$p \leq 0.001$).

Interestingly, quercetin supplements decreased bacterial maximum growth rate, but the achievement of a higher bacterial biomass could be attributed to a longer exponential growth phase and a delayed entrance in the stationary phase (Figure 1; Supplementary Table 1 and Supplementary Figure 1). Given these findings, it was inquired if flavonoids could be exploited as carbon sources since *bph*-encoded dioxygenases originally evolved to catabolize plant secondary metabolites (Singer et al., 2003). By cultivating the strain in mineral medium supplemented by flavonoids as a unique carbon source, we observed that only naringin and naringenin could support bacterial growth (Supplementary Figure 3).

3.2. Flavonoids affect *Paraburkholderia xenovorans* LB400 chemoattraction, motility, and biofilm formation ability

Chemotaxis and cell motility are considered essential features in the early phases of microbial colonization, leading to primary root surface attachment (Allard-Massicotte et al., 2016; Manner and Fallarero, 2018). Hence, we verified if flavonoids could modulate strain LB400 chemotactic responses, potentially influencing its root colonization ability. *In vitro* chemotaxis assay demonstrated that strain LB400 was attracted by 50 mM and 100 mM naringenin and by 50 mM quercetin (Figure 2A), whereas no statistically relevant effects were observed for the other flavonoid molecules assayed (data not shown). To evaluate if flavonoids regulate flagellar movement, an *in vitro* swimming assay was performed. While treatment with 50 μM and 100 μM naringin significantly increased the bacterial swimming halo, both 50 μM and 100 µM flavone and quercetin decreased strain LB400 swimming ability (Figure 2B). Biofilm formation is a fundamental feature for rhizospheric bacteria to ensure a stable attachment to the root surface (Zboralski and Filion, 2020): 10 µM and 100 µM naringin and naringenin promoted the ability of the bacterium to adhere and form a biofilm on the substrate (Figures 2C, D). These results suggest that the assayed flavonoids elicited different chemotactic motility and biofilm formation responses in *P. xenovorans* LB400, potentially influencing its recruitment by the plant through root exudation.

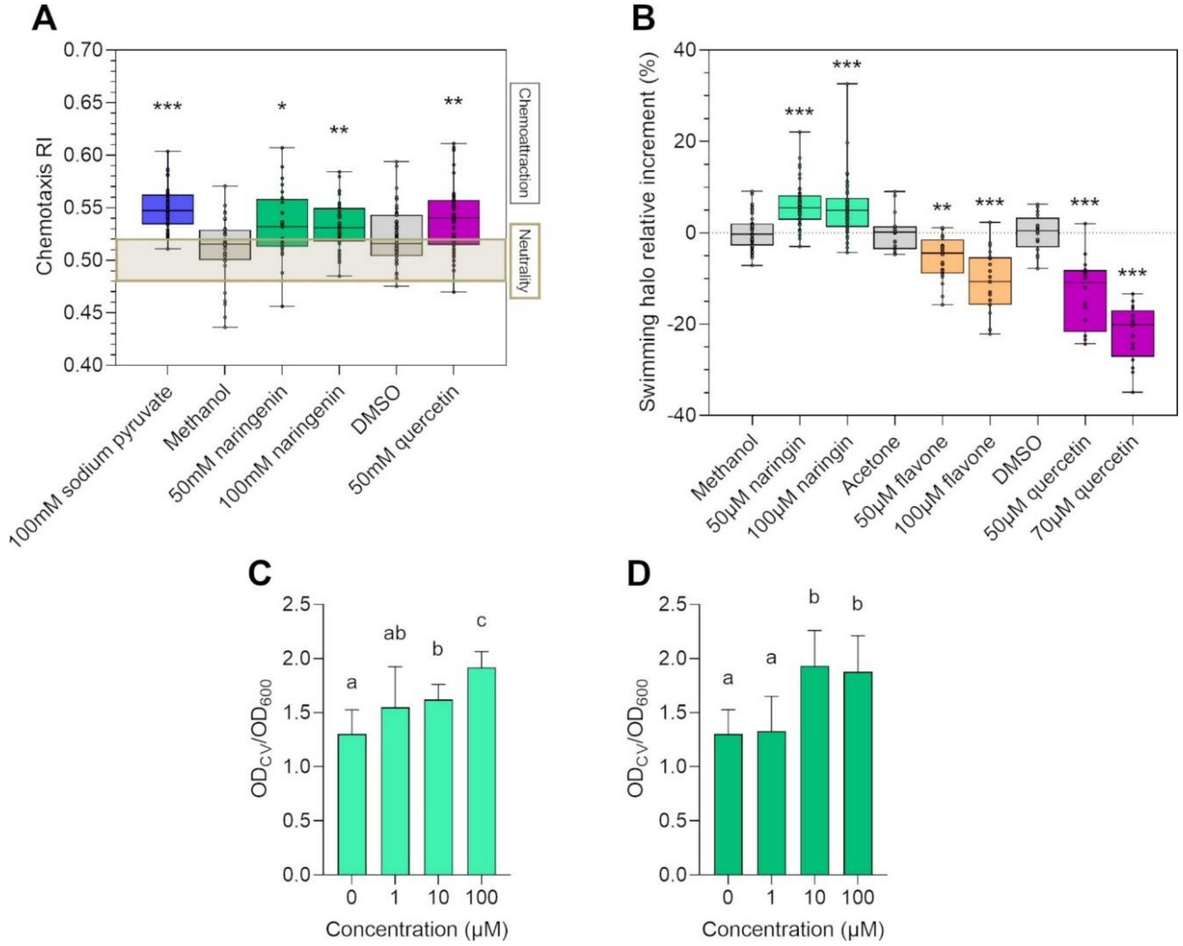

Figure 2. Flavonoids affect *P. xenovorans* LB400 chemotaxis, motility, and biofilm formation abilities. **(A)** Chemotaxis of strain LB400 toward flavonoids on gradient plates after 72 h of growth at 30°C is quantified by calculating the chemotaxis response index (RI). RI higher than 0.52 indicates chemoattraction, RI lower than 0.48 indicates repulsion, whereas $0.48 < RI < 0.52$ indicates neutral behavior. Statistical analysis was carried out on at least three independent experiments using ANOVA followed by Tukey–Kramer *post-hoc* test (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$). **(B)** Percentage of relative increase of the swimming halo diameter of strain LB400 was calculated as described by Wang et al. (Wang et al., 2019). Statistical analysis was carried out on at least three independent experiments using Dunn's *post-hoc* test (*$p \leq 0.01$; ***$p \leq 0.001$). The graphs **(C)** and **(D)** report the relative biofilm formation ability of strain LB400 in the presence of the flavonoids naringin **(C)** and naringenin **(D)** expressed as the ratio between the OD_{600} value for crystal violet staining and the OD_{600} indicating bacterial growth, as described in the material and methods section. Error bars represent the standard deviation of three independent experiments. Statistical analysis was performed using Dunn's *post-hoc* test, and letters indicate statistically different groups ($p \leq 0.05$).

3.3. Flavonoids induce the expression of the *bphA* gene in strain LB400

Flavonoids were demonstrated to influence the PCB degradative potential of soil microorganisms, acting as inducers or co-metabolites of the catabolic operon *bph* (Toussaint et al., 2012; Zubrova et al., 2021). The ability of individual flavonoid molecules to induce the transcription of the degradative machinery in strain LB400 was assessed by monitoring *bphA* gene relative expression by RT-qPCR. The relative induction rates indicated that two of the assayed flavonoids, namely, flavone and quercetin, were able to increase the levels of *bphA* transcripts after 8 h of incubation, similarly to biphenyl, the model inducer of the *bph* operon (Figure 3). These observations highlight flavonoid ability to activate the expression of PCB degradative traits in *P. xenovorans* LB400.

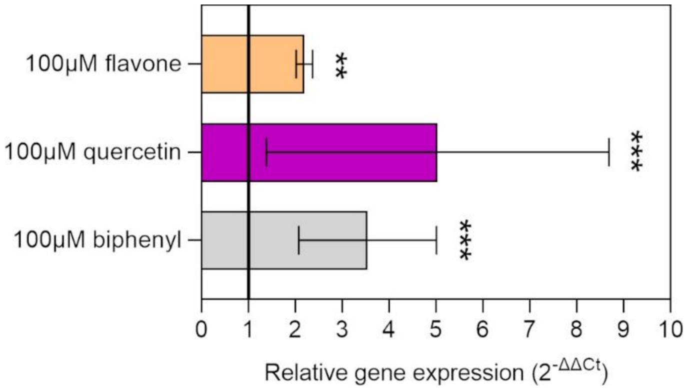

Figure 3. Relative gene expression of the *bphA* gene in strain LB400 exposed to flavonoids. The relative gene expression of the *bphA* gene was quantified via RT-qPCR, using the 16S rRNA gene as a housekeeping gene. The results of the relative expression of *bphA* transcripts are expressed as ΔΔCt, indicating the average fold change over the non-induced control. The black line (relative gene expression value = 1) represents the baseline of gene expression in the non-induced control. Biphenyl was used as positive control. Error bars represent the standard deviation of three independent experiments. Statistical analysis was performed using Dunn's *post-hoc* test and asterisks indicate significant differences from the control (**$p \leq 0.01$, ***$p \leq 0.001$).

3.4. Differential exudation of flavonoids affects *P. xenovorans* LB400 early adhesion on *Arabidopsis* roots

Given the results obtained with flavonoids as pure chemicals, we verified if differences in the amount and chemistry of flavonoids in the complex natural blend of exuded compounds released by plant roots could affect strain LB400 growth and root adhesion efficiency. For this purpose, the *Arabidopsis* mutant lines *tt4*, *tt8*, and *ttg*, differentially altered in flavonoid biosynthesis and exudation (Narasimhan et al., 2003), were adopted. To investigate the effect of root exudates on strain LB400 root adhesion ability, *Arabidopsis* plantlets of the different genetic backgrounds were exposed for 1 h to the bacterial culture before proceeding with the re-isolation of cells adhering on the rhizoplane, aiming to focus on the role of flavonoids in the early events of bacterial colonization. It was observed that in 1 h, strain LB400 colonized both WT and *tt4* roots at a similar rate (5.57 × 10^3 and 5.42 × 10^3 CFUs/mg of root fresh weight, respectively) whereas the bacterial density increased in the *tt8* line, which accumulates flavonoid aglycones (1.09 × 10^4 CFUs/mg of root fresh weight) (Figure 4A). On the contrary, roots of *ttg*, which overproduces flavonoid conjugates, were less colonized compared with the other lines (1.25 × 10^3 CFUs/mg of fresh root weight) (Figure 4A). These results suggest that two distinct flavonoid-overexpressing fingerprints differently affect strain LB400 colonization ability in the early events of root adhesion.

3.5. Flavonoid exudation affected the bacterial ability to exploit plant root exudates as nutrient sources

We tested whether the complex blend of root exudates produced by the mutants could sustain bacterial growth as a nutrient source and eventually provide signaling molecules for enhanced bacterial proliferation. WT and *ttg* mutant plant exudates showed a similar ability to support strain LB400 growth (Figure 4B, light blue bars). On the other hand, the exudates released by *tt4* and *tt8* relevantly contributed to enhance bacterial growth, allowing the formation of a higher bacterial biomass (4.66 × 10^7 CFUs/mL and 2.02 × 10^7 CFUs/mL for *tt4* and *tt8* lines, respectively).

Considering flavonoid involvement in the expression of the *bphA* gene (Figure 3), root exudates were also collected from plantlets subjected to PCB-18 stress, to assess if strain LB400 could be a target for a potential 'cry-for-help' strategy. The exudates collected from the WT and the different *Arabidopsis* mutants cultivated in presence of 70 µM PCB-18 showed the capacity to sustain strain bacterial growth (Figure 4B, red bars). Strain LB400 growth was unaffected by the presence of stress-triggered exudates released by WT and *ttg*, showing no major differences in using the root exudates released by these lines in the presence and absence of PCB stress. On the other hand, strain LB400 growth dropped of one order of magnitude with *tt4* exudates released upon PCB-18 stress (5.49 × 10^6 CFUs/mL) compared with the control conditions, and similarly, a decrease was also observed in the bacterial ability to exploit *tt8* exudates released under stress compared with the control.

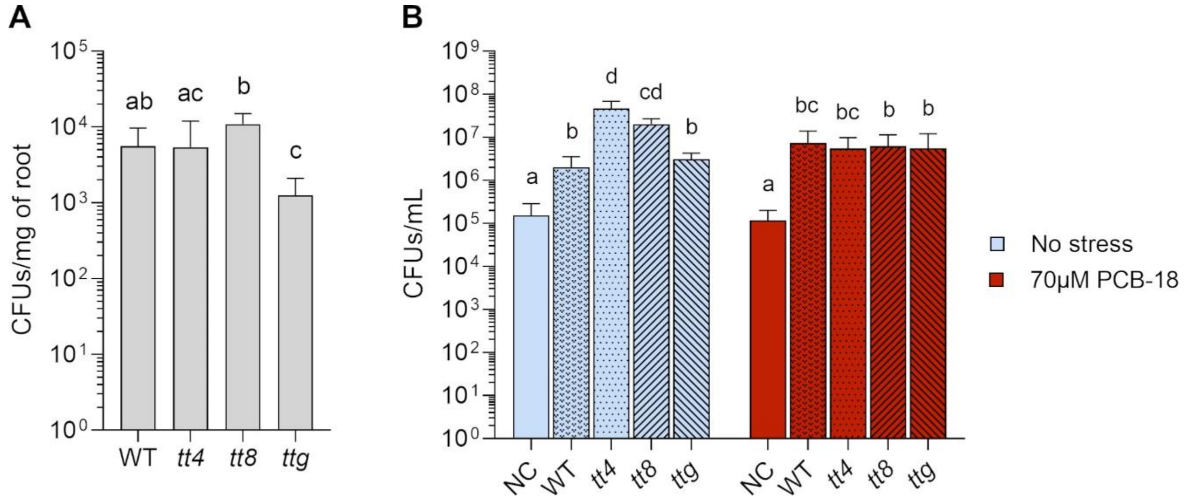

Figure 4. Root exudates from *Arabidopsis* mutants with distinct flavonoids exudation pattern affected *P. xenovorans* LB400 early colonization and were diversely exploited by the bacterium for growth. **(A)** Early colonization was measured via re-isolation of strain LB400 from *Arabidopsis* roots after 1 h of incubation. The graph reported the average and standard deviation values of three independent replicates for all the assayed *Arabidopsis* genotypes. Statistical analysis was performed using Dunn's *post-hoc* test, and letters indicate statistically different groups ($p \leq 0.05$). **(B)** Strain LB400 growth on root exudates collected from WT *Arabidopsis* and flavonoid metabolic mutant lines at day 7 of treatment under control conditions (plants exposed to acetone, the solvent used to dissolve PCB-18) or PCB stress induced by the treatment with 70 µM PCB-18. NC indicates 1/2 MS medium containing only acetone or 70 µM PCB-18, without plants, and was used as axenic control. Strain LB400 was inoculated at a concentration of 5×10^4 cells/mL and grown for 3 days before re-isolation. The bars represent the average and standard deviation values of three independent experiments. Statistical analysis was performed using Dunn's *post-hoc* test and letters indicate statistically different groups ($p \leq 0.05$).

3.6. *P. xenovorans* LB400-mediated plant growth promotion under control conditions and under PCB stress showed a similar pattern in *Arabidopsis* lines affected in flavonoid biosynthesis

The role of flavonoids in strain LB400–plant interaction was investigated *in planta* by taking advantage of a well-established microcosm system set up to assess the interaction between beneficial microbes and *Arabidopsis* plantlets (de Zélicourt et al., 2018; Rolli et al., 2022). In addition, a specific in-plate assay was developed in this study to simulate PCB-induced phytotoxicity in *Arabidopsis*. It was observed that the exposition to 20 µM PCB-18 dramatically affected plant growth and development and prompted a series of injuries attributable to PCB stress (Supplementary Figure 4). Indeed, when grown for 14 days in the presence of PCB-18, all the assayed *Arabidopsis* mutant lines reported a significant reduction in root fresh weight that was coupled also to a decrease in total plant biomass, depending on the genotypes (Figures 5A–D and Supplementary Figure 5). By analyzing root architecture, only slight differences were observed in terms of primary root length and number of secondary roots, suggesting that PCB-18 stress mainly affected root biomass rather than its morpho-phenotypic traits (Supplementary Figure 6). This assay was adopted to compare strain LB400-mediated growth promotion in the WT plants with *tt4*, *tt8*, and *ttg* mutant lines under control conditions and in the presence of PCB stress. Colonization with strain LB400 promoted plant growth by increasing plant biomass of both shoots and roots in WT and all mutant plant lines grown under control conditions (Figures 5A–D and Supplementary Figure 5). In particular, in the root system, strain LB400 induced the formation of a longer primary root, whereas the number of secondary roots showed a variation that was genotype-related (Supplementary Figure 6). These findings underlined that strain LB400 possesses plant growth promoting (PGP) traits and that the promotion activity was not altered in *Arabidopsis* backgrounds that are featured by null synthesis or overproduction of flavonoids.

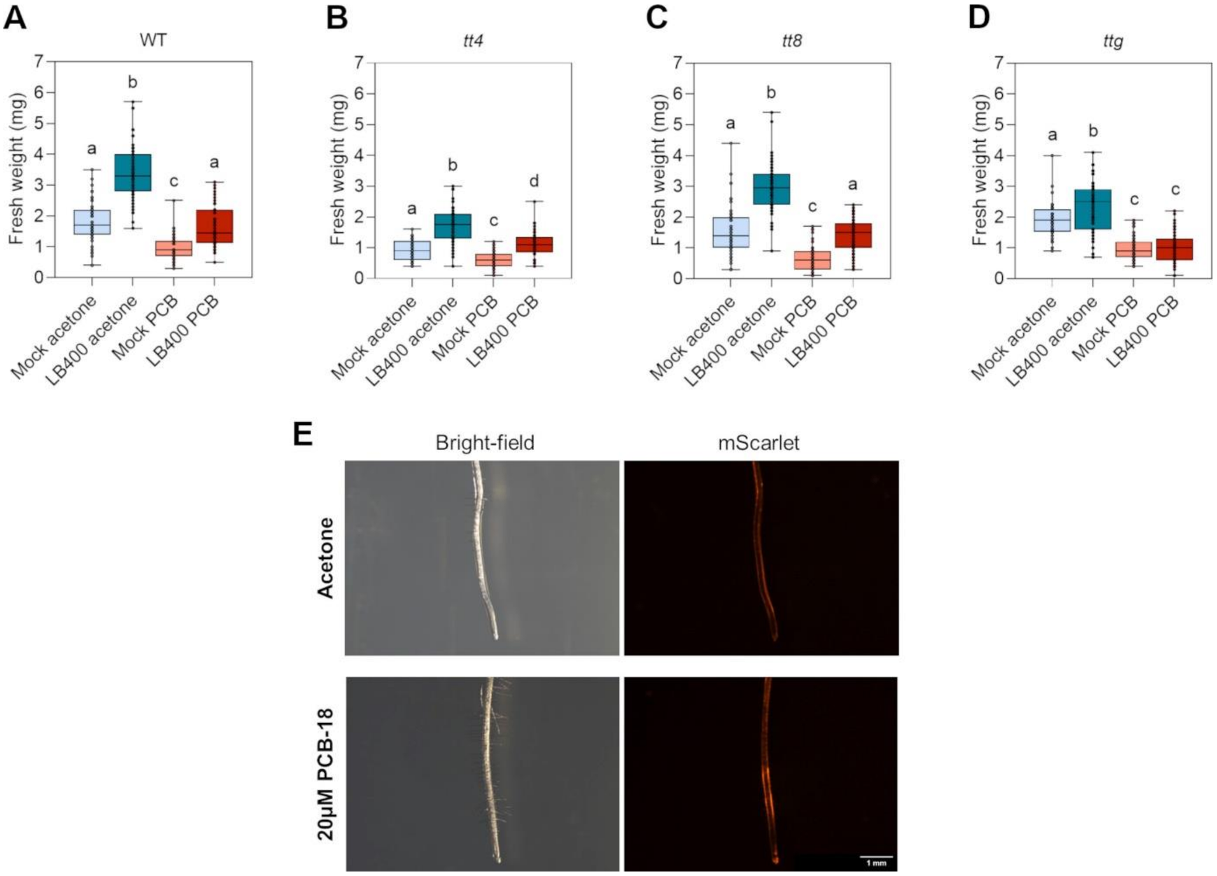

Figure 5. *P. xenovorans* LB400 growth promotion, colonization efficiency, and root adhesion profile were not differentially affected in *Arabidopsis* plants with diverse profiles of flavonoid biosynthesis and exudation. **(A–C)**, and **(D)** Root fresh biomass of sterile and strain LB400-colonized *Arabidopsis* plantlets grown under control conditions (acetone) and challenged by 20 µM PCB-18 treatment in WT **(A)**, *tt4* **(B)**, *tt8* **(C)**, and *ttg* **(D)** lines, respectively. The boxplots represent data from three independent experiments. Letters indicate statistically different groups (Dunn's *post-hoc* test with $p \leq 0.05$). **(E)** Fluorescence microscopy analysis of the mScarlet-labeled LB400 strain colonization pattern on the root system of WT plantlets 7 days after PCB treatment or in control conditions (acetone).

The PGP potential of LB400 was further investigated through quantitative *in vitro* tests, demonstrating that *P. xenovorans* LB400 possesses a large portfolio of PGP traits, including auxin synthesis (31.4 µg IAA/OD_{600}), ACC-deaminase activity (32.7 µg α-KB/OD_{600}), EPS production (2.08 µg bound Congo red/OD_{600}), siderophore release, and production of VOCs (Supplementary Figure 7). These beneficial features, linked with the PCB degradative activity of the strain, may explain strain LB400 ability to sustain plant growth both under control conditions and under PCB-18 stress. Remarkably, LB400 demonstrated to mitigate the toxicity stress in plants cultivated in the presence of 20 µM PCB-18 for WT and the *tt4* and *tt8* mutants whereas only the *ttg* mutant did not show the beneficial effect provided by the bacterial inoculant (Figures 5A–D and Supplementary Figure 5). No major changes in *Arabidopsis* colonization pattern were observed either by re-isolation (Supplementary Figure 8) and by fluorescence microscopy analysis (Figure 5E and Supplementary Figure 9 and 10).

4. Discussion

Root chemistry alteration by the components of the holobiont, either the host plant or the associated microbiota, is emerging as a valuable strategy for the recruitment and the preservation of the microbial functionalities that result in the consolidation of the holobiont fitness under stress, guaranteeing its useful services like rhizoremediation (Voges et al., 2019; Korenblum et al., 2020). The pillar of this process is the structural affinity between the contaminants and plant secondary metabolites that drive their degradation through the same enzymatic machinery of the microbial peripheral pathway. In this vision, the ecological services provided by the *bph* operon are hijacked from the degradation of biphenyl-like PSMs to also allow PCB catabolism, conferring a selective advantage to *bph*-equipped bacteria in polluted rhizosphere soil. Therefore, bioremediation relies on evolutionary-shaped processes, comprising the enzymatic versatility of microbial metabolism and the inter-kingdom trophic interactions among plants and microbes.

Among others, flavonoids constitute a broad group of specialized secondary metabolites (Vives-Peris et al., 2020), consistently abundant in the root exudates of several plant species including legumes (Leoni et al., 2021) and trees like *Acer saccharum*, *Alnus rugosa*, *Fagus grandifolia*, *Picea abies*, *Pinus strobus*, and *Quercus rubra* where catechin, naringenin, and taxifolin represented the most abundant exudates (Zwetsloot et al., 2018). Recent evidence supports the vision of a broader role for flavonoids in plant–microbe dynamics that could allow the development of beneficial relationships also in non-nitrogen-fixing crops and with non-rhizobial bacterial species (Yu et al., 2020; Kudjordjie et al., 2021; Yu et al., 2021; He et al., 2022). In this framework, our aim was to investigate whether flavonoids could affect the rhizocompetence and the degradation potential of the model PCB-degrader *P. xenovorans* LB400, thus contributing to the recruitment of a beneficial strain able to alleviate plants from the phytotoxic damages caused by these recalcitrant compounds (Rolli et al., 2021).

Our findings indicate that the exposure of strain LB400 to micromolar concentrations of plant flavonoids, supplied as pure chemicals, can selectively modulate the growth of the bacterium, affecting its proliferation, biomass yield, and maximum growth rate, both in relation to the specific flavonoid molecule type and its concentration. Similarly, some triterpene compounds like thalianin and arabidin previously demonstrated to specifically affect members of *Arabidopsis* root microbiota by promoting or inhibiting their growth and by serving as carbon sources (Huang et al., 2019). This flavonoid-triggered effect could drive relevant consequences in the proliferation ability of degrading bacteria in the rhizosphere of plants growing in PCB contaminated environments. Although considered the largest biome on earth, soil is an oligotrophic environment subjected to fluctuations in nutrient and water availability (Hartmann and Six, 2023). Endowed with a 15-carbon skeleton, flavonoids represent valuable nutrient sources for those rhizosphere microorganisms harboring appropriate catabolic enzymes, like *Rhizobium*, *Bradyrhizobium*, *Acinetobacter*, and *Pseudomonas* (Rao and Cooper, 1995; Pillai and Swarup, 2002; Arunachalam et al., 2003). *Pseudomonas putida* PLM2 showed to aerobically catabolize quercetin through oxidation and/or reduction reactions that led to the formation of catechol (Pillai and Swarup, 2002), and *P. xenovorans* LB400 itself was able to use morusin, morusinol, and kuwanon C, flavones produced by fine roots of mulberry, as sole carbon sources (Leigh et al., 2002). Therefore, the ability to exploit the flavonoid resource may represent an important selective value in plant–microbe interactions. It has been shown that the chemical variation of *Avena barbata* root exudates along the plant lifespan is coupled to the microbial succession of different phyla that encode for uptake systems and catabolic enzymes useful to exploit exudates as nutrient sources (Zhalnina et al., 2018). Indeed, micromolar supply of quercetin to an agricultural soil differentially affected specific microbial taxa, increasing up to 76% the relative abundance of *Proteobacteria* (Schütz et al., 2021). In agreement with our findings on flavonoid-triggered growth promotion in *P. xenovorans* strain LB400, *Bradyrhizobium* strain ORS285 can metabolize naringenin into a hydroxylated and methoxylated derivative that did not act as a Nod inducer. Instead, it stimulated bacterial growth presumably by regulating the glycerol and fatty acid metabolism (Nouwen et al., 2019). These results suggest that the assayed flavonoid compounds, being used as carbon sources or proliferation-stimulating agents, may contribute to fuel microbial growth and to increase *P. xenovorans* LB400 proliferation and persistence in the rhizosphere. Furthermore, our results demonstrated that naringin and naringenin enhance swimming motility, chemotaxis, and biofilm formation in strain LB400, traits that are often enriched in the genomes of plant-associated bacteria compared with soil-inhabiting ones (Levy et al., 2018) and that are believed to be involved in rhizosphere recruitment and colonization (Santoyo et al., 2021). Our observations support the evidence that root-secreted flavonoids constitute a carbon-rich reservoir that might induce priming mechanisms in soil microorganisms (Zwetsloot et al., 2018). This function is not necessarily related to the use of flavonoids as primary carbon sources but also to their role as intermediate substrates, signaling molecules, or attractants that can stimulate various metabolic activities, useful to support plant growth and survival in harsh conditions. Indeed, most of the bacteria isolated from the rhizosphere of maize, mustard cabbage, and lettuce grew similarly well on both host and non-host exudates, indicating a large metabolic versatility to exploit plant released compounds (Dhungana et al., 2023).

Studying the temporal dynamics of root exudation, McLaughlin and coauthors demonstrated that in few hours the main determinants of three distinct plant species exudate patterns were released by the roots and their concentration then increased with time (McLaughlin et al., 2023). This evidence motivated the use in this work of three *Arabidopsis* lines that are affected in flavonoid biosynthesis to verify whether strain LB400 was differentially recruited in the rhizoplane. *Arabidopsis thaliana* stands as a proficient model for holobiont dynamics under abiotic stresses (Poupin et al., 2023) and offers a large portfolio of well-characterized genetic and metabolic resources, especially for flavonoid biosynthesis, regulation, and exudation (Nakayama et al., 2019). It was observed that in 1 h, the WT and the null-producer *tt4* line were similarly colonized, *tt8* roots were colonized at higher efficiency whereas *ttg* roots were far less colonized. In the assayed mutant lines, flavonoids are the root exudates mainly affected and therefore they vary largely in quantity and composition. Nevertheless, in the complex blend of exudates released by these mutant lines, also other molecules of the phenylpropanoid pathway result to be differentially exuded (Narasimhan et al., 2003), thus making it intricate to distinguish an effect mediated undoubtedly by flavonoids. The scenario is even more complex, considering that different flavonoids have been showed to exert contrasting functions in the recruitment of microbial taxa in the rhizosphere (Wang et al., 2022). Indeed, naringenin strongly improved *Aeromonas* sp. chemotaxis and motility, potentially explaining the higher colonization rate in *pap1-D* mutant that overaccumulates anthocyanins and flavonols (He et al., 2022). On the other hand, daidzein, that possesses antimicrobial activity, decreased the α-diversity of the microbial community in soybean rhizosphere, suggesting a potential role as chemorepellent (Okutani et al., 2020). Tadra-Sfeir and colleagues reported a transcriptomic study on the diazotrophic bacterium *Herbaspirillum seropedicae*, highlighting that naringenin repressed bacterial motility while enhancing early root colonization and activating the expression of genes related to the degradation of aromatic compounds (Tadra-Sfeir et al., 2015). Similarly, we observed that different flavonoid molecules can exert specific effects: while quercetin and flavone did not contribute to bacterial swimming motility, they acted as inducers of the PCB degradative pathway. Some of the observed effects are also concentration-dependent, as in the case of flavone-mediated promotion of strain LB400 growth. No precise information is available about flavonoid concentration in the rhizosphere, also considering the impact of abiotic processes like adsorption to soil particles or biological degradation by the soil microbiota (Shaw et al., 2006; Del Valle et al., 2020). Therefore, there is the possibility that flavonoid concentrations used for the *in vitro* experimental setup are higher than those available in the rhizosphere. In axenic systems for root exudate collection, for instance, flavonoids were estimated in micromolar ranges (Toussaint et al., 2012), suggesting that the concentrations used in the present study can indeed affect bacterial physiology and plant–bacteria interactions mimicking the real conditions that could be found in soil.

Our interest in testing the selected *Arabidopsis* lines was also coupled to their previous exploitation in a rhizo-engineering strategy for PCB removal by the degrading strain *Pseudomonas putida* PML2 (Narasimhan et al., 2003). Importantly, all the different blends of exudates released by the *Arabidopsis* lines used in this study could successfully support *P. xenovorans* growth, independently from their over- or underproduction of flavonoids given by the genetic mutation. Presumably, some of the aromatic organic acids that are overaccumulated in the absence of flavonoids in *tt4* exudation, like cinnamic acid and indole-3-acetic acid (Narasimhan et al., 2003), which were previously highlighted as preferentially consumed by rhizospheric microorganisms (Zhalnina et al., 2018), could contribute to the enhanced ability of sustaining strain LB400 growth under control conditions. The changes in the exudation pattern induced by the 'cry-for-help' in the presence of PCB stress are still unknown. Here, we observed that no major changes occurred in strain LB400 growth in the presence of exudates from WT plants cultivated with or without PCB, thus excluding a prompt 'cry-for-help' effect, as already observed for *Aeromonas* sp. H1 under dehydration stress (He et al., 2022). In any case, the growth of strain LB400 on exudates released under PCB-18 stress decreased for *tt4* and *tt8* mutants, exhibiting absent (*tt4*) or modified flavonoid biosynthesis (*tt8*), thus potentially supporting a role of flavonoids and/or their aglycones in nurturing degrading bacteria. Bacterial growth was not affected in the presence of the flavonoid-hyperproducing *ttg* mutant exudates, independently from the treatment. This mutant line releases conjugated flavonoids, which are more hydrophilic, mobile, and bioavailable molecules. Therefore, they are considered short-lived forms that can be potentially rapidly degraded by plant and microbial glucosidases (Hartwig et al., 1991), leading to formation of byproducts that are still poorly characterized for their effect on soil microbiota and influence in the rhizosphere dynamics (Shaw et al., 2006).

Based on previous research on PCB-18 toxicity in *Arabidopsis* (Bao et al., 2013), an *in vitro* assay was developed that simulated the phytotoxic effects induced by PCBs that led to reduced plant growth and development, with the aim to assess *P. xenovorans* LB400–plant interaction under PCB stress. As a model strain for PCB removal (Chain et al., 2006), strain LB400 was previously used mainly in bioaugmentation approaches for contaminated sediments (Tehrani et al., 2014; Le et al., 2015; Bako et al., 2022), whereas its association with plant roots is still largely overlooked. Recently, *Paraburkholderia* phylotypes, matching with *P. xenovorans* LB400, were retrieved in forest soil with sugar maple, red pine, and black locust trees as the dominant p-hydroxybenzoic acid responder, supporting the notion of the presence of a specialized metabolism to degrade phenolics upon priming (Wilhelm et al., 2021). Therefore, the present study contributes to widen the knowledge on *P. xenovorans* LB400 beneficial contribution to plant growth showing that, in addition to its well-documented PCB degradation ability (Ponce et al., 2011), this bacterial strain is also endowed with a large portfolio of PGP traits, potentially contributing to further boost plant fitness under stress (Marasco et al., 2012; Vigani et al., 2019). Strain LB400 administration to the plant contributed to improve both shoot and root biomass, with the root weight being the growth parameter that was mostly affected by PCB-18 stress in the developed assay. *P. xenovorans* LB400 exerted beneficial effects both under control conditions and under stress, in WT and *Arabidopsis* mutants with null (*tt4*) or overexpressing (*tt8*) flavonoid metabolism, whereas it did not show to improve the growth of the overproducer *ttg* mutant line when growing under PCB stress. Considering also that the degrading strain *Pseudomonas putida* PML2 performed the most remarkable PCB depletion (more than 90%) in the WT *Arabidopsis* roots rather than in the mutants (Narasimhan et al., 2003), these observations may suggest that the bacterial-driven plant growth promotion under control conditions and under PCB stress could be achieved through a flavonoid-independent mechanism.

Our results delineate a scenario in which these secondary metabolites play a pivotal role in facilitating the early bacterial recruitment in the rhizoplane. Indeed, once the bacterium is settled on the root surface, strain LB400 colonization efficiency (at 7 and 14 DAT) and adhesion pattern (at 7 DAT) did not differ among the investigated *Arabidopsis* genotypes. A similar trend was also observed for *P. putida* PML2, which colonized with a similar efficiency the three *Arabidopsis* lines that produce flavonoids, although the *tt4* line presented less bacterial cells (Narasimhan et al., 2003).

Remarkably, a fluorescence-labeled version of strain LB400 was developed within this study, contributing to specifically visualize the colonization profile on the root system. It was observed that strain LB400 established homogeneously on the primary roots from the differentiation zone to the apical region, whereas secondary roots were not colonized 7 DAT. Nevertheless, it was previously observed that *Bacillus subtilis* established early in the root elongation zone of *Arabidopsis*, a potential hotspot for exudation, but this event preceded the colonization over the entire root length (Knights et al., 2021). Interestingly, this colonization pattern complied with the bacterial-mediated remodeling of root architecture that mainly affected primary root length and biomass. Often, bacterial-triggered remodeling of root architecture is mediated by the alteration of plant hormone homeostasis. The PGP strain *Achromobacter* sp. 5B1, for instance, influenced the growth and branching pattern of *Arabidopsis* roots through a redistribution of auxins in the primary roots, with the result of improving salt resistance (Jiménez-Vázquez et al., 2020). Strain LB400 was shown to be able to produce IAA and VOCs and express the ACC-deaminase enzyme, having thus the potential to interfere with the plant hormone homeostasis.

In addition to PGP traits, bacterial degradative ability is an essential beneficial factor in plant–microbe interaction in contaminated soils. *P. xenovorans* LB400 is a well-known PCB degrader, and our study contributes to further explore its degradation potential by demonstrating the ability of flavone and quercetin to activate the transcription of the *bph* operon at similar levels as biphenyl. Although possessing genetic differences (Hirose et al., 2019), isoflavone and quercetin, among other flavonoids, acted as co-metabolites by inducing the *bph* operon expression in the degrading strains *Rhodococcus erythropolis* U23A and *Pseudomonas alcaliphila* JAB1 (Pham et al., 2015; Zubrova et al., 2021).

To summarize, our results are in line with recent evidence claiming a broader role for flavonoids in the recruitment and assembly of the plant microbiota with regard to non-rhizobial strains (He et al., 2022). This role includes the early sustainment of bacteria in the rhizoplane in two ways: (i) recruitment by stimulating chemotaxis and motility processes, resulting in root attachment through biofilm formation and (ii) induction of proliferation of the bacteria established on the root by their action as nutrients or growth stimulator signals. Furthermore, flavonoids improve strain LB400 degradative functionalities for PCB removal, with the potential effect of reducing soil phytotoxicity. These aspects are crucial in rhizo-remediation strategies, whose success rely in the synergistic activities performed by the host plant and the degrading microbiota. Nevertheless, our results specifically highlight that flavonoids are important to initiate *Arabidopsis* root colonization, but once a baseline colonizing community is established, at longer time intervals, presumably other mechanisms and/or other plant exuded metabolites occur in plant–bacteria interaction to sustain bacterial growth and colonization pattern, both under control conditions and PCB stress.

More research is needed to disclose the differential composition of root exudation under PCB stress and identify the key secondary metabolites that are released by the plant following the ‘cry-for-help’. Although single-strain studies in axenic plant systems contribute to determine the causative mechanisms, exploiting PCB-degrading synthetic communities would be useful to address the complexity of interactions among bacteria and host plants in contaminated environments. With this knowledge, it would be possible to mechanistically demonstrate the impact of stress-triggered root exudation in recruitment, colonization, and functional features of degrading bacteria, with the ultimate goal to exploit them to sustain rhizoremediation services in contaminated soils.

In the perspective of a field approach, the use of plant secondary metabolites as specific biostimulants to shape the microbiome toward desired services is an attractive strategy for rhizoremediation (Koprivova and Kopriva, 2022). Such approach was applied to facilitate the mineralization of organic phosphorus, showing that soil amendments with quercetin, naringenin, or luteolin increased the relative abundance of *Micrococcaceae* and *Nocardioidaceae*, bacterial families that positively correlated with enhanced alkaline phosphatase activity, in rhizospheric soil (Wang et al., 2023). A major limitation to this kind of approach is the reduced lifetime of flavonoids in soil, estimated to decrease up to 63%, due to sorption or other reactions with dissolved organic carbon (Del Valle et al., 2020). On the other side, foliar application of rutin significantly enhanced *Amaranthus hypochondriacus* phytoremediation efficiency to remove cadmium by favoring its immobilization in the cell wall rather than in the vacuole (Kang et al., 2022).

The other crucial players in rhizoremediation are plants: so far, species showing resilience, high biomass production, resistance to pollutants’ toxic effects, and easy-to-grow ability have been identified (Kafle et al., 2022). This is the case of *Festuca arundinacea* that was broadly applied for land reclamation of soils exhibiting a diverse array of pollution profiles, including hydrocarbons, heavy metals (Khashij et al., 2018), and PCBs as well (Terzaghi et al., 2019). Improving this knowledge would allow, for instance, the selection of plants exhibiting specific host phenotypes, including an enriched exudation of specific metabolites, to select for more efficient PCB degrading strains through host-mediated microbiome engineering.

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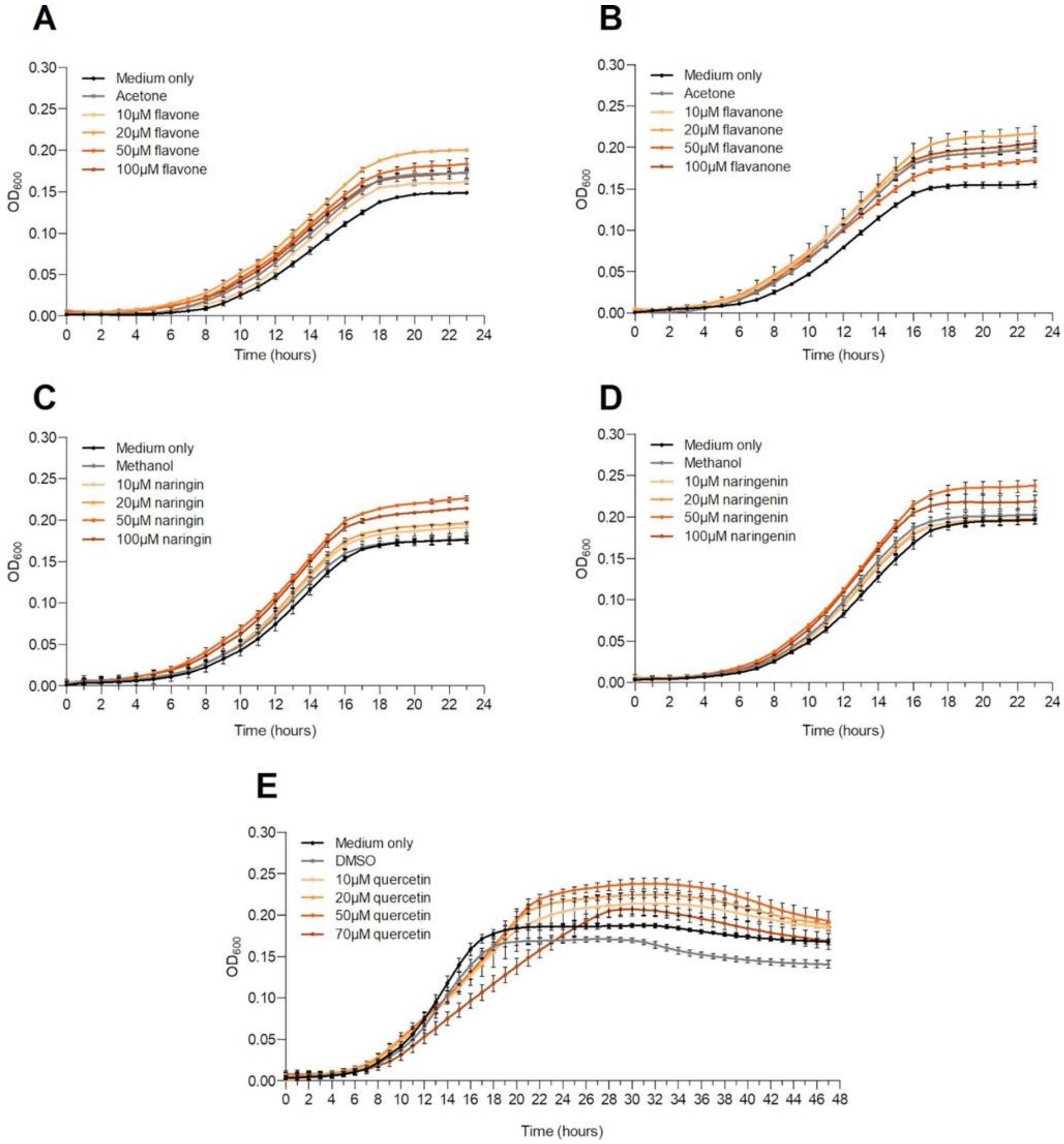
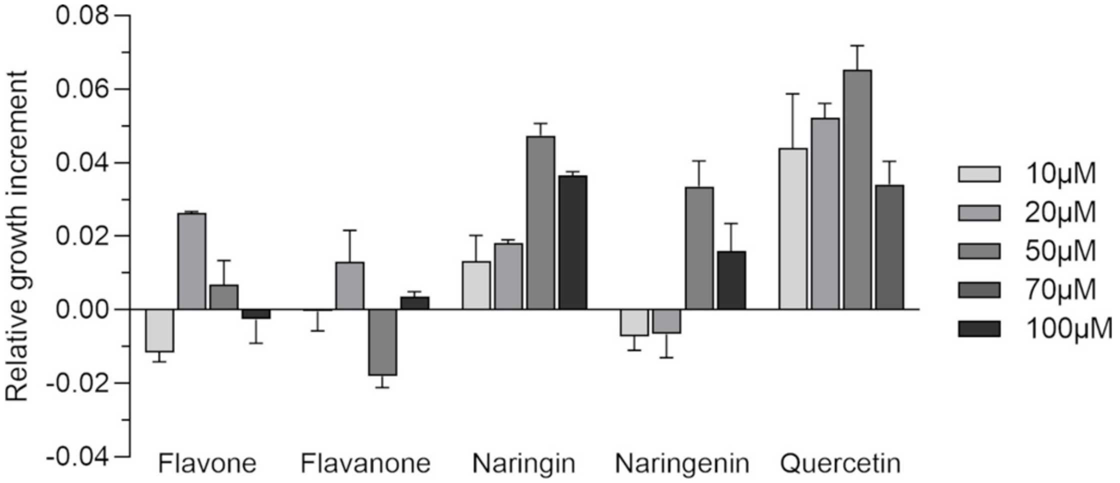
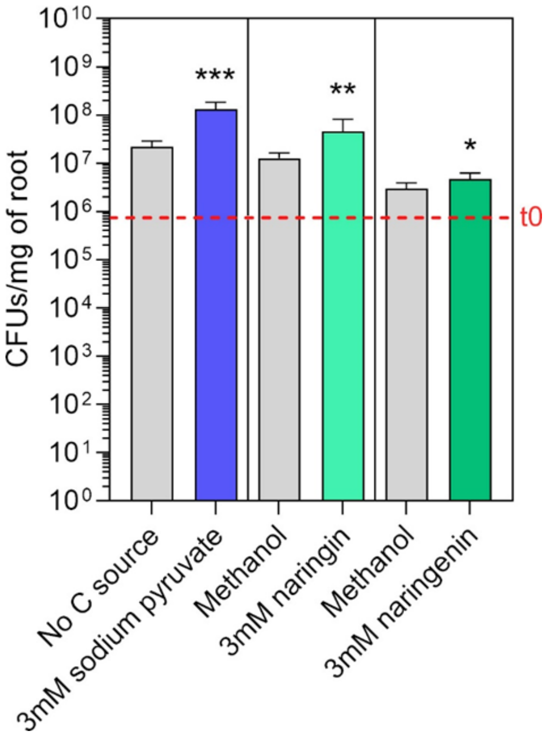
Supplementary materials



Supplementary Figure 1. Growth curves of the strain *P. xenovorans* LB400 in presence of flavonoids. The bacterium was assayed for growth in 1/10 TSB medium supplemented with the following flavonoids: (**A**) flavone, (**B**) flavanone, (**C**) naringin, (**D**) naringenin and (**E**) quercetin at final concentrations of 10, 20, 50, 70 and 100 µM. "Medium only" indicates growth in presence of 1/10 TSB with no additions, while "acetone", "methanol" and "DMSO" indicate growth in 1/10 TSB plus the solvents in which flavonoids were dissolved. The average and standard deviation values of three biological replicates are reported for each treatment and for each time point.

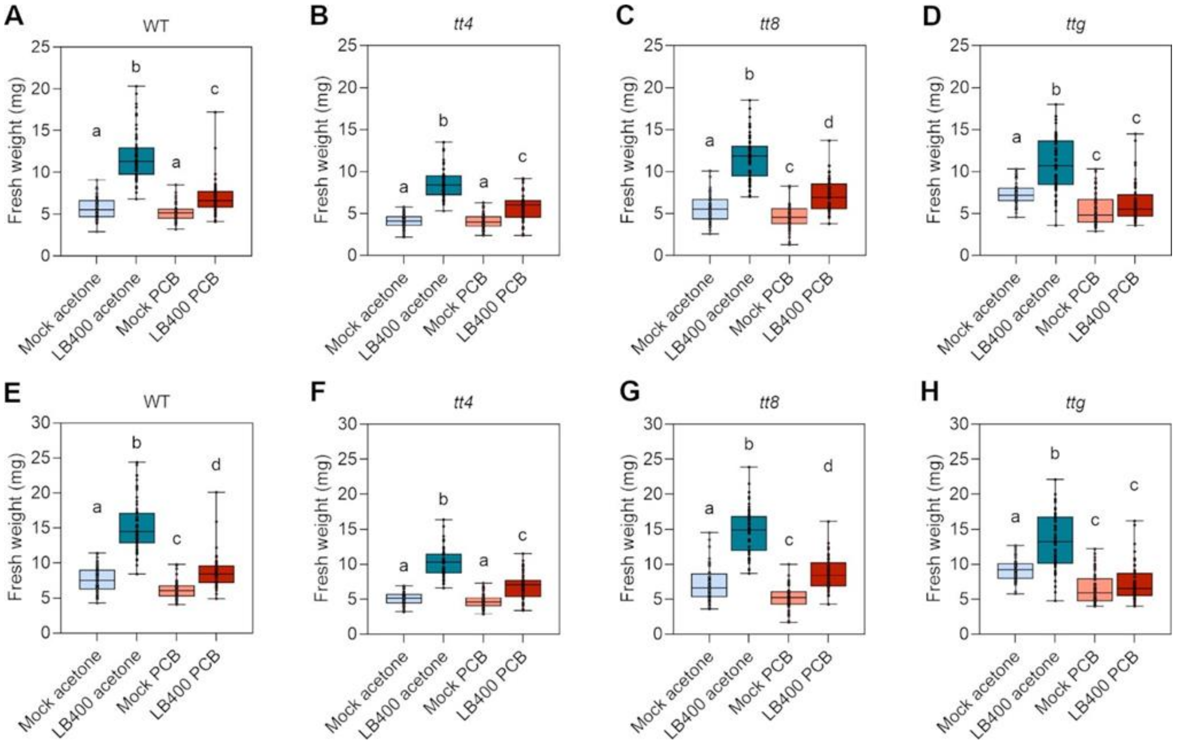


Supplementary Figure 2. Relative growth increment of strain LB400 at stationary phase. The relative growth increment was calculated as the ratio of the bacterial growth in 1/10 TSB medium supplemented with different concentrations of flavonoids (10, 20, 50, 70 and 100 µM) and the growth in presence of the respective solvents in which flavonoids were dissolved. The average and standard deviation values of three biological replicates are reported for each treatment.

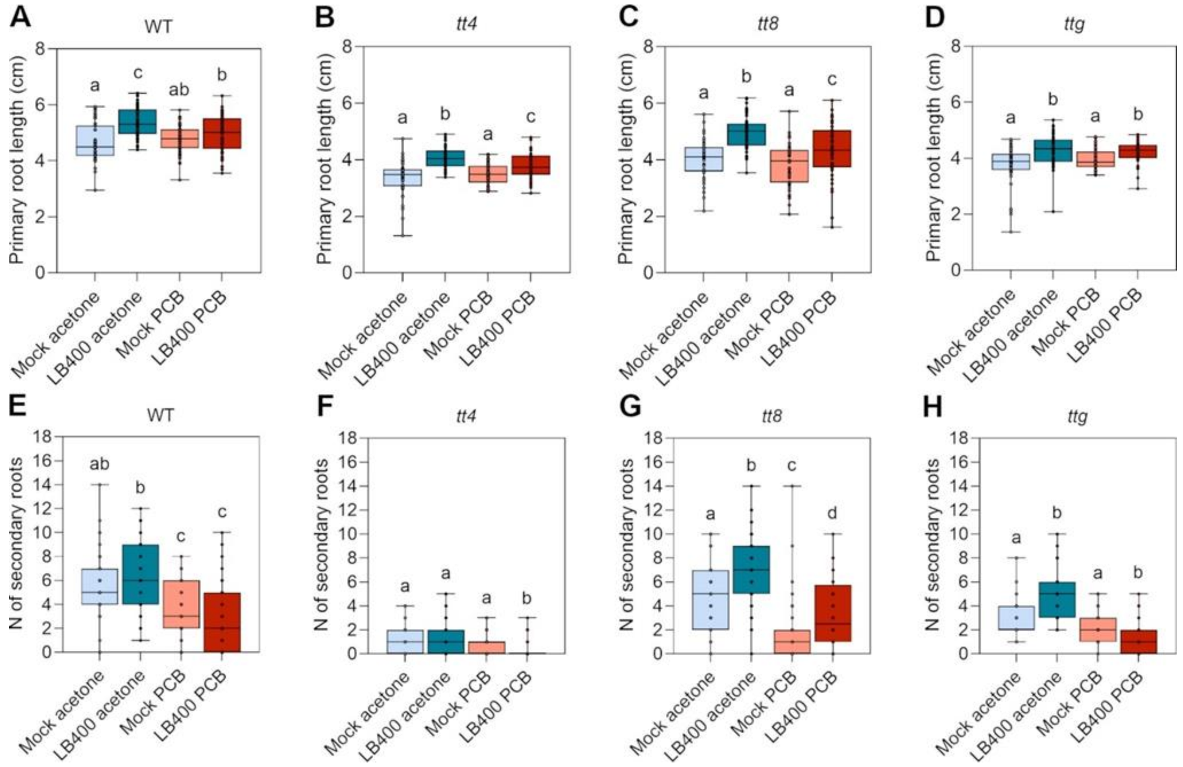


Supplementary Figure 3. *P. xenovorans* LB400 uses the flavonoids naringin and naringenin as carbon sources. The bacterium was grown for 6 days in mineral medium supplemented with either 3 mM sodium pyruvate as positive control or 3 mM naringin and naringenin. A corresponding amount of the respective solvent was used as control. The dashed red line represents the cell density that was inoculated in each vial at the beginning of the experiment (t0). Average and standard deviation values of two biological replicates, each with three technical replicates, are reported for each treatment. Statistical analysis was performed using the Mann-Whitney test (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001).

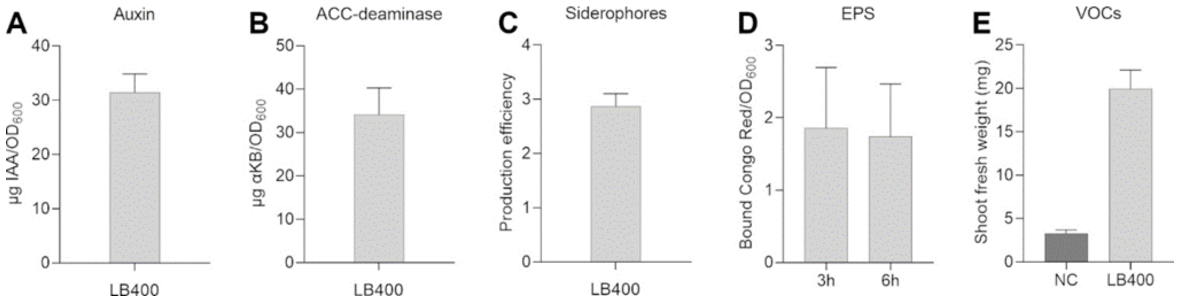
Supplementary Figure 4. *In vitro* PCB-18-induced stress in *Arabidopsis thaliana* wild type (WT) L*er* ecotype. In the present work, an *in vitro* PCB-18-induced stress experiment was set-up as described in the material and methods section. In the image, two representative plants showing the effects on *Arabidopsis* growth in presence of 20 µM PCB-18. In this case, the plantlets were deliberately grown for 28 days, a longer period compared the time points analyzed in this study (7 and 14 days) to clearly observe the PCB-18-induced phytotoxic effect on plant growth and development. PCB-18 phytotoxic effect causes a reduction in shoot and root biomass (plant on the right) if compared with a plant grown under control conditions in 1/2 MS supplemented with acetone as untreated control (plant on the left). Size of the bar: 1 cm.



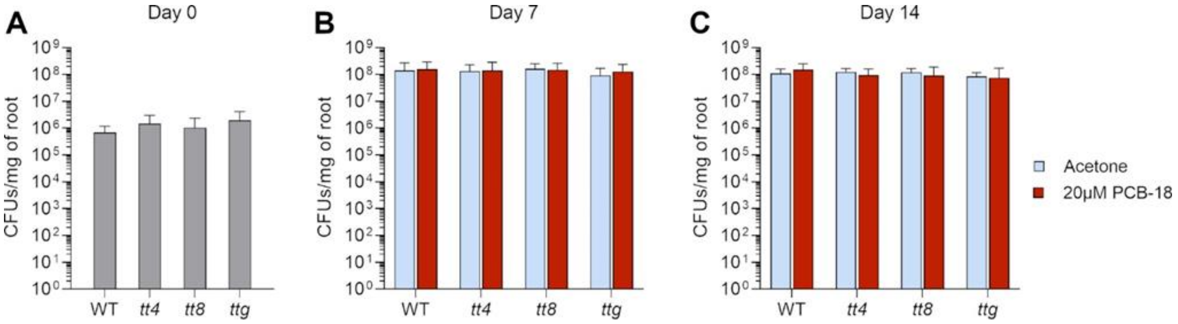
Supplementary Figure 5. PCB-18-induced decrease in plant fresh weight in WT plants and *Arabidopsis* lines affected in flavonoid biosynthesis and exudation. (**A**), (**B**), (**C**) and (**D**): shoot fresh weight in axenic (mock) or *P. xenovorans* LB400-colonized plants grown under control conditions (acetone) or exposed to PCB-18 in WT, *tt4*, *tt8* and *ttg* genotypes. (**E**), (**F**), (**G**) and (**H**): total plant fresh weight in axenic (mock) or *P. xenovorans* LB400-colonized plants grown under control conditions (acetone) or exposed to PCB-18 in WT, *tt4*, *tt8* and *ttg* genotypes. The boxplots represent data from three independent experiments. Letters indicate statistically different groups (Dunn’s post-hoc test with $p \leq 0.05$).



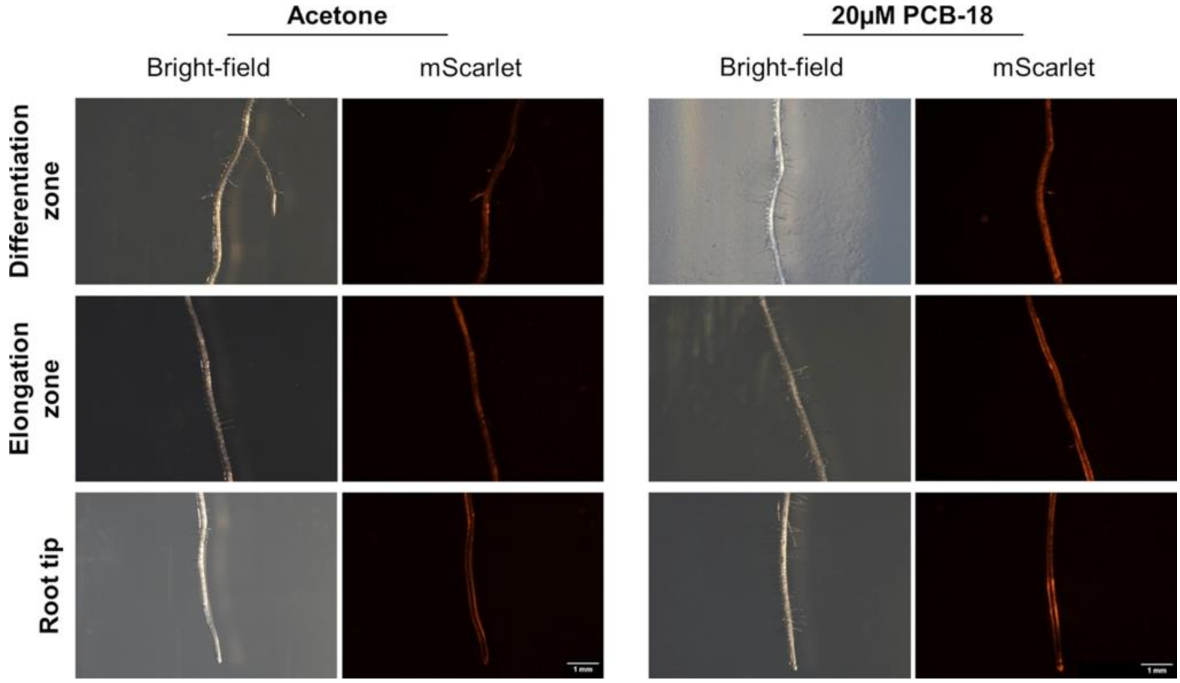
Supplementary Figure 6. PCB-18-induced remodeling of root architecture traits in WT plants and *Arabidopsis* lines affected in flavonoid biosynthesis and exudation. (**A**), (**B**), (**C**) and (**D**): primary root length in axenic (mock) or *P. xenovorans* LB400-colonized plants grown under control conditions (acetone) or exposed to PCB-18 in WT, *tt4*, *tt8* and *ttg* genotypes. (**E**), (**F**), (**G**) and (**H**): number of secondary roots in axenic (mock) or *P. xenovorans* LB400-colonized plants grown under control conditions (acetone) or exposed to PCB-18 in WT, *tt4*, *tt8* and *ttg* genotypes. The boxplots represent data from three independent experiments. Letters indicate statistically different groups (Dunn's post-hoc test with $p \leq 0.05$).



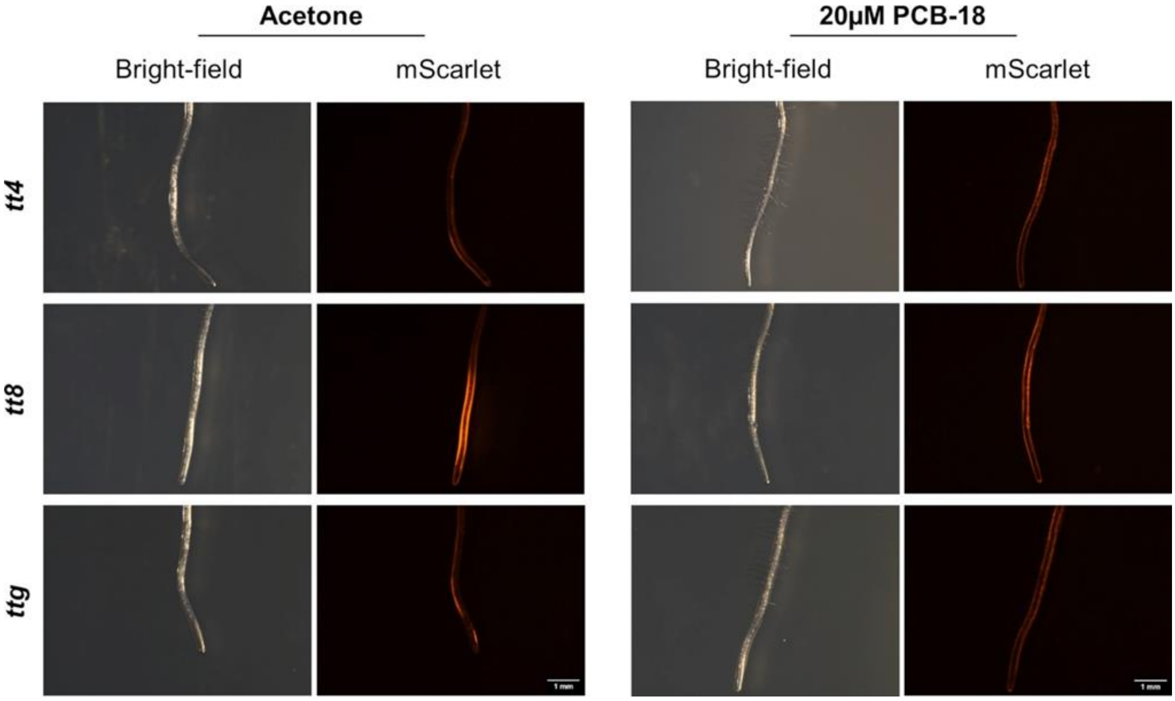
Supplementary Figure 7. *P. xenovorans* LB400 exhibited multiple plant growth promoting traits *in vitro*. The bacterium was assayed for auxin biosynthesis (**A**), expression of ACC-deaminase activity (**B**), siderophore release (**C**), EPS production (**D**) and VOCs release (**E**).



Supplementary Figure 8. Strain LB400 colonization efficiency in *Arabidopsis* plantlets at day 0, day 7 and day 14 after transfer on PCB-stressed plates in the *in vitro* assay to evaluate PCB-18-induced stress. It was investigated whether the differential biosynthesis of flavonoids in the different *Arabidopsis* mutant lines could affect strain LB400 colonization ability and root adhesion pattern on a longer time frame. The Day 0 graph (**A**) shows the colonization efficiency of strain LB400 in plantlets germinated for 5 days in presence/absence of the bacterium in the agar medium (day 0 of the assay). It represents the baseline colonization of the plantlets before transferring them to new plates to test resistance to PCB-18 stress or to control plates with acetone. Graphs (**B**) and (**C**) represent LB400 root colonization efficiency on *Arabidopsis* plantlets respectively 7 or 14 days after the transfer on plates supplemented with acetone or 20 µM PCB-18. Strain LB400 showed similar levels of colonization in the different genotypes of *Arabidopsis*. The barplots represent data from three independent experiments. Statistical analysis was performed using one-way ANOVA.



Supplementary Figure 9. Fluorescence microscopy analysis of *mScarlet*-tagged LB400 strain colonization profile on the root system of *Arabidopsis* WT. A fluorescent *mScarlet*-labeled *P. xenovorans* LB400 strain was developed in this study and fluorescence microscopy analyses were performed to elucidate the bacterial adhesion pattern in root colonization of the different *Arabidopsis* lines under control conditions (acetone) and in presence of 20 µM PCB-18. The panels illustrate the pattern of colonization in the different regions of the root system (differentiation zone, elongation zone and root tip). Plantlets were analyzed at 7 DAT and it was observed that *mScarlet*-labelled LB400 strain largely decorated *Arabidopsis* primary root and the root tip, while secondary roots were not colonized. These findings suggest that 7 days after transfer and exposure to PCB stress, *P. xenovorans* LB400 colonization patterns are not diversely affected in *Arabidopsis* mutant plantlets with distinct flavonoid exudation.



Supplementary Figure 10. Fluorescence microscopy analysis of *mScarlet*-tagged LB400 strain colonization profile on the apical region (root tip) of roots of flavonoids-affected *Arabidopsis* mutant lines *tt4*, *tt8* and *ttg* under control conditions (acetone) and under PCB-18 stress. The fluorescence microscopy analysis was performed to unravel *mScarlet*-tagged LB400 strain colonization pattern on the root system of the *Arabidopsis* lines affected in flavonoid biosynthesis and accumulation under control conditions (acetone) and in presence of 20 µM PCB-18. The panels illustrate the pattern of colonization in the root tip region for the *Arabidopsis* mutant lines *tt4*, *tt8* and *ttg*.

Treatment	Concentration (μM)	Maximum growth rate	Increase (%)	Entrance in stationary phase (hours)	OD$_{600}$ at entrance in stationary phase
Flavone	20	0.0206 ± 0.0005***	+9.6%	18	0.1876
	50	0.0197 ± 0.0007^{ns}	+4.8%	18	0.1714
Flavanone	20	0.0214 ± 0.0008^{ns}	+2.4%	17	0.2034
	100	0.0205 ± 0.0012^{ns}	-1.9%	17	0.1913
Naringin	10	0.0229 ± 0.0006***	+6.0%	17	0.1781
	20	0.0235 ± 0.0001***	+8.8%	17	0.1830
	50	0.0245 ± 0.0009***	+13.4%	17	0.2077
	100	0.0244 ± 0.0009***	+13.0%	17	0.1989
Naringenin	50	0.0272 ± 0.0011***	+9.2%	17	0.2266
	100	0.0263 ± 0.0011^{ns}	+5.6%	17	0.2135
Quercetin	10	0.0158 ± 0.0022***	-20.2%	21	0.1891
	20	0.0169 ± 0.0001***	-14.6%	22	0.2100
	50	0.0163 ± 0.0004***	-17.7%	23	0.2238
	70	0.0114 ± 0.0005***	-42.4%	28	0.2048

Supplementary Table 1. Values of strain LB400 maximum growth rate when exposed to growth-stimulating plant flavonoids. In the table, the maximum growth rate was calculated for the flavonoid molecules and for the concentrations that induced a growth promotion as indicated in Figure 1 of the manuscript. The % increase in growth rate compared to the solvent control, the time of entrance in stationary phase and the OD$_{600}$ reached in the moment of entrance in stationary phase are also reported. Statistical analysis was performed using the Mann-Whitney test, by comparing the flavonoid treatment with the respective solvent control. ***p ≤ 0.001; ns: non-significant (p > 0.05).

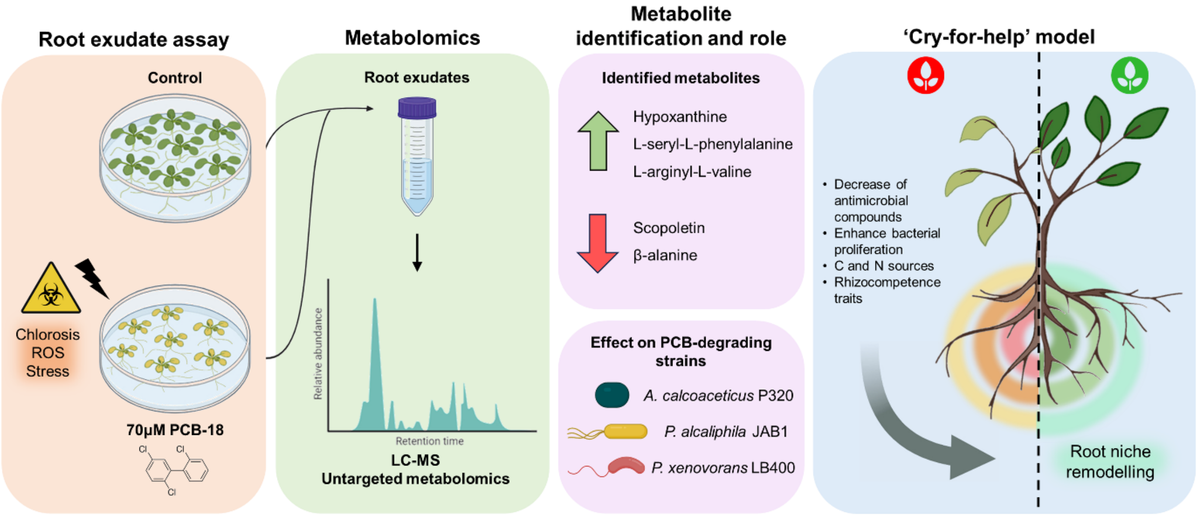
CHAPTER IV

Polychlorinated biphenyls induce a stress-triggered shift in *Arabidopsis* root exudation pattern as a ‘cry-for-help’ for degrading microbes

Abstract

The importance of plant rhizodeposition to sustain microbial growth and induce xenobiotic degradation in polluted environments is increasingly recognized. Here, the ‘cry-for-help’ hypothesis, consisting in a root chemistry remodeling upon stress, was investigated in presence of polychlorinated biphenyls (PCBs), highly recalcitrant and phytotoxic compounds, highlighting its role in reshaping the nutritional and signaling features of the root niche to accommodate PCB degrading microorganisms. *Arabidopsis* exposure to 70 µM PCB-18 triggered plant detrimental effects and, based on stress-related traits and PCB-responsive gene expression, the root exudates (REs) of plantlets exposed for 2 days to the pollutant were characterized through metabolomics analysis by LC-MS. Principal component analysis disclosed a different root exudation fingerprint in PCB-18 exposed plants, potentially contributing to a ‘cry-for-help’ event. To investigate this aspect, the five compounds identified in the exudate metabolomics analysis, namely scopoletin, β-alanine, hypoxanthine, L-arginyl-L-valine and L-seryl-L-phenylalanine were assayed for their influence on the physiology and functionality of the three PCB degrading strains *Pseudomonas alcaliphila* JAB1, *Paraburkholderia xenovorans* LB400 and *Acinetobacter calcoaceticus* P320. Scopoletin, which relative abundance decreased in PCB-18 stressed plant exudates, hampered the growth and proliferation of strains JAB1 and P320, presumably due to its antimicrobial activity, and reduced the beneficial effect of *Acinetobacter* P320, showing a higher degree of growth promotion in the scopoletin-depleted mutant *f6’h1* compared to *Arabidopsis* WT plants exposed to PCB. Nevertheless, scopoletin induced the expression of the *bph* catabolic operon in strain JAB1. The primary metabolites hypoxanthine, L-arginyl-L-valine and L-seryl-L-phenylalanine, that increased in relative abundance upon PCB-18 stress, were preferentially used as nutrients and growth stimulating factors by the three degrading strains and showed a variable ability to affect rhizocompetence traits like motility and biofilm formation. These findings expand the knowledge on PCB-triggered ‘cry-for-help’ and its role in steering the PCB degrading microbiome to boost the holobiont fitness in polluted environments.

Graphical abstract



1. INTRODUCTION

By modulating their root chemistry, plants modify the composition of the exudation pattern to actively recruit beneficial microorganisms to facilitate adaptation and/or protection from stresses (Afridi et al., 2024). Such event, referred to as 'cry for help' (Rolfe et al., 2019), was originally documented as a plant response to herbivores and phytopathogen attacks (Berendsen et al., 2018; Yi et al., 2011), although mounting evidence suggests that it could have broader implications in plant functional requirements (Feng et al., 2023) and be deployed also under abiotic stresses (Rizaludin et al., 2021). The 'cry-for-help', for instance, was claimed to be involved in plant survival strategy in contaminated soils, especially when pollution is caused by poorly phyto-degradable, highly recalcitrant and phytotoxic xenobiotics like polychlorinated biphenyls (PCBs) (Rolli et al., 2021).

PCBs are a class of 209 congeners that, despite having been banned from production since 1980, continue to pose a threat to human, animal and ecosystem health and persist at sites of former industrial production and in their neighborhoods (Xiang et al., 2020). This is the case of the Brescia-Caffaro site in Italy, that encompasses a ~100 ha restricted area where activities have been prohibited since 2001 because of the hazardous PCB concentrations largely exceeding law thresholds (Di Guardo et al., 2017). The biological degradation of PCBs occurs through both anaerobic dechlorination, preferentially on highly chlorinated congeners (Xiang et al., 2020), and aerobic cleavage of the biphenyl ring, mainly on low chlorinated congeners, performed by a variety of microorganisms (Suman et al., 2024, 2021), including bacteria and fungi, inhabiting polluted soils (Kumari and Das, 2023). These microbial functionalities are crucial for plant fitness since PCB pollution dramatically affects plant growth and development (Vergani et al., 2017b, 2017a) due to inefficient plant detoxification systems and lack of specific enzymatic activities for their degradation (Lin et al., 2020). The beneficial association between host plants and degrading bacteria could therefore be a consequence of PCB-induced detrimental effects on plant physiology and stress response. According to this hypothesis, PCB exposure would induce a change in plant rhizodeposition with the potential to support the recruitment of PCB-degrading bacteria and boost their enzymatic degradation activities to catabolize pollutants and in turn alleviate the pollutant stress (Musilova et al., 2016; Rolli et al., 2021; Uhlik et al., 2013). As a consequence, long-term exposure to PCBs would have driven a Darwinian selection of holobionts (Osmanovic et al., 2018), constituted by the host plant and the associated microbiomes that adapted as a unit to thrive in presence of the pollutants (Mapelli et al., 2022; Vergani et al., 2022). In this scenario, the 'cry-for-help' mechanism would contribute to determine the structure and functionality of the holobiont (Rolli et al., 2021). Acknowledged as mediators between plants and the surrounding root microbiome, flavonoids are highly abundant plant exudates with multifaceted capabilities to support plant fitness under stress (Ghitti et al., 2022). Due to structural similarity with the PCB backbone, flavonoids were reported to act as co-metabolites and inducers for the expression of *bph*-encoded dioxygenases, responsible for the aerobic degradation of PCB congeners to 4-chlorobenzoate in different bacterial species including *Pseudomonas putida* PML2 (Narasimhan et al., 2003) and *Rhodococcus erythropolis* U23A (Pham et al., 2015; Toussaint et al., 2012). More recently, *bphA* gene expression in *Pseudomonas alcaliphila* JAB1 was demonstrated to be induced by a wide range of secondary plant metabolites, including flavonoids and monoterpenes (Zubrova et al., 2021). We demonstrated that flavonoid pure compounds can act as nutrients and signaling factors, differentially affecting bacterial growth, motility, chemotaxis and early plant colonization in the PCB degrader *Paraburkholderia xenovorans* LB400 (Ghitti et al., 2024): through these effects, flavonoids contribute to recruit and sustain the proliferation of strain LB400 in the plant rhizosphere. Nevertheless, our findings on the study of the interaction of *Paraburkholderia* strain LB400 with *Arabidopsis* lines affected in flavonoid biosynthesis and exudation, demonstrated that other non-flavonoid metabolites could be responsible for stimulating strain LB400-triggered plant growth promotion and colonization under PCB stress (Ghitti et al., 2024). Despite metabolomics and exo-metabolomics represent key –omics approaches to investigate how root chemistry affects microbiome structure and functionality under adverse environmental conditions (van Dam and Bouwmeester, 2016), the framework of xenobiotic stress is still scarcely investigated. Such investigation would contribute to define key determinants of the holobiont dynamics in contaminated environments (Kour et al., 2021) and, from an applicative perspective, would lead to improved bioremediation strategies by allowing, for instance, the selection of plant species with the ability to boost the degradative potential of rhizosphere-associated microbiome more efficiently (Terzaghi et al., 2018), or the identification of biostimulating compounds to be applied in bio-enhancement processes.

This study aimed to identify the PCB-induced modifications in root exudation fingerprint and consequently determine the role of specialized metabolites, differentially produced under PCB stress, on the growth and activity of degrading bacteria. We used *Arabidopsis thaliana* as model plant to investigate PCB phytotoxic effects and the versatile PCB degrading strains *Acinetobacter calcoaceticus* P320 (Vergani et al., 2019), *Paraburkholderia xenovorans* LB400 (Chain et al., 2006) and *Pseudomonas alcaliphila* JAB1 (Zubrova et al., 2021). The trichlorobiphenyl congener PCB-18 was used as paradigm of this class of pollutants, considering that low-chlorinated congeners can be more easily up-taken by plant roots (Asai et al., 2022) and that this molecule was proved to exert detrimental effects on the growth of *Arabidopsis* (Bao et al., 2016) and other crops (Hao et al., 2020). Our findings suggest that the components of root chemistry that are specifically regulated under PCB stress can affect the growth and/or functionality of specific bacterial PCB-degraders, potentially remodeling the rhizosphere niche to favor the establishment of beneficial associations with bacteria able to catabolize this class of xenobiotics.

2. MATERIALS AND METHODS

2.1. Bacterial strains, plant material, culture media and chemicals.

Three PCB-degrading bacterial strains were employed in this study to assess their metabolic response to *Arabidopsis thaliana* root exudates. The strains were *Acinetobacter calcoaceticus* P320 (Vergani et al., 2019, 2017a), *Pseudomonas alcaliphila* JAB1 (Ridl et al., 2018) and the model PCB degrading strain *Paraburkholderia xenovorans* LB400 (Chain et al., 2006). Depending on the assay, the strains were grown either in Tryptic Soy Broth (TSB, Merck, Darmstadt, Germany), Luria-Bertani (LB) broth or in Mineral Medium Brunner (MMB, DSMZ, Germany) supplemented with 30 mM sodium pyruvate as carbon source. Bacterial cells were stored in glycerol stocks at -80°C and periodically revitalized on LB agar plates. *Arabidopsis thaliana* Col-0 was used for the described assays and the *f6'h1* mutant, depleted in scopoletin biosynthesis (Stringlis et al., 2018), was used in the present study. The root-exuded compounds identified by metabolomics were used as pure compounds in *in vitro* assays, solubilized in the respective solvents and prepared as concentrated stocks. Hypoxanthine (Merck, Germany) was solubilized in 1 N NaOH solution, scopoletin (Merck, Germany) in 80% methanol solution, β-alanine (N-(2-Hydroxyethyl)-beta-alanine, Merck, Germany) and the dipeptides L-seryl-L-phenylalanine (SF) and L-arginyl-L-valine (RV) (Twin Helix) were solubilized in ultrapure MilliQ water.

2.2. Evaluation of *Arabidopsis thaliana* phytotoxicity to PCB-18 stress.

This miniaturized assay allowed to evaluate the phytotoxic effect exerted by increasing concentrations of PCB-18 on the fresh weight of *Arabidopsis* plantlets. *Arabidopsis thaliana*

Col-0 seeds were surface sterilized in a buffer containing 70% ethanol and 0.05% SDS and shaked for 10 min. Seeds were then washed twice with 95% ethanol and spread on Whatman paper previously sterilized by dipping in ethanol. Sterile seeds were spread on square petri dishes containing 1/2 Murashige and Skoog (MS) medium (2.2 g/L Murashige and Skoog Basal Medium, 0.5 g/L MES hydrate, pH=5.4; 9 g/L agar type E). The seeds were vernalized for 48 h at 4°C in the dark and then transferred in a growth cabinet under long day conditions (16 h light, 8 h darkness) at 22°C, 50% humidity and 120-150 µmol/m light intensity. Six days after germination, three plantlets were transferred in the well of a 24-well plate filled with 2 mL of MS medium containing 1% sucrose supplemented with increasing concentrations of PCB No. 18 (2,2',5-trichlorobiphenyl, LCG Standards), solubilized in acetone. Three technical replicates were used for each treatment and an equal volume of acetone was used for plantlets growing under control conditions. After 7 days of growth, the fresh weight of the plantlets was quantified using a high sensibility scale and used to assess the phytotoxic effect induced by PCB-18 supplement compared to mock treatment with acetone. The 70 µM concentration was selected to be used for further experiments (Supplementary Figure 1).

2.3. Root exudates collection assay form *Arabidopsis* plantlets exposed to PCB-18.

A time-course assay was developed to collect root exudates (REs) and plant tissues and evaluate the PCB-induced phytotoxicity for the choice of the most appropriate time point to be analyzed via metabolomics. *Arabidopsis thaliana* Col-0 seeds were surface sterilized as described previously and about 30 seeds were distributed in a 4.5 cm diameter petri dish containing 10 mL of liquid 1/2 MS medium with 1% sucrose. After vernalization, the plates were transferred in the growth cabinet and grown for 11 days. The liquid nutrient medium was removed and the plantlets were gently washed twice with 5 mL of 1/2 MS medium. After the washes, 10 mL of 1/2 MS medium was added to the petri dishes, containing either 70 µM PCB-18 to induce PCB stress or an equal volume of acetone as control. At this stage, sucrose was not added to the MS medium to prevent interference in the subsequent GC-MS analyses of root exudates. The plants were then grown for 7 days. Four biological replicates were collected for PCB and mock treatments at the different time points of 2, 4 and 7 days (T2, T4 and T7) after PCB-18 treatments. The collected samples consisted in (i) the medium in which the plants were floating, that was considered as REs (Pantigoso et al., 2020) and was further subjected to metabolomic analysis and (ii) ~30 plantlets for RNA extraction and RT-qPCR quantification of the expression level of target genes responsive to PCBs. The root exudates were filtered through a 0.45 µm filter (Millipore) to remove root debris, lyophilized, and stored at -80˚C for further analyses. The plant material was rapidly frozen in liquid nitrogen and stored at -80°C for further analysis.

2.4. Evaluation of PCB-18 effect on plant growth

After 7 days of growth, the fresh weight of the plant shoots was quantified to verify the detrimental effect on plant growth exerted by the treatment with 70 µM PCB-18 compared to an equal volume of acetone as mock treatment. The plant fresh weight was measured by using a high sensibility scale. N° of plant shoots analyzed = 30, in three biological replicates.

2.5. Reactive oxygen species (ROS) staining in *Arabidopsis* leaves.

ROS staining of collected *Arabidopsis* leaves was performed using 3,3'-diaminobenzidine (DAB) as described by Daudi and O'Brien (Daudi and O'brien, 2012).

2.6. Ability of *Paraburkholderia xenovorans* LB400 to grow on Arabidopsis REs released under PCB-18 stress or under mock treatment.

The collected root exudates from *Arabidopsis* Col-0 plants exposed to PCB stress or to mock treatment with acetone at the different time points were used to assess their potential to sustain the growth of the model PCB degrader *P. xenovorans* LB400. This analysis was used to select the most appropriate time point of the plant root exudation profile to be further analyzed through the metabolomic approach. Strain LB400 was inoculated in 1/2 TSB liquid medium and incubated overnight at 30°C on a shaker (150 rpm). Subsequently, cells were collected and washed twice in physiological buffer by centrifugation (5 min, 4000 rpm). Cells were then inoculated at a final concentration of 10^5 cells/mL in triplicates in a 96-well plate using the collected root exudates as culture media. Negative controls without root exudates were set using 1/2 MS medium containing only 70 µM PCB-18 or an equal amount of acetone. The plate was incubated on a rotatory shaker at 30°C for four days. Bacterial cultures were then re-isolated and quantified by plating serial dilutions, obtaining the number of CFUs/mL.

2.7. RNA extraction from *Arabidopsis* seedlings and RT-qPCR to evaluate the relative expression of *Arabidopsis* genes that are responsive to PCB stress.

Seedlings were harvested at the indicated times (T2, T4 and T7 after PCB addition or mock treatment) and frozen in liquid nitrogen. Total RNA extractions were performed using the NucleoSpin RNA kit (Macherey-Nagel), including DNase treatment, following the manufacturer's recommendations. RNA quantification and quality were measured using a NanoDrop (Thermo Scientific). The cDNA was synthesized from 1 µg total RNA using oligo(dT) and RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, USA) in a total volume of 20 µL. For cDNA synthesis, RNA was heated at 65°C for 5 min, the reverse transcription (RT) mix was added and the reaction proceeded at 42°C for 1 hour. cDNA was then heated at 70°C for 5 min and stored at 4°C. qPCR reactions were performed in 12 µL final volume with 1 µL RT reaction product, 250 nM final concentration of each primer pair and SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, USA). The following genes were analyzed and the primer sequences are indicated by the literature references: *ABCG40* (Wrzaczek et al., 2009), *UGT73D1* (Langlois-Meurinne et al., 2005), *Germin like protein subfamily 1 member 15* (Subramanian et al., 2018), *XTR8* and *CYP707A3* (Rai et al., 2016). POLYUBIQUITIN 10 (*UBIQ10*, at4g05320) was used as reference gene. Based on literature data, *UGT73D1* and *Germin-like protein subfamily 1 member 15* are PCB-responsive genes that are up-regulated in presence of tetrachlorobiphenyl (Subramanian et al., 2018) and dichlorobiphenyl (Jin et al., 2011), respectively. *ABCG40* is a xenobiotic-responsive gene, involved in *Arabidopsis* resistance to lead contamination (Fan et al., 2016). Since flavonoids can act as inducers of the PCB catabolic pathway in bacterial cells (Narasimhan et al., 2003), flavonoid marker genes were also monitored, including TT8, the transcription factor for flavonoid biosynthesis and its target genes *XTR8* and *CYP707A3* (Rai et al., 2016). All reactions were performed in a CFX Connect Real-Time PCR Detection System (Bio-Rad) as follows: 95 °C for 30 s, × 40 95 °C for 5 s and 60 °C for 20 s; a dissociation step was programmed to validate PCR products. Results were analyzed using the CFX Manager Software (Bio-Rad).

2.8. Metabolomics analysis of root exudate composition.

The metabolomics analysis was performed at MetaSysX (Germany). Sample preparation was performed according to MetaSysX standard procedure, a modified protocol from Salem et al. (Salem et al., 2016). The analysis was originally performed by both LC-MS (Liquid Chromatography-Mass Spectrometry) and GC-MS (Gas Chromatography-Mass Spectrometry), but statistically relevant results were obtained only with the LC-MS approach. For the LC-MS measurements of hydrophilic analytes, the samples were measured with a Waters ACQUITY Reversed Phase Ultra Performance Liquid Chromatography (RP-UPLC) coupled to a Thermo-Fisher Exactive mass spectrometer. C18 columns were used for the hydrophilic measurements. Chromatograms were recorded in Full Scan MS mode (Mass Range [100-1500]). All mass spectra were acquired in positive and negative ionization modes. For LC-MS data processing, the data extraction was accomplished with the software PeakShaper (MetaSysX GmbH). Alignment and filtration of LC-MS data were completed using in-house software. After extraction from the chromatograms, the data was processed, aligned and filtered for redundant peaks. The alignment of the extracted data from each chromatogram was performed according to the criteria that a feature had to be present in all replicates of at least one of the groups. At this stage, the average RT and m/z values are given to the features. The alignment was performed for each type of measurement independently. The alignment of the data was followed by the application of various filters to refine the dataset, which included the removal of (i) isotopic peaks, (ii) in-source fragments of the analytes (due to the ionization method), and (iii) redundant peaks like additional less intense adduct of the same analyte and redundant derivatives, to guarantee the quality of the data for further statistical analyses. For the annotation, the in-house MetaSysX database of chemical compounds was used to match features detected in the LC-MS polar platform. The annotation of the content of the sample was performed by database query of mass-to-charge ratio and the retention time of detected features within certain criteria, corresponding to 6 ppm and 0.1 min deviation from the reference compounds mass-to-charge ratio and retention time, respectively for the polar and non-polar platforms. Coeluting compounds with the same mass-to-charge ratio were all kept. The normalization was performed for each platform separately for the median of intensities of each sample. The normalized intensities were merged as a final data matrix. For the statistical analysis all normalized intensities were $\log_2$ transformed. For heatmap visualization the logarithmically transformed values were scaled by median-centering. The missing values were not replaced and color coded with white. The test was conducted on the $\log_2$ transformed intensities. The statistical test was computed with a two-tailed t-test assuming unequal variance. The p-values were corrected using the Benjamini-Hochberg (BH) method.

2.9. *In vitro* assay for the putative antimicrobial effect of scopoletin.

The putative antimicrobial effect induced by the presence of scopoletin was tested and quantified *in vitro* by employing the microplate assay proposed by Stringlis et al. (Stringlis et al., 2018) with modifications. In brief, 96-well plates (VWR, USA) were filled with 100 µL of TSB supplemented with different concentrations of root exudates (0.25, 0.5, 1 and 2 mM). Negative controls contained equal amounts of the respective solvents (80% methanol for scopoletin and water for β-alanine) and the antibiotic tetracycline (100 µg/mL, Merck, Germany) was used as positive control for the inhibition bacterial growth. The bacterial strains were grown overnight in 1/2 TSB medium until late-log phase and 2 mL of bacterial culture were harvested, washed twice and resuspended in physiological buffer. To start the growth curves, 100 µL of the bacterial cell suspension was added to each well containing the TSB supplemented with the REs to reach a final concentration of 10^8 cells/mL in the well. The growth was monitored by measuring OD_{600} every hour for 24 h using a 96-well plate reader (Tecan, Switzerland), keeping the plate incubated at 30°C and shaking for 7 seconds before each measurement. Each condition was tested with three biological replicates and, respectively, three technical replicates. Relative growth to express bacterial sensitivity to the REs was calculated as reported by Harbort et al. (Harbort et al., 2020) by dividing the final OD_{600} measurement (at time 24 h) of each concentration assayed by the OD_{600} obtained in the root exudate-free control. Doubling time and maximum growth rate were calculated as specified by Navarro Perez et al. (Navarro-Pérez et al., 2022).

2.10. Evaluation of scopoletin on *Acinetobacter* P320-plant interaction under control conditions and in presence of PCB-18 stress.

Acinetobacter P320, the scopoletin-most affected bacterial strain, was evaluated for its performance in plant-bacteria interaction in wild type (WT) *Arabidopsis* plants and in *f6'h1* mutant, depleted in scopoletin biosynthesis (Stringlis et al., 2018). Sterilized seeds were sown on 1/2 MS agar plates (50 mL) supplemented with *Acinetobacter* P320 at a concentration of 2 x 10^5 cells/mL or without the bacterial inoculum in case of control seeds, that were prepared by adding an equal volume of physiological buffer. The plates were vernalized for 2 days at 4°C in the dark and then placed vertically in a growth cabinet for 5 days (22°C, 50% humidity, long day conditions with light intensity of 120-150 µmol/m). Five days after germination, *Arabidopsis* plantlets were transferred onto fresh 1/2 MS plates containing 20 µM PCB-18 (treated) or an equal volume of acetone (mock treatment), as described in Ghitti et al., 2024. The plates were incubated for a total of 14 days in vertical position in a growth cabinet. At the end of the experiment, the fresh weight of the plantlets was measured by using a precision scale. All measurements were performed on 3 independent experiments and on at least 7 plants per condition. The beneficial index, meaning the growth promotion ability exerted by *Acinetobacter* P320, was calculated as the ratio between the fresh weight of *Acinetobacter* P320-colonized plants and non-inoculated seedlings and was expressed in percentage in the same genetic background. The beneficial index was used as a tool to compare *Acinetobacter* P320 improving performances under PCB-18 stress in the *Arabidopsis* backgrounds used in the present assay. To evaluate the bacterial colonization efficiency, the root systems were collected, placed in pre-weighed Eppendorf tubes and their fresh weight measured. The roots were then homogenized with a TissueLyser II (QIAGEN, Germany) using the following protocol: 2 cycles at 20 Hz frequency for 20 seconds and, after adding 900 µL of physiological buffer, 2 cycles at 15 Hz for 1 minute. The smashed root suspension obtained was used as 10^{-1} suspension to prepare serial dilutions for the drop-plate count method, that were plated on LB plates. After an overnight incubation at 30°C, bacterial colonies were counted and the root colonization efficiency expressed as CFUs/mg root fresh weight.

2.11. Induction of *bphA* gene expression in *Pseudomonas* JAB1 by scopoletin.

Adapting the protocol used by Zubrova and collaborators (Zubrova et al., 2021), *Pseudomonas* JAB1 cells were grown in MMB supplemented with 30 mM sodium pyruvate on a rotatory shaker at 30°C until late-log phase. The culture was then harvested by centrifugation, washed in physiological buffer and resuspended at 1 OD/mL in fresh MMB supplemented with 30 mM sodium pyruvate. Aliquots of the bacterial suspension were divided into glass vials, previously amended with 0.25 mM scopoletin and biphenyl, the latter used as positive control for *bphA* induction. Media supplemented only with the solvents in which scopoletin and biphenyl were solubilized, respectively 80% methanol and acetone, were used as negative controls. The solvents were evaporated for 15 min prior addition of the bacterial suspension. The cultures were incubated at 30°C on a rotatory shaker and for each condition 200 µL of culture were sampled in triplicate at time points 0 and 2 h, then pelleted by centrifugation (4000 rpm, 4°C, 5 min). The pellet obtained was stored at -20°C for subsequent RNA extraction steps. Total RNA extraction and RT-qPCR was performed as previously reported (Ghitti et al., 2024). In brief, RNA was extracted using the Nucleospin RNA kit (Macherey-Nagel, Germany) and subsequently residual gDNA was removed using the NucleoSpin RNA XS kit (Macherey-Nagel, Germany). Reverse transcription of RNA was then carried out was then carried using 1 µg of RNA with the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, USA). qPCR was finally performed using the CFX Connect Real-Time PCR Detection System (Bio-Rad, USA) and the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, USA). The reaction volume was 12 µL and contained 1 µL template cDNA (5 ng/µL) and 0.25 µM primers for the amplification of JAB1 *bphA* gene (F: 5'-GAGATCCAGAAGGGGCTAC-3'; R: 5'-GCGCATCCAGTGGTGATA-3'), as target gene, and the reference gene *infB* (F: 5'-AGTGACCGATAGTGAGAAAC-3'; R: 5'-AACACTGATGGTCTTGCTAC-3'). Thermal protocol was set up with a 10-min incubation at 95°C, then 40 cycles at 95°C (10 s) and 60°C (40 s). Data analysis to calculate the relative abundance of *bphA* gene expression was performed as reported previously (Ghitti et al., 2024).

2.12. *In vitro* growth on pure root exudates as unique carbon or nitrogen sources.

The ability of the bacterial strains to use pure plant root exudates (hypoxanthine, L-seryl-L-phenylalanine and L-arginyl-L-valine), that increased their abundance upon PCB-18 stress, as unique sources of carbon or nitrogen was tested *in vitro* in liquid culture. The bacterial strains were cultivated overnight in MMB medium supplemented with 30 mM sodium pyruvate at 30°C on a rotatory shaker (150 rpm) and cells harvested by centrifugation (10 min, 4000 rpm). To test the ability to use REs as carbon sources, bacterial cultures were washed twice in physiological buffer and resuspended at a final concentration of 5 x 10^5 cells/mL in MMB supplemented with 10 mM root exudates instead of sodium pyruvate. MMB with no carbon source was used as negative control while MMB supplemented with 10 mM sodium pyruvate was used as positive control for bacterial growth. For growth on REs as unique nitrogen sources, bacterial cultures were washed twice in physiological buffer and resuspended at a final concentration of 5 x 10^5 cells/mL in MMB deprived of the nitrogen source normally required by the medium and supplemented with 4 mM root exudates as carbon source. MMB with no nitrogen source was used as negative control while the conventional composition of MMB as positive control for bacterial growth. The cultures were aliquoted (200 µL per well) into a transparent 96-well plate (VWR, USA). Abiotic controls were analyzed as blanks to subtract the absorbance background given by the medium. Each condition was tested with three biological replicates and, respectively, three technical replicates. Bacterial growth was monitored by measuring optical density at 600 nm every hour for 48 h using a 96-well plate reader (Tecan, Switzerland), keeping the plate in incubation at 30°C and shaking for 7 seconds before each measurement.

2.13. *In vitro* biofilm formation assay.

Biofilm formation was estimated *in vitro* by quantifying the bacterial cell adhesion to a solid surface (polystyrene 96-well plate, VWR, USA) using crystal violet (CV) staining, following the method applied by Yoshioka and Newell (Yoshioka and Newell, 2016) with few adaptations. The bacterial strains were grown overnight at 30°C on a rotatory shaker (150 rpm) in liquid K10T-1 medium composed by 50 mM Tris-HCl (pH 7.4), 0.2% bacto tryptone, 0.15% glycerol, 0.61 mM $MgSO_4$, and 1 mM K_2HPO_4 (Yoshioka and Newell, 2016). Two mL of bacterial culture were harvested, washed twice in physiological buffer and finally resuspended in fresh K10T-1 medium. Ten µL of the resulting culture were added to each well of the plate, which had been pre-filled with 200 µL of K10T-1 supplemented with 5, 50 and 500 µM of the root exudates, to obtain a final concentration of 10^6 cells/mL. The respective solvents were added to the medium as negative controls and non-inoculated medium was aliquoted as blank. The plate was then incubated statically for 24 h (P320 and JAB1) or 48 h (LB400) at 30°C. After incubation, OD_{600} of the bacterial cultures was measured using a 96-well plate reader (Tecan, Switzerland). The liquid culture was then carefully removed from the plate and the wells washed twice with phosphate buffered saline (PBS). The cells adhering to the well were stained for 15 min with 0.5% (weight/volume) CV solution in 20% ethanol. CV solution was removed and the plate rinsed twice with distilled water and air dried for 15 min. The remaining CV was solubilized for 30 min using 200 µL/well of 97% ethanol and the OD_{600} of the resulting solution was measured using a 96-well plate reader (Tecan, Switzerland). The CV optical densities obtained were then normalized using OD_{600} values measured previously for bacterial growth. Each condition was tested with 3 independent biological replicates.

2.14. *In vitro* swimming motility assay.

The effect of pure root exudates on bacterial swimming motility was tested following the indications of Kearns (Kearns, 2010) and Bartolini and Grau (Bartolini and Grau, 2019). Soft agar plates with 1/10 TSB medium supplemented with 0.25% (w/v) agar were prepared, adding 50 and 100 µM root exudates before pouring. The appropriate solvent for each effector molecule was added in equal volume as negative control. Plates were dried with the lid for 2 h under laminar flow hood. Motile bacterial strains (JAB1 and LB400) were grown overnight at 30°C on a rotatory shaker (150 rpm) in 1/2 TSB to late-log growth phase. Cultures were then washed by centrifugation (5 min, 1670 g, 4°C) and diluted in physiological buffer to a concentration of 0.5 OD/mL. Two µL of the bacterial suspension were spotted at the center of the plate and dried for 30 min with the lid and 10 min without the lid under laminar flow hood. After 24 h of incubation at 30°C, the plates were scanned and the colony diameter measured using ImageJ software (https://imagej.nih.gov). Each condition was tested 3 times, each with 3 technical replicates.

2.15. Bacterial growth assay in presence of increasing concentrations of pure root exudate compounds.

For this *in vitro* growth assay the protocol used by Huang et al. (Huang et al., 2019) was applied with some adaptations. Briefly, the strains were grown overnight on a rotatory shaker (150 rpm) at 30°C in 1/2 TSB liquid medium diluted with ultrapure MilliQ water until the bacterial strains reached late-log growth phase. The cultures were centrifuged (5 min, 4000 rpm), washed in physiological buffer (9 g/L NaCl) and diluted 1000-fold in the various media used for the bioassay, consisting in the medium 1/10 TSB supplemented with the compounds that were enriched in *Arabidopsis* REs secreted under PCB stress (hypoxanthine, L-seryl-L-phenylalanine and L-arginyl-L-valine) at final concentrations of 10, 20, 50 and 100 µM. The diluted cultures were then aliquoted (200 µL per well) into a transparent 96-well plate (VWR, USA). Cultures diluted in 1/10 TSB supplemented only with the REs solvents were used as negative controls. Abiotic controls were aliquoted as blanks to subtract the absorbance background given by the media. Each condition was tested with three biological replicates and, respectively, three technical replicates. Bacterial growth was monitored by measuring optical density at 600 nm every hour for 24 h using a 96-well plate reader (Tecan, Switzerland), keeping the plate in incubation at 30°C and shaking for 7 seconds before each measurement.

2.16. Statistical analysis.

Statistical analyses were performed using R and Graphpad. Normal data were tested using ANOVA followed by unpaired t-test for multiple comparisons (confidence interval 95%). For non-normal data, Kruskal-Wallis test was adopted, followed by Dunn's post-hoc test (confidence interval 95%). To compare non-normal distributions with small sample size ($n < 30$), Mann–Whitney non-parametric test was used.

3. RESULTS

3.1. *In vitro* assay to collect PCB-18 triggered 'cry-for-help' signature in *Arabidopsis thaliana* exudation pattern.

Based on the reduction in *Arabidopsis* plantlets fresh biomass in presence of PCB-18 (Supplementary Figure 1), a miniaturized *in vitro* assay was developed to evaluate PCB phytotoxic features in *Arabidopsis thaliana*. Seven days after 70 µM PCB-18 application, stressed plants showed leaf chlorosis (Figure 1A), a 44% reduction in fresh biomass (Figure

1B) and enhanced ROS accumulation in leaves (Figure 1C), indicating that the assay was effective in recapitulating the main hallmarks of PCB stress. Plantlets and the root exudates enriched medium were analyzed at different time points (2, 4, 7 days after PCB addition) to select the most suitable one for collecting REs for metabolomics analysis. Furthermore, RT-qPCR analysis was applied on cDNA obtained from *Arabidopsis* plantlets to monitor the relative expression of PCB-stress responsive genes (Subramanian et al., 2018) and flavonoid responsive genes (Rai et al., 2016), knowing the interconnection between these plant secondary metabolites and the stimulation of microbial degraders (Ghitti et al., 2022). All the analyzed genes showed a statistically relevant increase in their expression level upon PCB-18 treatment at T2; in the case of the PCB-responsive genes *UGT73D1* and *Germin-like protein subfamily 1 member 15* this increase was observed also at T4 in PCB-treated plantlets (Figure 1E).

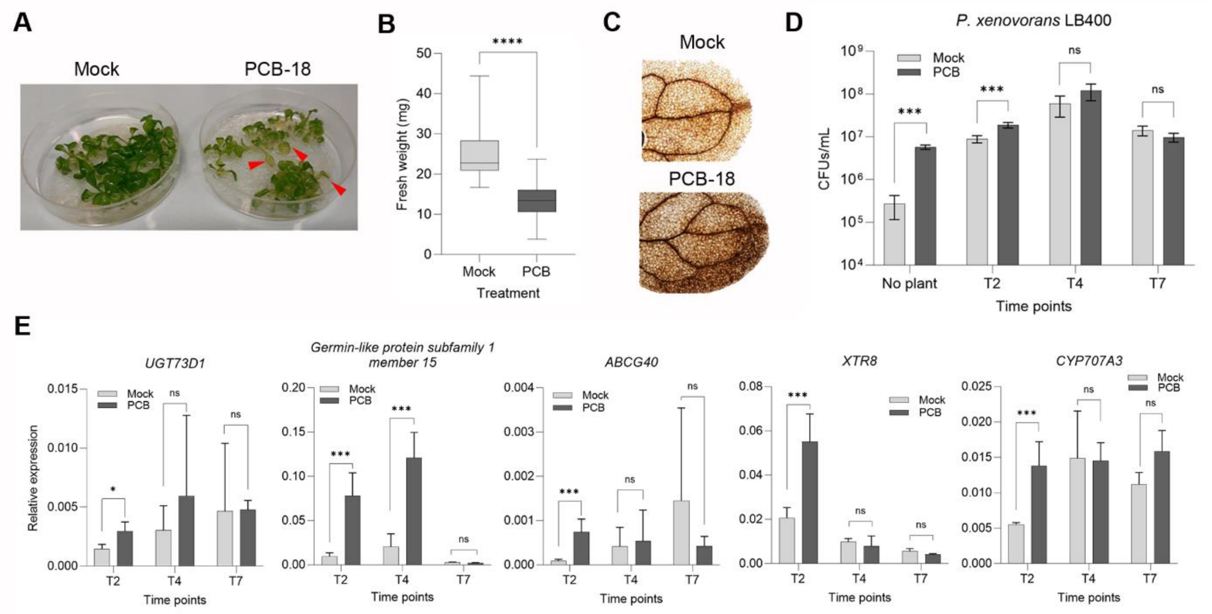


Figure 1. Validation of the *in vitro* assay to simulate PCB stress in *Arabidopsis thaliana* and selection of the appropriate time point for metabolomic analysis of REs. (A) Upon exposure for seven days (T7) to 70 µM PCB-18, *Arabidopsis* plantlets showed marks of chlorosis on leaves, as indicated by the red arrows. **(B)** PCB-18-induced stress showed a severe decrease in fresh biomass for plantlets at T7 treated with 70 µM PCB-18 compared to plants exposed to an equal volume of acetone (mock). Statistical analysis was performed by applying the unpaired t-test, *****p* ≤ 0.0001, n=30. **(C)** ROS staining on leaves of plantlets at T7 exposed to PCB stress or mock treatment. **(D)** Ability of the PCB degrader strain *Paraburkholderia xenovorans* LB400 to grow by exploiting the root exudate-enriched medium released by *Arabidopsis* plantlets exposed to PCB-18 or mock treatment at different time points. The medium for the sample indicated as "no plant" corresponded to 1/2 MS supplemented with 70 µM PCB-18 (dark-grey bar) and acetone (light grey bar). Statistical analysis was performed by applying the Mann-Whitney test (n=3): ****p* ≤ 0.001, ns: non statistically relevant. **(E)** RT-qPCR representing the relative expression level of PCB-, xenobiotics- and flavonoid-responsive genes. The plantlets were collected at different time points upon PCB or mock treatment, at T2, T4 and T7. Statistical analysis was performed by applying the Mann-Whitney test (3 < n > 4): **p* ≤ 0.05; ****p* ≤ 0.001, ns: non statistically relevant.

The model PCB-degrader *Paraburkholderia xenovorans* LB400 was than incubated on REs collected at the different time points. The strain demonstrated enhanced growth ability in presence of the medium enriched with the T2 root exudates, while no statistically relevant differences were observed for T4 and T7 time points by comparing the root exudates released by *Arabidopsis* plantlets in presence of acetone or PCB-18 (Figure 1D). The developed assay demonstrated to represent a valuable set-up to simulate PCB stress in *Arabidopsis* and the T2 time point was selected for subsequent RE metabolomics analysis, potentially representing the root exudation fingerprint specifically triggered by PCB-18 and affecting microbial degraders.

3.2. Untargeted metabolomics analysis of *Arabidopsis* PCB-18-triggered root exudates.

The metabolomics analysis detected 2513 metabolite features comprising 200 annotated ones (Supplementary Figure 2 and Supplementary Figure 3). Principal component analysis (PCA) of metabolite fingerprint was performed to identify similarities/dissimilarities among REs exuded with PCB-18 or with acetone as mock treatment in the plant medium. The PCA plots indicated that the exudates released by PCB-exposed plantlets diverged from those exudated by mock-treated ones, as observed in sample separation along axis 1 that explains 66.9% and 58.2% of the variance respectively for the metabolites detected in positive mode and negative mode in the LC-MS analysis (Figure 2A, B). In the metabolite dataset, 65 features showed a statistically different relative abundance between the REs of mock-treated and PCB-18 exposed plants (Figure 2C). These features may represent the PCB-18 induced 'cry-for-help' in *Arabidopsis* exudation pattern and the variation in their relative abundance among treatments is represented in the heatmap (Figure 2C). Only five of these compounds were identified and corresponded to: scopoletin and N-hydroxyethyl-beta-alanine (β-alanine), which showed a decrease upon PCB-18 treatment; and hypoxanthine, L-seryl-L-phenylalanine (SF), L-arginyl-L-valine (RV) that increased their relative abundance in PCB-exposed REs (Figure 2D). In the vision that the identified exudates are part of the 'cry-for-help' strategy exerted by the host plant under PCB stress, these metabolites were further assayed, as pure compounds, to investigate their putative role on the physiology of three selected PCB-degrading bacteria.

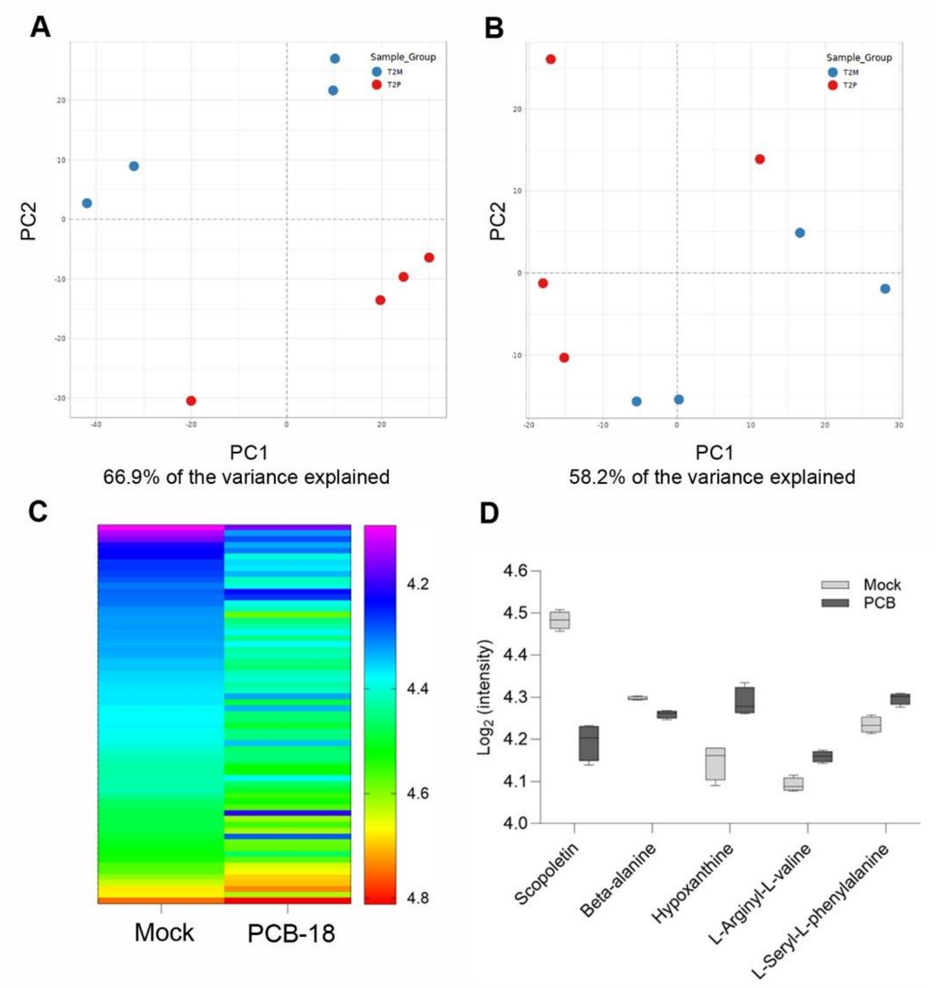


Figure 2. *Arabidopsis* root exudate compounds diverge in response PCB-18. **(A), (B)**: PCA analysis of the detected metabolite features corresponding to the rhizodeposition released by plantlets exposed for 2 days (T2) to 70 µM PCB-18 (T2P, in red in the graph) or mock-treated with an equal amount of acetone (T2M, in blue in the graph) detected by LC-MS analysis in positive and negative ionization modes, respectively. **(C)** Heatmap of the 65 metabolic features that showed differential relative abundancies among PCB-18 treated REs compared to acetone-treated ones. The metabolites have a mean ratio fold-change ≥ 1 and a p-value ≤ 0.05, corrected using the Benjamini-Hochberg (BH) method. In the acetone-treated sample, metabolites were ordered from the one with the lowest relative intensity (top of the graph) to the one with the highest relative intensity (bottom of the graph). In the PCB-18 bar, the corresponding relative intensity for each metabolite is shown, according to the heat-color map. **(D)** Boxplot showing the relative intensity of the annotated metabolites, expressed as $\log_2$ of the normalized intensity: scopoletin, β-alanine, hypoxanthine, L-arginyl-L-valine and L-seryl-L-phenylalanine in the root exudates released by *Arabidopsis* plantlets under PCB-18 stress or in the mock treatment.

3.3. Scopoletin negatively influenced the growth of PCB-degrading bacteria.

Scopoletin is a well-described plant secondary metabolite that mediates host-microbe interactions, commonly due to its antimicrobial properties (Stringlis et al., 2019; Voges et al., 2019). Since the relative abundance of this compound decreased in REs of PCB-stressed plants (Figure 2C), we verified if it could negatively affect the growth of PCB degrading bacteria, based on previous observations of scopoletin antimicrobial activity on beneficial/pathogenic microbes (Stringlis et al., 2018). *Acinetobacter* P320 was highly inhibited by scopoletin even at the lowest tested concentration of 500 µM (Figure 3A and Supplementary Figure 4A), showing a severe decrease in biomass accumulation already after 6 h upon scopoletin supplementation (Figure 3B) and an overall decrease of its relative growth after 24 h of exposure to this plant secondary metabolite (Figure 3C). Furthermore, 1 mM and 2 mM scopoletin negatively affected the doubling time of the bacterium (Supplementary Table 1) causing also a concentration-dependent reduction of the maximum growth rate ranging from 25.5% at 500 µM to 56% at 2mM (Figure 3C). Scopoletin inhibitory effect was less prominent on *Pseudomonas* JAB1, whose growth was hindered only at the highest assayed amounts (Figure 3D and Supplementary Figure 4B): 1 mM and 2 mM scopoletin supplies caused a decrease in biomass formation (Figure 3E) and a reduction in the bacterial maximum growth rate (Figure 3F). On the other hand, *P. xenovorans* LB400 growth was unaffected by scopoletin (Supplementary Figure 4C).

To counteract the deleterious effects of toxic metabolites, a protection strategy is embedding bacterial cells in a biofilm matrix. Therefore, we further verified if scopoletin could affect the biofilm formation ability in our model bacteria. In agreement with the observed reduction in bacterial growth, biofilm formation was stimulated in *Acinetobacter* P320 and *Pseudomonas* JAB1 when exposed at the highest assayed concentrations (Figure 3G, H), while in *Paraburkholderia* LB400 it was observed a reduction in the ability to build the biofilm matrix (Figure 3I). As scopoletin, also β-alanine showed a reduction in the root exudation profile of PCB-18 stressed plantlets. It was observed that at the highest assayed concentration, β-alanine stimulated *Paraburkholderia* LB400 biofilm formation, potentially pointing to a deleterious effect for bacterial cells (Supplementary Figure 5).

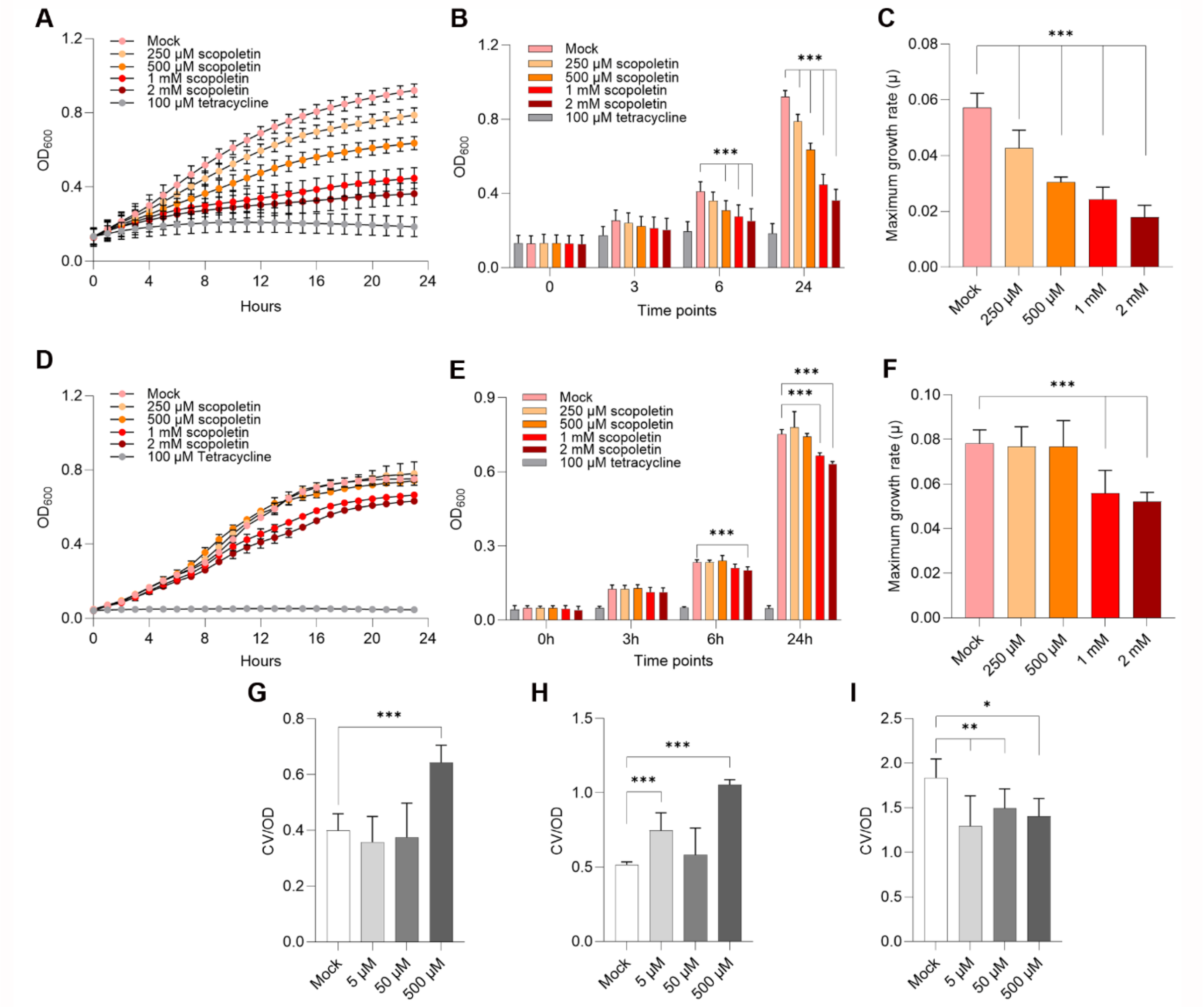


Figure 3. **Scopoletin negatively affected the growth of *Acinetobacter* P320 and *Pseudomonas* JAB1**. **(A)** and **(D)**: growth curves of *Acinetobacter* P320 and *Pseudomonas* JAB1, respectively, at increasing concentration of scopoletin. In the assay, 100 µM tetracycline was used as positive control for bacterial growth inhibition. **(B)** and **(E)**: bacterial biomass accumulation at different time points during the growth of *Acinetobacter* P320 and *Pseudomonas* JAB1, respectively, at increasing concentration of scopoletin. **(C)** and **(F)**: values of *Acinetobacter* P320 and *Pseudomonas* JAB1 maximum growth rate when exposed to increasing concentrations of scopoletin. For all the graphs, statistical analysis was performed using the Mann-Whitney test, by comparing the scopoletin treatment with the mock solvent control. ***$p \leq 0.001$. **(G)**, **(H)**, **(I)**: influence of scopoletin on biofilm formation ability of *Acinetobacter* P320, *Pseudomonas* JAB1 and *Paraburkholderia* LB400, respectively, represented as the ratio between the crystal violet OD (CV) of the stained biofilm and the optical density of the culture at 600 nm (OD). Statistical analysis was performed using the Mann-Whitney test (n = 3), by comparing the scopoletin treatment with the mock solvent control. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

3.4. Scopoletin negatively affected the functionality of the PCB-degrading strain *Acinetobacter* P320.

Based on previous observations, reporting that the growth of the strain *Acinetobacter* P320 was the most hampered by scopoletin, we investigated its influence on *Acinetobacter* P320-plant interaction under PCB-18 stress by using the *Arabidopsis f6'h1* mutant that is impaired in scopoletin biosynthesis. In mock-treated plantlets, *Acinetobacter* P320 stimulated plant growth in both WT e *f6'h1* lines without significant differences (Figure 4A and Supplementary Figure 6). On the contrary, upon PCB-18 stress the bacteria-triggered growth promotion was enhanced in the scopoletin-depleted plants compared to the wild type ones (Figure 4B and Supplementary Figure 6), although colonization efficiency was not altered (Figure 4C). These results indicate that scopoletin negatively affected the *Acinetobacter* P320-mediated ecosystem services provided to the plant under PCB stress. Scopoletin marginally affected *Pseudomonas* JAB1 functionality, being able to trigger the expression of the PCB degradative operon similarly to biphenyl (Supplementary Figure 7).

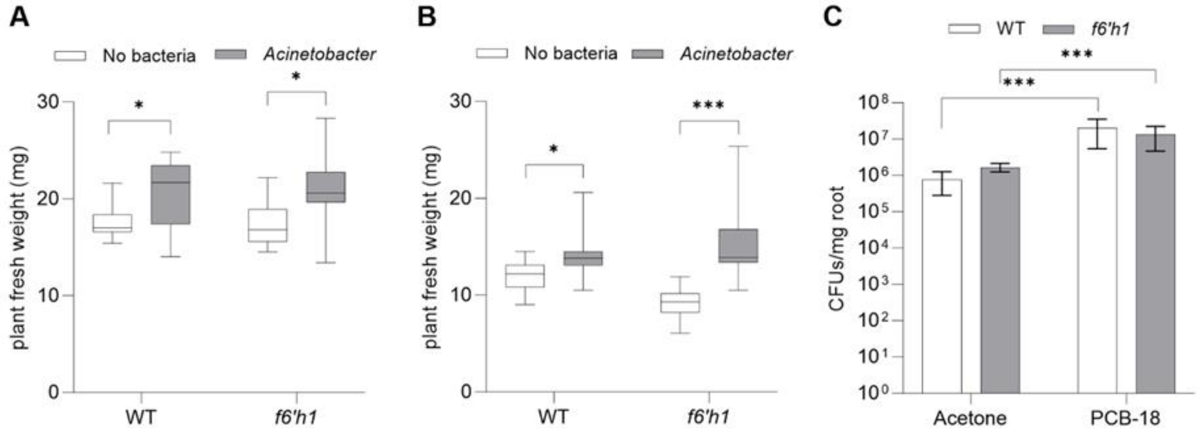


Figure 4. Scopoletin reduced *Acinetobacter* P320-triggered plant growth promotion under PCB stress. (A) Fresh weight of *Arabidopsis* plantlets exposed to mock treatment with acetone showed that *Acinetobacter* P320 is able to trigger a similar degree of growth promotion in both WT and in the scopoletin-depleted *f6'h1* line. **(B)** Fresh weight of *Arabidopsis* plantlets stressed by treatment with 20 µM PCB-18 indicated that *Acinetobacter* P320-induced growth promotion is enhanced in *f6'h1* plantlets (83%) compared to WT (18%), suggesting that scopoletin interferes with the bacterial promotion of plant growth under PCB stress. Statistical analysis was performed by applying the Mann-Whitney test (n = 21): *$p \leq 0.05$; ***$p \leq 0.001$. **(C)** Root colonization efficiency of *Acinetobacter* P320 in WT and *f6'h1* plantlets exposed to mock treatment or under PCB-18 stress. Statistical analysis was performed by applying the Mann-Whitney test (3 biological replicates with 3 technical replicates each): ***$p \leq 0.001$.

3.5. Metabolites which accumulation was enhanced under PCB stress serve as nutrients for PCB-degrading bacteria and diversely affected rhizocompetence traits.

Based on their increase in relative abundance in PCB-treated REs, it was verified if hypoxanthine, L-seryl-L-phenylalanine and L-arginyl-L-valine can act as carbon/nitrogen sources, proliferation molecules or signaling compounds that affect the physiology of the selected degrading bacteria. Hypoxanthine was exploited as carbon source by *Paraburkholderia* LB400 (Figure 5A) and by *Pseudomonas* JAB1 (Figure 5B). In the latter strain this compound provided a nitrogen source for growth (Figure 5C) and enhanced bacterial swimming motility (Figure 5D). The dipeptide SF was used by strain LB400 as nitrogen source (Figure 5E) and acted as growth promoting factor by enhancing *Acinetobacter* P320 biomass formation and total proliferation when supplied at the highest concentrations of 50 and 100 µM

(Figure 5F). Biofilm formation showed contrasting results depending on the bacterial strain, the root exudate compounds and assayed concentrations (Supplementary Figure 8).

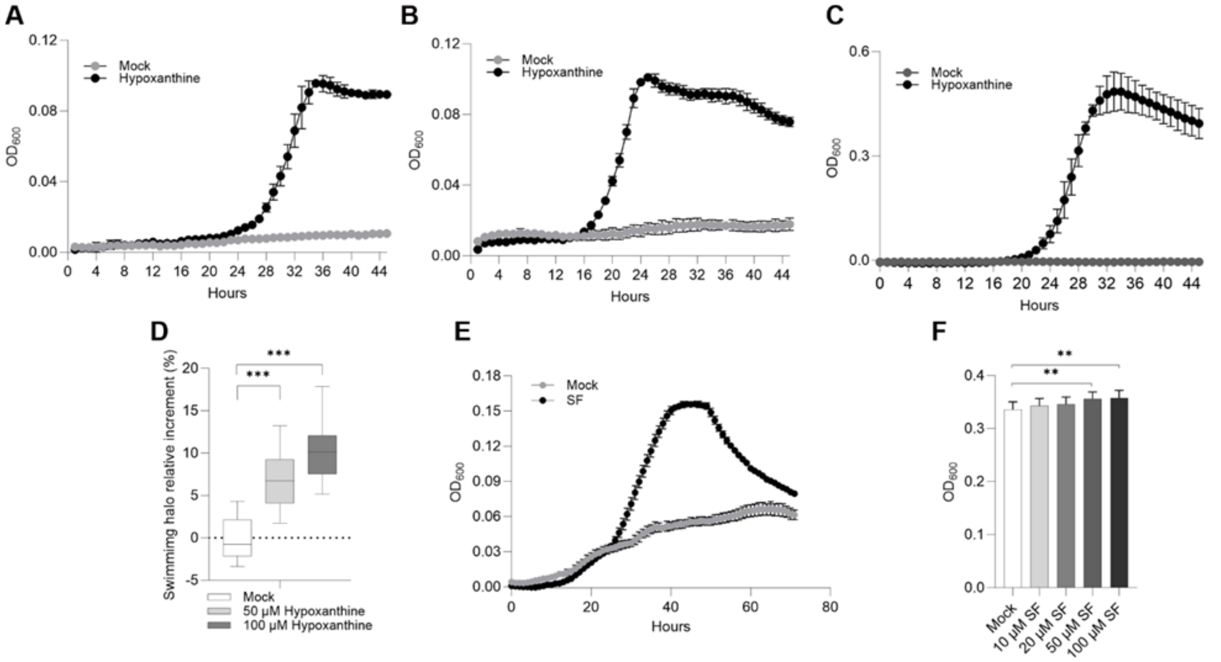


Figure 5. Hypoxanthine and L-seryl-L-phenylalanine (SF) are exploited as nutrient sources and signaling molecules by PCB-degrading bacteria. (A), (B): Growth curves of strain LB400 and strain JAB1, respectively, in mineral medium in presence of hypoxanthine as carbon source. **(C)** Growth curve of strain JAB1 in mineral medium in presence of hypoxanthine as nitrogen source. **(D)** Swimming ability of strain JAB1 in presence of hypoxanthine. Statistical analysis was carried out on at least 3 independent experiments using Dunn's *post-hoc* test; $***p \leq 0.001$. **(E)** Growth curve of strain LB400 in mineral medium in presence of L-seryl-L-phenylalanine as nitrogen source. **(F)** Growth modulation activity of L-seryl-L-phenylalanine supplemented as pure chemicals to 1/10 TSB liquid medium in *Acinetobacter* P320. In the graph, bacterial biomass reached at stationary phase at 23 hours is reported, expressed as OD_{600}. The bars represent the average ± standard deviation of three independent replicates. Statistical analysis was performed using the Mann-Whitney test by comparing the exudate concentrations with the respective solvent control ($*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$).

4. DISCUSSION

Due to their former industrial production, soil pollution by PCBs is a worldwide issue and still nowadays the toxicity and recalcitrance of these persistent organic pollutants pose a thread for human, animal and ecosystem health and compromise plant growth and development in agricultural land. A sustainable solution particularly suited for large areas contaminated by these xenobiotics is rhizoremediation, which exploits the plant holobiont as a unit of Darwinian selection composed of plants that can thrive in contaminated environments strictly associated with PCB-degrading microbes. Plant-associated microorganisms are intriguing biological resources for PCB remediation: from an evolutionary perspective, the *bph* operon served for the degradation of biphenyl-like plant secondary metabolites and it was hijacked for PCB catabolism due to the structural resemblance between these two classes of molecules (Singer et al., 2004). This merged metabolism is the result of the enzymatic versatility of the microbial peripheral pathway that shows no strict substrate preference, allowing the catabolism of compounds with a similar backbone (Ren et al., 2018). Although in contaminated environments degrading bacteria have the selective advantage to exploit pollutants for their metabolism, often pollutants are poorly bioavailable or their high concentrations can be deleterious also for the microbial cells themselves. The growth of plants in polluted sites contributes to remedy this issue since root exudation is believed to affect the pollutant mobilization and therefore this effect positively impacts the degrading functionality of the associated microbiome. Indeed, it was demonstrated that root exudation affects the fractions of dissolved and particulate organic carbon that mediate the movement of hydrophobic compounds in soil (Terzaghi et al., 2020): the “rhizosphere effect” contributed to de-adsorb from soil particles and to mobilize up to 62% of the initial PCB contamination (Chekol et al., 2004). Furthermore, root exudation performs a biostimulating role by providing nutrients, signaling molecules and co-metabolites to significantly increase the biological activity (growth and enzyme activities) of degrading microorganisms (Lu et al., 2017; Vaidya et al., 2022). In phenanthrene-spiked microcosms cultivated with clover, *Novosphingobium* sp.HR1a showed a two-fold higher growth in terms of CFUs compared to unvegetated soil, because clover root exudates were exploited for carbon and nitrogen supply and enhanced the expression of the bacterial dioxygenase involved in the degradation of PAHs, favoring plant release from stress (Molina et al., 2021). Plant primary and secondary metabolites are in particular valuable candidates to support the microbial aerobic cleavage of biphenyls and anaerobic dechlorination of PCBs (Vergani et al., 2017b). In this study, we explored the hypothesis that the PCB-induced phytotoxic effects could drive the plant to modify its root chemistry in a ‘cry-for-help’ strategy to recruit, feed and sustain PCB-degrading microbes in the rhizosphere. By using *Arabidopsis thaliana*, a model plant for holobiont studies (Poupin et al., 2023), we developed an experimental set-up to recapitulate the main hallmarks of PCB phytotoxicity, including reduced plant growth (Subramanian et al., 2018), chlorosis (Liu and Schnoor, 2008), overproduction of ROS (Wang et al., 2021) and alteration of plant homeostasis (Ren et al., 2022). Although the so-called “green liver” model is designated to detoxify root-absorbed PCBs through oxidation, conjugation with glutathione and storage in the vacuole (Coleman et al., 1997), the system results inefficient (Schäffner et al., 2002) because PCB catabolism *in planta* is hampered by several factors that can dramatically influence their recalcitrance (Aken et al., 2010; Rezek et al., 2007). Under PCB-18 stress, the metabolomic investigation performed in this work demonstrated that the root exudation pattern was dramatically altered compared to mock-treated plantlets, potentially supporting a ‘cry-for-help’ hypothesis triggered by PCB detrimental effects. Upon plant exposure to different external stress factors, root exudation previously showed to be tuned both quantitatively and qualitatively as a developmental strategy to cope with unfavorable conditions (Rizaludin et al., 2021). In aluminum contaminated soil, the release of malate was indeed increased in maize and soybean exudates since the high metal chelating ability of this molecule reduces aluminum uptake by plant roots (Liang et al., 2013). Similarly, over-production of proline and pinitol in soybean root exudates contributed to plant adaptation to drought through the osmolyte properties of these compounds (Canarini et al., 2016). In this study our hypothesis is that the change in the root exudate fingerprint is not correlated to a direct effect toward the plant, rather to an indirect effect mediated by the biostimulation of degrading microbes in the rhizosphere.

Plant metabolic and physiological responses to xenobiotic exposition, including PCBs, is still poorly characterized (Lin et al., 2020; Subramanian et al., 2018) and would deserve increasing attention by the research community to drive knowledge-based solutions for rhizoremediation in terms, for instance, of plant species selection, in order to cope with stress and biostimulate PCB degradation as observed for *Festuca arundinacea* in a semi-field trial (Terzaghi et al., 2019).

The majority of metabolites detected in our root exudate analysis could not be identified: despite the technical improvement of metabolomics approaches in recent years (Patel et al., 2021), the expected repertoire of metabolites in plants is extremely complex and uncharted (Alseekh and Fernie, 2018), pushing the limits in terms of molecule identification (Aharoni et al., 2023). Among the compounds identified, scopoletin was the unique secondary metabolite. Being one of the best characterized *Arabidopsis* exudate components (Stringlis et al., 2019), it is produced under iron deficiency and it selectively impacts the assembly of the rhizosphere microbial community (Voges et al., 2019), leading to enhanced plant growth due to the generation of conditions that favor the establishment of beneficial bacteria like *Pseudomonas simiae* WCS417 (Stringlis et al., 2018). The ability of scopoletin to sculpt the root-associated microbiome is linked to its differential antimicrobial features, inhibiting the growth of phytopathogenic fungi like *Fusarium* and *Verticilloides* spp, while being ineffective on beneficial bacteria like *Pseudomonas capeferrum* WCS358 (Stringlis et al., 2018), supposedly insensitive to its ROS-induced stress (Voges et al., 2019). In our study we observed that, in PCB-18 stressed plants scopoletin relative abundance decreased in the root exudates, potentially remodeling the rhizosphere niche with more suitable conditions to accommodate degrading bacteria. Indeed, it was observed that both *Acinetobacter* P320 and *Pseudomonas* JAB1 were sensitive to scopoletin antimicrobial activity, although at different levels, while only *P. xenovorans* LB400 was resistant. Scopoletin negatively affected different growth parameters of sensitive bacterial strains, decreasing their viability, biomass accumulation and maximum growth rate with the ultimate effect to unbalance their fitness and services provided to the plant host. For *Acinetobacter* P320, indeed, it was observed that the bacterium-triggered growth enhancement of the plant under PCB-18 stress was restricted by scopoletin, as indicated by the higher promotion in the scopoletin-depleted mutant *f6’h1* compared to scopoletin-producing WT plantlets.

The coumarin umbelliferone has been previously demonstrated to induce the expression of the *bph*-encoded degradative enzymes in *Pseudomonas* JAB1 (Zubrova et al., 2021) and a similar effect was observed for scopoletin in the present study. The ability to act as *bph*-transcriptional inducer is nevertheless not a generalized common trait for the same class of secondary metabolites. Coumarin, for instance, could not trigger *bph* expression in strain JAB1 and a similar differential activity for compounds belonging to the same class was observed also for flavonoids with catechin and quercetin acting as *bph* transcriptional inducers while flavone did not (Zubrova et al., 2021). Collectively, these findings indicate a regulated release of scopoletin by *Arabidopsis* roots exposed to different stresses, like Fe deficiency and PCB pollution, reinforcing a highly specific role for this molecule in mediating plant abiotic resistance through the recruitment and sustainment of a beneficial microbiota.

β-alanine is part of the 250 non-proteinogenic amino-acids produced by plants to perform anti-herbivory and anti-microbial actions and to mediate response to abiotic stresses (Parthasarathy et al., 2019). The decrease of its relative abundance in root exudates under PCB-18 stress may appear controversial, since its accumulation is documented under a diverse array of stresses, including drought and heavy metal contamination. Nevertheless, β-alanine is a structural component of pantothenate that contributes to coenzyme A backbone. The TCA cycle, in which the coenzyme A shuttle is fundamental, is a major target of PCB stress in rice, leading to a higher energy demand that dramatically affects plant growth (Lin et al., 2020). Therefore, the observed pattern in PCB-exposed plantlets may be part of plant response to PCB stress. β-alanine-mediated effect on the physiology of PCB degrading bacteria is largely cryptic and the only effect we have observed was linked to *P. xenovorans* LB400 decreased biofilm formation only at the intermediate assayed concentration. Biofilm formation on the rhizoplane is considered an early determinant in successful plant–microbe interactions: β-alanine, and scopoletin as well, both reduced in PCB stressed plants, seem to affect this trait in strain LB400, potentially decreasing its fitness. Regarding scopoletin, coumarins have been identified as effective antibiofilm and anti-quorum sensing compounds in medical/pharmaceutical fields against human pathogens (Reen et al., 2018), while few reports are available in the environmental microbiology context as antagonist molecules able to block *Ralstonia solanacearum* outbreak (Yang et al., 2017, 2016). Being sensitive to scopoletin, in presence of this compound *Acinetobacter* P320 and *Pseudomonas* JAB1 showed an increased ability to form biofilm, that could potentially act as a barrier against penetration and contact with toxic compounds (Shree et al., 2023).

The identified compounds that increased their relative abundance in root exudates upon PCB-18 treatment are all primary metabolites, an abundant and heterogenous class of molecules that are supposed to be released by the root tip through the action of efflux transporters (Canarini et al., 2019). Primary metabolites demonstrated to exert a strong selective pressure on microbial diversity (Shi et al., 2011; Steinauer et al., 2016) that, in turn, affects exudates concentration in the rhizosphere through their preferential microbial utilization (Zhalnina et al., 2018).

Hypoxanthine is a purine catabolic product that is then converted to allantoin and allantoate, metabolites that can act as ROS scavengers, and showed to attenuate stress symptoms under darkness stress and senescence in *Arabidopsis* (Brychkova et al., 2008). Its overproduction in root exudates in the presence of PCB-18 can be interpreted as a response to PCB phytotoxicity. In the rhizosphere, hypoxanthine is generally used by soil microorganisms as a nitrogen source (Izaguirre-Mayoral et al., 2018) and a specific chemoreceptor for its translocation has been identified in *P. putida* KT2440, a well-known plant growth promoting strain (Fernández et al., 2016). In our study, it was observed that hypoxanthine can be exploited as both N and C sources by *Pseudomonas* JAB1 and that it could, moreover, improve the bacterium swimming motility. Hypoxanthine depletion in *Pseudomonas fluorescens* Pf0-1 led to a reduced performance in the rhizosphere, due to an altered transition from planktonic cells to biofilm attached cells (Yoshioka and Newell, 2016). In our study, hypoxanthine inhibition of biofilm formation for *Pseudomonas* JAB1 and *Paraburkholderia* LB400 was concentration dependent. Overall, these results indicate that hypoxanthine can be exploited as nutrient and can diversely affect rhizosphere colonization traits in the selected PCB-degrading bacteria.

Low molecular weight peptides, amino acids and derivatives produced from the cleavage of larger proteins are typical constituents of plant root exudation (McLaughlin et al., 2023) and different dipeptides were identified in *Arabidopsis* root secretome (Strehmel et al., 2014). In our work, the identified dipeptides overproduced under PCB stress showed both a capacity to serve as nitrogen sources and as stimulators of bacterial proliferation as putative co-metabolites, in agreement with previous findings (Moormann et al., 2022). Furthermore, we suggested a possible role as mediators of processes necessary for root colonization as biofilm formation and swimming motility. Such aspects are largely overlooked, although sparse evidence supports the involvement of amino acids and peptides in regulatory processes as signal molecules in plant-bacteria interactions (Minen et al., 2023).

5. CONCLUSIONS

The analyses, performed on the developed *in vitro* set up that mimics PCB phytotoxic effects in plants, delineate the scenario of a shift in *Arabidopsis* exudation pattern upon stress. Exuded metabolites were collected in liquid medium, using a widely adopted approach for root metabolomics studies that, nevertheless, represents an approximation of the exudation process occurring in soil. Furthermore, the selection of the liquid media and the incubation time can have a large impact on exudation rates and composition (Maurer et al., 2021). Although by using three model bacterial degraders a comprehensive postulate on the role of the identified root exudates cannot be completely ruled out, a potential 'cry-for-help' effect could be envisaged in terms of chemistry remodeling of the root niche to recruit degrading bacteria. In soil, roots interact simultaneously with a plethora of microorganisms (Rolli et al., 2022) and the outcome of these associations in PCB polluted sites have been reported to be influenced by both xenobiotic concentrations and soil edaphic factors (Mapelli et al., 2022). Rhizodepositions are crucial players in microbiome dynamics, although contrasting effects exerted by pure root metabolites, depending on their concentration and on the bacterial species involved, can hamper the comprehension of clear causation effects (Ghitti et al., 2024; He et al., 2022; Huang et al., 2019). Based on evidence obtained *in vitro* on model plants and microorganisms, further studies are claimed since the identification of the root metabolic drivers of bacterial assembly in polluted environments is of paramount importance to steer the soil microbiome, potentially enriching those populations endowed with the highest bioremediation potential.

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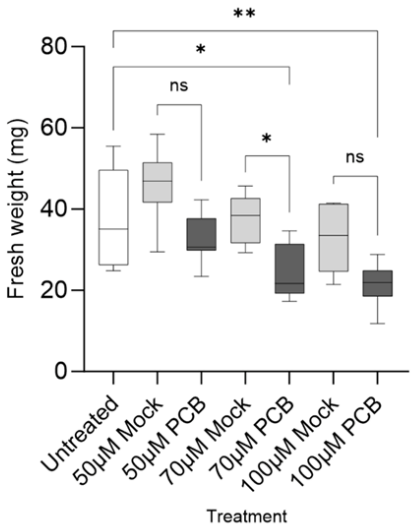
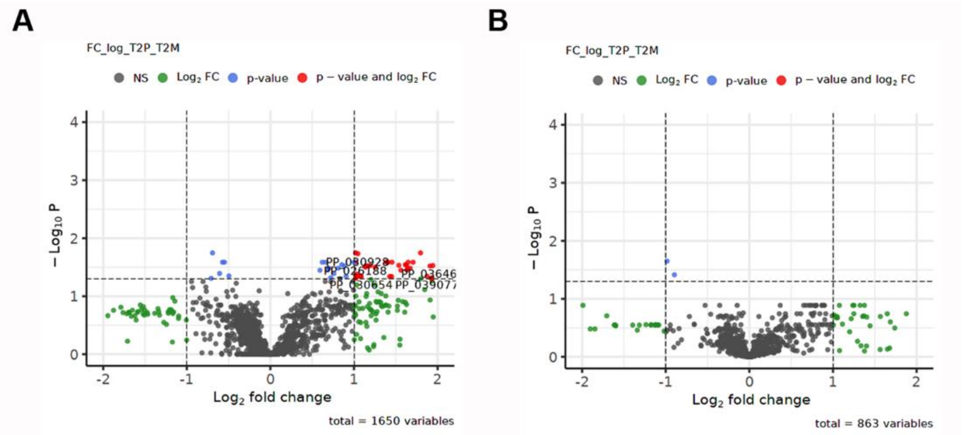
Supplementary materials

Bacterium	**Treatment**	**Concentration**	**Doubling time**	**Statistical analysis**
Acinetobacter P320	MetOH	80%	3:14 ± 0:33	
	Scopoletin	250 µM	3:37 ± 0:46	ns
		500 µM	4:12 ± 0:45	ns
		1 mM	4:19 ± 0:27	***
		2 mM	5:32 ± 0:23	***
Pseudomonas JAB1	MetOH	80%	2:36 ± 0:24	
	Scopoletin	250 µM	2:36 ± 0:34	ns
		500 µM	2:47 ± 0:37	ns
		1 mM	3:25 ± 0:20	***
		2 mM	3:30 ± 0:37	ns

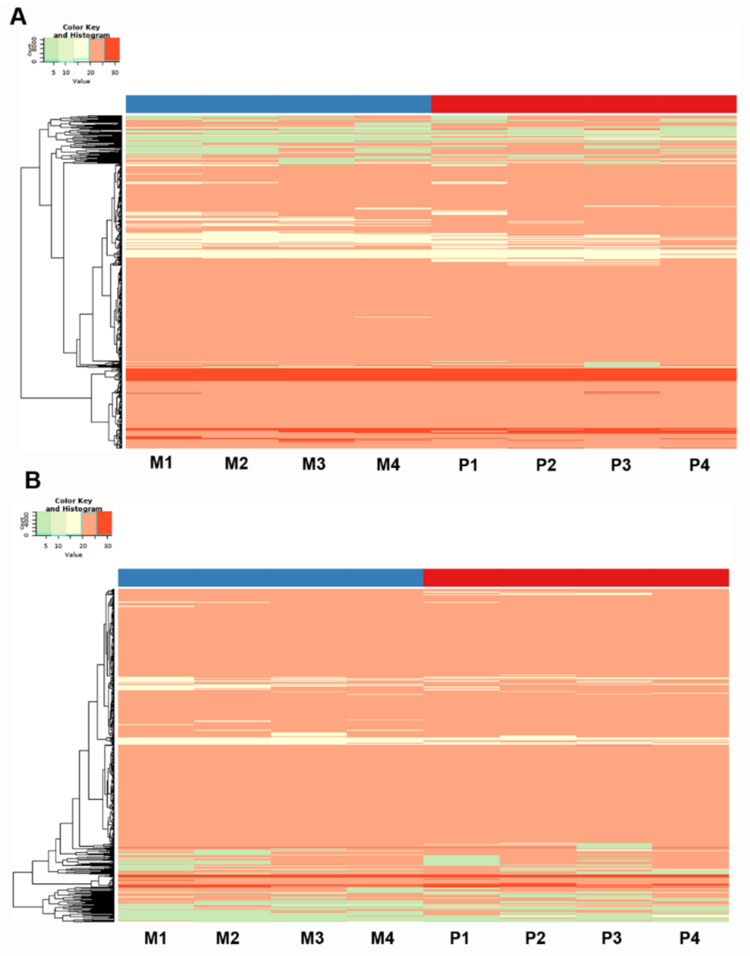
Supplementary Table 1. Values of *Acinetobacter* P320 and *Pseudomonas* JAB1 doubling time when exposed to increasing concentrations (250 µM to 2 mM) of scopoletin. The doubling time was indicated as hours:minutes. The plant secondary metabolite was dissolved in 80% methanol, indicated here as control. Statistical analysis was performed using the Mann-Whitney test, by comparing the scopoletin treatment with the mock solvent control. ***$p \leq 0.001$; ns: non-significant ($p > 0.05$).



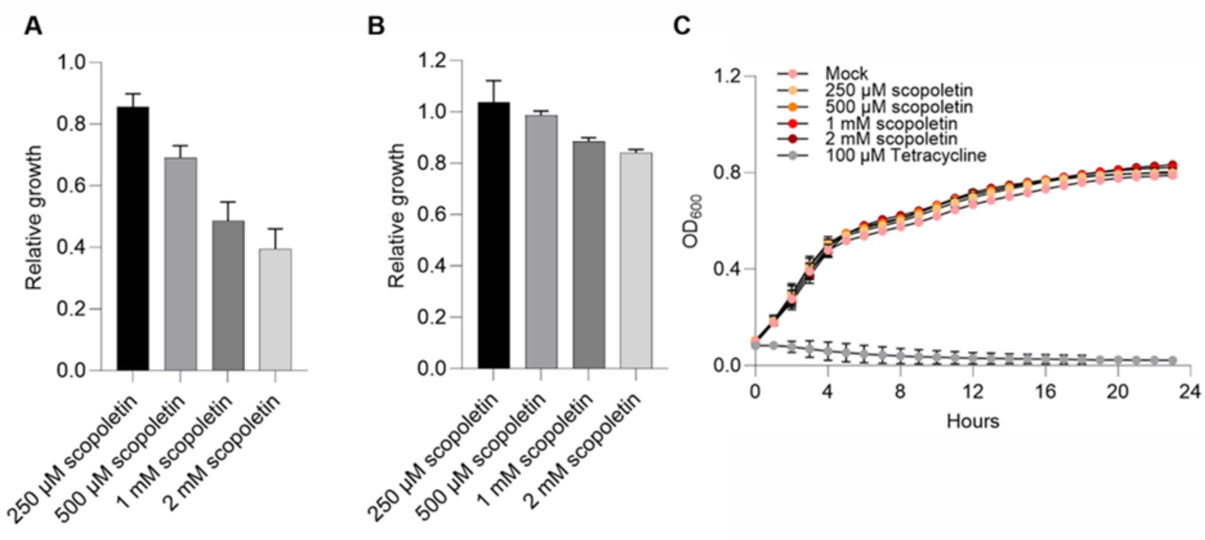
Supplementary Figure 1. *Arabidopsis thaliana* sensitivity to PCB-18 phytotoxic effects. This assay was fundamental to establish the PCB-18 concentration to be used to trigger *in vitro* PCB stress. Seven days post-germination in plate, *Arabidopsis* plantlets were transferred in a 24-well plate containing 1/2 MS supplemented with increasing concentrations of PCB-18 or an equal volume of acetone, the solvent in which the pollutant was resuspended. Each well contained 3 plantlets, 3/4 technical replicates were used and the experiment was repeated twice. The plantlets in each well were pooled and weighed by using a precision scale. Acetone application in mock treated samples did not interfere with plant growth, showing similar plant fresh weight if compared with untreated plantlets. It was observed that 50 µM PCB-18 application caused a 28.4 ± 1.8% decrease compared to the mock treatment. At 70 and 100 µM concentrations, a strongest PCB-18-triggered phytotoxic effect was observed compared to 50 µM, although the percentage decrease was quite similar and corresponded to 35.8 ± 4.5% and 34.7 ± 11% respectively. Considering a lower sample variability, 70 µM concentration was selected to identify the PCB-triggered 'cry-for-help' exudation pattern.



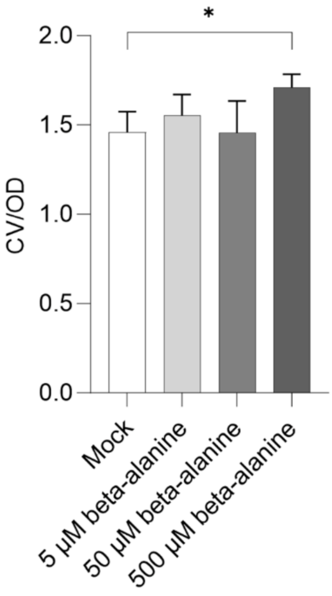
Supplementary Figure 2. Volcano plots of the untargeted metabolomic analysis to investigate PCB-triggered changes in *Arabidopsis* root exudation pattern. (A), (B): volcano plots of the detected metabolite features obtained by LC-MS in the positive polarity and in the negative polarity, respectively. These data represent all metabolite features with a fold change that was >$\log_2|1|$ and for which the p-values of the two-tailed t-test was corrected using the Benjamini-Hochberg (BH) method.



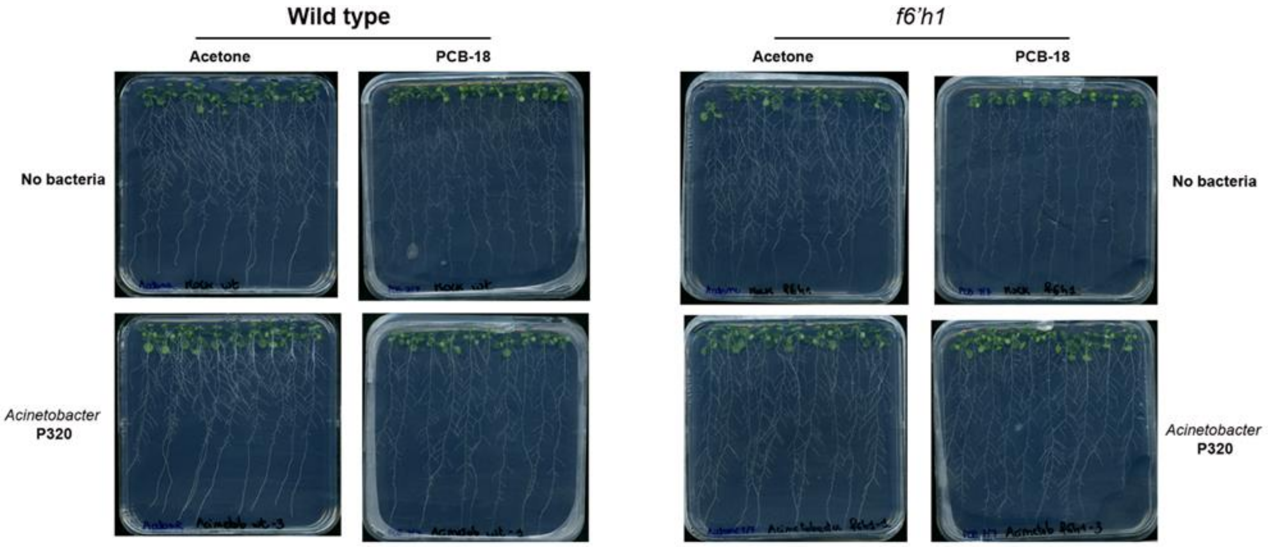
Supplementary Figure 3. (A), (B) Heatmaps of the hydrophilic metabolites detected through LC-MS in positive and negative polarity mode, respectively. The graphs represent the root exudates released by plantlets exposed for 2 days (T2) challenged with 70 µM PCB-18 (P, in red in the graph) or mock-inoculated with an equal amount of acetone (M, in blue in the graph).



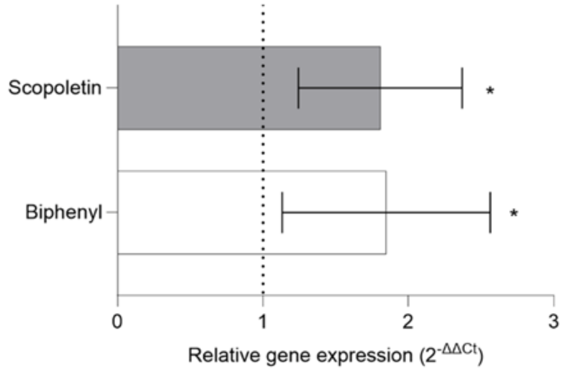
Supplementary Figure 4. Scopoletin affected the growth of the selected PCB-degrading strains. (A) and **(B)** *Acinetobacter* P320 and *Pseudomonas* JAB1 relative growth, respectively, at increasing concentrations of scopoletin. The bars represent the ratio between the OD_{600} value of the culture in presence of scopoletin and the OD_{600} of the control culture grown in presence of the solvent used to resuspend the plant secondary metabolite. **(C)** *P. xenovorans* LB400 growth is not affected by scopoletin supplements to the medium. In the assay, 100 µM tetracycline was used as positive control for growth inhibition.



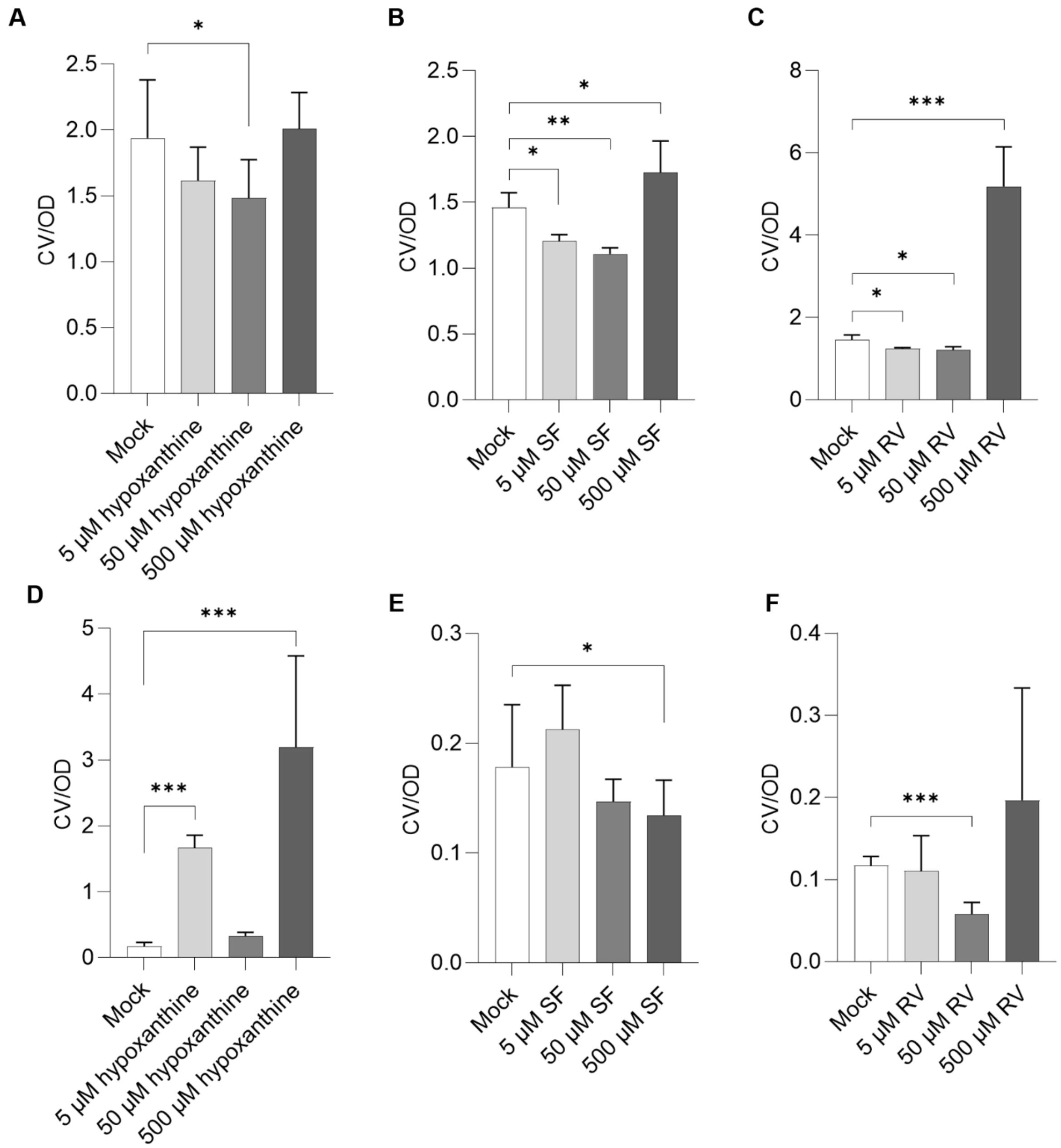
Supplementary Figure 5. β-alanine positively affected strain LB400 ability to form a biofilm. Biofilm formation ability of the strain was represented as the ratio between the crystal violet OD (CV) of the stained biofilm and the optical density of the culture at 600 nm (OD). Statistical analysis was performed using the Mann-Whitney test (n=3), by comparing the beta-alanine treatment with the mock solvent control. **p* ≤ 0.05.



Supplementary Figure 6. Representative images of *Acinetobacter* P320-induced growth promotion in *Arabidopsis* plantlets in WT and *f6'h1* line under mock treatment (acetone) and PCB-18 induced stress. The brightness of the images was enhanced for all the displayed samples (value 84 by using the contrast in Photosphop CS5 Extended v.12) to improve the visibility of the root system.



Supplementary Figure 7. Scopoletin acts as inducer of the *bphA* gene for PCBs catabolism in *Pseudomonas* JAB1. The relative gene expression of *bphA* gene was quantified via RT-qPCR, using the *infB* gene as housekeeping gene. The results of the relative expression of *bphA* transcripts are expressed as ΔΔCt, indicating the average fold change over the non-induced control. The black line (relative gene expression value = 1) represents the baseline of gene expression in the non-induced control. Biphenyl was used as positive control. Error bars represent the standard deviation of 3 independent experiments. Statistical analysis was performed using the Mann-Whitney test (n=3), confidence interval 95%. **:* $p \leq 0.05$.



Supplementary Figure 8. Effects of hypoxanthine, seryl-L-phenylalanine (SF) and L-arginyl-L-valine (RV) on biofilm formation ability of *Paraburkholderia* LB400 and *Pseudomonas* JAB1. (A), **(B)** and **(C)** biofilm formation ability in *Paraburkholderia* LB400 at increasing concentrations of hypoxanthine, SF and RV dipeptides. **(D)**, **(E)** and **(F)** biofilm formation ability in *Pseudomonas* JAB1 at increasing concentrations of hypoxanthine, SF and RV dipeptides. Biofilm formation ability of the strains was represented as the ratio between the crystal violet OD (CV) of the stained biofilm and the optical density of the culture at 600 nm (OD). Statistical analysis was performed using the Mann-Whitney test (n=3), by comparing the compound treatment with the mock solvent control. *$p \leq 0.05$; **$p \leq 0.01$, ***$p \leq 0.001$.

CHAPTER V

Development of a bacterial biosensor using the PCB degrader *Pseudomonas alcaliphila* JAB1: a promising tool to study plant-bacteria interactions upon PCB stress

Abstract

Rhizoremediation is a sustainable technique for soil clean-up from recalcitrant xenobiotics like polychlorinated biphenyls (PCBs) and exploits the complex frame of interactions occurring in the plant holobiont. Plant-bacteria crosstalk is mediated by root exudation of primary and secondary metabolites (PSMs) which composition is reshaped under stress condition through a mechanism known as ‘cry-for-help’. Previous reports demonstrated that PSMs, flavonoids among others, induced the activation of the biphenyl oxidative pathway in PCB degrading bacteria due to molecular structural analogy, thus boosting their degradative capacity. In this study, a bacterial biosensor was developed using the PCB-degrading strain *P. alcaliphila* JAB1 that was tagged with (i) a chromosomal constitutive mScarlet protein to identify the root colonization pattern and (ii) a plasmidic inducible eGFP protein, regulated by the putative *bph* operon promoter, to monitor the activation of the PCB catabolic pathway. This approach is useful to determine the topography of bacterial colonization on plant roots and the activation of the PCB catabolic pathway, thus elucidating the role of PCB-induced root exudates in modulating the PCB degradation potential of strain JAB1. The biosensor was applied in a plant-bacteria interaction assay by exposing *Arabidopsis thaliana* plants to PCB-18 stress. Root exudates (REs) released by *Arabidopsis* cultivated upon PCB stress sustained the growth of the strain more than untreated plants’ REs, indicating a putative ‘cry-for-help’ mechanism. Moreover, JAB1 strain root colonization of *tt8* flavonoid over-accumulating *Arabidopsis* mutant line was favored when the plant was subjected to PCB stress, and its REs effectively promoted the expression of PCB degradation genes in JAB1, thus confirming an involvement of flavonoids in inducing the pathway. The developed biosensor was a valuable tool for unveiling the crosstalk between plants and beneficial bacteria mediated by REs in holobionts challenged by PCB stress, providing information useful for a future set up of soil rhizoremediation approaches.

1. Introduction

Developing and improving sustainable techniques for the remediation of soils contaminated by persistent and poorly biodegradable xenobiotics is a major challenge when addressing the issue of mitigating environmental pollution (Thijs et al., 2016; Arora, 2018). In the frame of polychlorinated biphenyls (PCBs) soil pollution, degrading bacteria that adapted to contaminated environmental niches possess the genetic background essential for the complete mineralization of PCBs. Therefore, the utilization of PCB degrading strains for soil clean-up through rhizoremediation approaches has gained increasing interest (Zenteno-Rojas et al., 2020; Vergani et al., 2022). PCB-degrading bacteria are commonly chromosomally endowed with the *bph* operon, the gene cluster that encodes for the enzymes necessary for aerobic biphenyl/PCB catabolism (Vergani et al., 2019). The expression of this operon permits the catabolism of low chlorinated PCB congeners, with up to 6 chlorine atoms, through a composite pathway where the action of dioxygenases, dehydrogenase and hydrolase enzymes leads to the mineralization of PCB molecules to benzoic acid and acetyl CoA (Vergani et al., 2017; Hirose et al., 2019). Many PCB-degrading bacterial strains possessing this enzymatic arsenal have been isolated from historically contaminated soils (Ridl et al., 2018; Vergani et al., 2019; Garrido-Sanz et al., 2020). *Pseudomonas alcaliphila* JAB1 was isolated from a PCB-contaminated soil in Czech Republic and selected for its versatility in the degradation of several PCB congeners. Strain JAB1 genome is available and annotated and features the presence of a gene cluster for biphenyl/PCB degradation with identical structure compared to the *bph* operon of the known PCB-degrader *P. furukawaii* KF707 (Ridl et al., 2018; Suman et al., 2021). Strain JAB1 was recently employed to investigate the role of a selection of root exuded plant secondary metabolites (PSMs) on the induction of the biphenyl degradation pathway (Zubrova et al., 2021). Indeed, it has been demonstrated that root exudates that share structural similarity with biphenyl can trigger the expression of the PCB catabolic genes by acting as co-metabolites or inducers and improve the expression of the enzymatic machinery (Singer et al., 2003; Toussaint et al., 2012; Uhlik et al., 2022). In a study conducted by Pino and colleagues, flavonoids naturally exuded by a selection of plants (*Avena sativa*, *Brachiaria decumbens*, *Medicago sativa* and *Brassica juncea*) increased the PCB degradation potential of a microbial consortium constituted by *Pseudomonas* spp. and *Stenotrophomonas* spp. (Pino et al., 2016). Plant materials containing high concentrations of terpenes showed to increase the degradation of a PCB mixture mediated by a *Pseudomonas stutzeri* strain isolated from a PCB-contaminated soil (Dudášová et al., 2012). Further studies demonstrated also that PSMs-induced biodegradation of PCBs was tied to an increased expression of the *bph* operon genes, thus helping to clarify the fine mechanism regulating this interaction. As previously mentioned, flavonoids like flavone and quercetin and terpenes like limonene, carvone and pinene were seen to activate the expression of *bphA* gene in *P. alcaliphila* JAB1 (Zubrova et al., 2021) and Pham and colleagues demonstrated that isoflavone boosted the expression of *bphA* in *Rhodococcus erythropolis* U23A (Pham et al., 2015).

A still poorly exploited experimental technique, applicable for the improvement and fine-tuning of PCB rhizoremediation strategies, is the utilization of bacterial biosensors (bioreporters). Biosensors are synthetic biology tools for the real-time and rapid detection of specific molecules in the environment, to analyze and quantify their presence with a technology that is generally less laborious than chemical analyses (Dash and Osborne, 2023). Most importantly, biosensors quantify the bioavailable fraction of the target compound, essential to determine its realistic biological effects, toxicity and biodegradability, and translate it into easily measurable signals (e.g. luminescence or fluorescence) (van der Meer and Belkin, 2010; Bilal and Iqbal, 2019). Indeed, the use of bioreporters can be advantageous for rapid and cost-effective detection of contaminants and their bioavailability (Zeng et al., 2021) as well as for studying the molecular mechanisms inducing the degradation pathways in rhizoremediation, with the perspective of unveiling the functioning of plant-bacteria crosstalk mediated by root exudates in a contaminated environment (Trögl et al., 2012; Pini et al., 2017). Many works highlighted the importance of bioreporter technology to monitor environmental contamination, for instance to detect the amount of bioavailable heavy metals like Cr(IV) in environmental matrices (Francisco et al., 2019), but also hydrocarbons like petrol alkanes (Sevilla et al., 2015) and phenantrene (Wei et al., 2014). Furthermore, biosensors utility can be broadened by their application in plant-bacteria interaction studies, to visualize bacterial distribution on plant roots determined by the exudation of specific metabolites and observe the activation of contaminant-degrading pathways. A Lux-based biosensor was recently developed using the strain *Pseudomonas syringae* pv. *phaseolicola* RJ3 to analyze its spatial distribution and the temporal dynamics of colonization of *P. vulgaris* leaves during systemic infection (Soldan et al., 2021). Pini and collaborators reported a study where an engineered *Rhizobium leguminosarum* biosensor strain was used to detect the root exudation of legume signal molecules like sugars, organic acids and flavonoids, to elucidate the steps required in the establishment of legume-rhizobia symbioses (Pini et al., 2017). In the frame of PCB rhizoremediation, *P. fluorescens* F113rifpcb bioreporter strain was constructed, coding for a GFP which expression is inducible by 3-chlorobenzoate, a PCB degradation product. F113rifpcb was tested on *M. sativa* roots in presence of PCB-2 and helped identify root areas where co-metabolites could potentially induce the degradation pathway. In this case, GFP expression was visible on *M. sativa* roots mainly on the mucilage present near the root tip (Boldt et al., 2004). Deeper investigation is needed to understand the complexity of plant-bacteria crosstalk, in particular concerning plant root exudates' involvement in the expression and regulation of bacterial *bph* operon, in modulating bacterial physiology and metabolism and directing root colonization upon PCB contamination. Therefore, in the present study, we designed a fluorescence-based bacterial bioreporter to observe the induction of the biphenyl degradation pathway using the gram-negative PCB degrading strain *P. alcaliphila* JAB1. The use of the JAB1 biosensor strain allowed the identification of differential responses to root exudation induced by PCB stress, useful to recognize a potential 'cry-for-help' response of the plant challenged by the presence of PCBs.

2. Materials and methods

2.1. Bacterial strain, plant material, culture media and chemicals

The bacterial strain used in this study is *Pseudomonas alcaliphila* JAB1, a PCB-degrading strain isolated from an historically contaminated soil in Czech Republic (Ridl et al., 2018) and was kindly provided by Pr. Ondřej Uhlík (UCT Prague). For the assays, the strain was grown in tryptic soy broth (TSB, Condalab, Spain) or in mineral medium Brunner (MMB; DSMZ, Germany) supplemented with 30 mM sodium pyruvate as carbon source. The bacterium was maintained in a glycerol stock at -80°C and weekly revitalized on Luria-Bertani (Merck, Germany) agar plates. Antibiotics were added to the growth medium to select the fluorescence tagged JAB1 strains at the following concentrations: 250 µg/mL chloramphenicol, 15 µg/mL gentamicin and 100 µg/mL rifampicin. The chemicals used were biphenyl (Merck, Germany), solubilized in a 0.5 M stock in acetone, and PCB No. 18 (PCB-18, 2,2',5-trichlorobiphenyl, LCG Standards), solubilized in a 200 mM stock in acetone. The plant material employed in the study were *Arabidopsis thaliana* accessions Col-0 and L*er*, comprised the mutants for flavonoid biosynthetic pathway *tt4*, *tt8* and *ttg* (NASC, Nottingham *Arabidopsis* Stock Centre). *Arabidopsis* plantlets were cultivated on half-strength Murashige and Skoog (1/2 MS) medium (2.2 g/L MS basal salt mixture, 0.5 g/L MES hydrate, pH 5.4), supplemented when needed with 9 g/L agar type E. Before the experiments, *Arabidopsis* seeds were surface sterilized using a solution containing 0.05% SDS and 70% ethanol under shaking conditions for 10 min and subsequently washed twice with 95% ethanol.

2.2. Generation of a bacterial biosensor to detect the induction of the PCB degradation pathway

The strain *P. alcaliphila* JAB1 was engineered with the plasmid pUCP18-CmR-IR_GntR-*egfp*, that was constructed by Dr. Jáchym Šuman, in the framework of a scientific collaboration with the laboratory of Pr. Ondřej Uhlík (UCT Prague). The aim was the generation of a bacterial biosensor (from now referred to as JAB1 biosensor strain) in which the expression of the GFP reporter protein is under the control of the putative promoter of the *bph* operon. Through this biosensor it is possible to track the induction of the PCB degradation pathway. The plasmid harbored an inducible *gfp* gene putatively regulated by the same transcription mechanism as the *bphA* gene, that encodes for the protein involved in the first step of the biphenyl aerobic degradation pathway. The transcription of the *bph* operon in JAB1 is likely controlled via a GntR-like transcription factor that is encoded upstream *bphA* within an intergenic region of 397 bp and that convergently controls the expression of *gntR*, an intergenic region of 91 bp and *bphA*. The putative promoter was identified *in silico* via PromotorHunter. The strain was made competent and transformed via electroporation following the protocol of Choi and collaborators (Choi et al., 2006). For electroporation, 100 ng of plasmid pUCP18-CmR-IR_GntR-*egfp* were mixed to 20 µL of electrocompetent cells (~10^9 cells/mL). Electroporation was carried out using the Electroporator 2510 apparatus (Eppendorf, Germany) and cells pulsed at 2.5 kV, 200 Ω and 25 µF, immediately followed by 1-hour incubation at 37°C with LB medium. Transformants were then selected by plating onto LB agar supplemented with 250 µg/mL chloramphenicol.

2.3. *In vitro* biosensor validation

A dose-response assay was carried out with increasing concentrations of biphenyl to validate the biosensor and determine its sensitivity. Adapting the protocol used by Zubrova and collaborators (Zubrova et al., 2021), the JAB1 biosensor strain was grown overnight in 100 mL MMB supplemented with 30 mM sodium pyruvate and 250 µg/mL chloramphenicol at 30°C on a rotatory shaker. The culture was then harvested at 0.4 OD_{600}, corresponding to late logarithmic growth, by centrifugation (4000 rpm for 10 min, 4°C) and washed twice with physiological buffer (0.9% NaCl) before concentrating in fresh MMB with sodium pyruvate at $OD_{600} = 1$. The bacterial suspension was then aliquoted in 50 mL glass vials supplemented with biphenyl, to obtain final concentrations of 1, 10, 100 and 250 µM, or with an equal amount of acetone as negative control. The vials were incubated at 30°C on a rotatory shaker and the culture sampled at time points 0 and 2 h from induction to measure the optical density (600nm) using a transparent microtiter plate and to quantify GFP fluorescence (excitation wavelength 485 nm, emission wavelength 535 nm) using a black microtiter plate through a 96-well plate reader (Tecan, Switzerland). Samples from vials containing only the growth medium without bacterial cells were used as blanks. All conditions were tested in biological duplicates with 4 technical replicates. GFP fluorescence values were then normalized to obtain the relative fluorescence unit (RFU/OD_{600}) by calculating the ratio between the fluorescence values obtained and the OD_{600} at every time point. The induction ratio (Sevilla et al., 2015) was then calculated as the ratio between RFU/OD_{600} of the induced samples and the one of the samples before the treatment.

2.4. Chromosomal tagging of the JAB1 biosensor with the *mScarlet* fluorescent protein

The JAB1 biosensor strain was labeled also with a constitutively expressed mScarlet-I fluorescent protein via conjugation in order to detect the strain in all experimental settings, like for instance observing its root system colonization profile. The protocol followed for chromosomal insertion of the gene was adapted from Schlechter and Remus-Emsermann (Schlechter and Remus-Emsermann, 2019). The donor strain used was *E. coli* S17-1, containing the Tn5 transposon delivery plasmid pMRE-Tn5-145 (Addgene, USA) coding for the mScarlet-I protein. JAB1 biosensor, naturally rifampicin resistant, was used as recipient strain. In this way, it would be possible to counter-select for the biosensor strain against the *E. coli* donor strain, by using the following combination of antibiotics: chloramphenicol (250 µg/mL), gentamicin (15 µg/mL) and rifampicin (50 µg/mL). The two strains were grown in LB medium (supplemented with 250 µg/mL chloramphenicol and 50 µg/mL rifampicin for JAB1 biosensor and 50 µg/mL ampicillin for *E. coli*) as illustrated in the protocol: *E. coli* was used at $OD_{600} = 0.5$ while JAB1 biosensor strain was collected at a $OD_{600} = 0.3\text{-}0.4$ corresponding to logarithmic growth. The bacterial cultures were then combined at 1:1 donor:recipient ratio to mix similar amounts of donor and recipient cells. Bacterial mating was then carried out on nitrocellulose filters on an LB plate incubated overnight at 30°C. Trans-conjugants were gently re-suspended from the filters using physiological buffer and plated on LB agar containing 250 µg/mL chloramphenicol, 100 µg/mL rifampicin and 15 µg/mL gentamicin. Plates were incubated at 30°C for four days and the colonies re-streaked on LB agar containing antibiotic selection. Single colonies were then tested for *E. coli* donor cells contamination through ITS-PCR (primers ITS-F: 5'-GTCGTAACAAGGTAGCCGTA-3'; ITS-R: 5'-GCAAGGCATCCACC-3'). Furthermore, the insertion of the target gene was determined by multiplex PCR following the protocol (Schlechter and Remus-Emsermann, 2019) and using the primers FWD_Tn5_gt (5'-CTGAGTAGGACAAATCCGCCG-3'), REV_Tn5_gt (5'-GCCTCGGCAGAAACGTTGG-3'), FWD_Tn5/7_gt (5'- ATGGTGAGCAAGGGCGAG-3') and REV_Tn5/7_gt (5'-CAACAGGAGTCCAAGCTCAG-3'). Samples containing the plasmid would yield two electrophoretic bands (~700 bp and ~400 bp), thus suggesting *E. coli* contamination or defective insertion, while samples containing only the fluorescent protein coding sequence inserted in the genome would yield only the larger band (~700 bp). Positive colonies were also observed by fluorescence microscopy to observe the red fluorescence emission.

2.5. Induction assay of JAB1 biosensor strain in presence of biphenyl and PCB-18

The assay was carried out using the same protocol reported in paragraph 2.3. In this case, the glass vials were supplemented with 20 µM PCB-18, with 500 µM biphenyl as positive control and with an equal amount of acetone as negative control. At time points 0 and 2 h from induction cultures were sampled as previously reported to calculate the RFU/OD_{600} and the induction ratio. Bacterial culture aliquots of 1 mL were also pelleted at the same time points by centrifugation at 4000 rpm for 5 min at 4°C and stored at -20°C for subsequent RNA extraction steps.

2.6. Total RNA extraction and RT-qPCR on *bphA* and *gfp* genes

Total RNA was extracted, purified and concentrated following the same protocol reported in Chapter III of the present thesis by using the Nucleospin RNA kit and the NucleoSpin RNA XS kit (Macherey-Nagel, Germany). Reverse transcription was also performed as reported in Chapter III of the present thesis using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, USA). The cDNA obtained was used as a template for qPCR, performed via the CFX Connect Real-Time PCR Detection System (Bio-Rad, USA) using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, USA). The reaction volume was 12 µL containing 1 µL template cDNA (5 ng/µL) and 0.25 µM primers for the amplification of JAB1 *bphA* (F: 5'-GAGATCCAGAAGGGGCTA-3'; R: 5'-GCGCATCCAGTGGTGATA-3') and *gfp* (F: 5'-GACCACTACCAGCAGAAC-3'; R: 5'-GCTCAGGTAGTGGTTGTC-3') as genes of interest, or of *infB* as reference gene (F: 5'-AGTGACCGATAGTGAGAAAC-3'; R: 5'-

AACACTGATGGTCTTGCTAC-3’). Thermal protocol was set up as follows: 95°C (10 min), then 40 cycles at 95°C (10 s) and 60°C (40 s). A negative control without template cDNA was added and all reactions were performed in triplicates. The relative abundance of *bphA* and *gfp* expression was obtained by subtracting the threshold cycle (Ct) of the reference gene to the Ct of *bphA* or *gfp* and obtaining a ΔCt value. The ΔCt of the *bphA* or *gfp* in presence of the inducer was then further compared to the ΔCt in control conditions without the inducer. The relative expression values of *bphA* and *gfp* were then calculated as $2^{-\Delta\Delta Ct}$ as reported in Chapter III.

2.7. Bacterial growth on *Arabidopsis* root exudates released under PCB-18 stress

For root exudate collection, surface-sterilized seeds of *Arabidopsis thaliana* Col-0 were cultivated on 1/2 MS liquid medium supplemented with 1% sucrose for 11 days. The medium was subsequently removed, and the plants washed twice with 1/2 MS. Ten mL of fresh 1/2 MS medium were added, supplemented with 70 µM PCB-18 to induce the stress or with the same volume of acetone as mock treatment. Root exudates from 3 independent biological replicates were collected, pooled together and filtered at day 0 and day 7 after stress induction and stored at 4°C. *P. alcaliphila* JAB1 was inoculated in 1/2 TSB medium and incubated overnight at 30°C on a shaker (150 rpm). The next day, cells were collected in exponential growth phase, washed twice in physiological buffer by centrifugation (5 min, 4000 rpm) and counted. Cells were then inoculated in 3 technical replicates in a 96 well plate at a final concentration of 10^4 cells/mL, using the *Arabidopsis* root exudates collected at day 0 (T0) and 7 as media. 1/2 MS medium containing only 70 µM PCB-18 or acetone was used as negative control without *Arabidopsis* root exudates. The plate was incubated on a rotatory shaker at 30°C for 4 days. Bacterial cells were then re-isolated and quantified by plating serial dilutions on LB agar using the drop plate count method, obtaining the number of CFUs/mL.

2.8. Plant-bacterium interaction assay under PCB stress

An interaction assay was set up using the bacterial strain and *Arabidopsis* L*er* WT and mutant lines to investigate the root colonization pattern and the bacterial catabolic activity upon PCB stress. The bacterial strain (*P. alcaliphila* JAB1 WT or the biosensor strain) was grown overnight in 1/2 TSB liquid medium (supplemented with 250 µg/mL chloramphenicol for the biosensor strain) on a rotatory shaker at 30°C. Bacterial cells were then collected in exponential phase, washed twice in physiological solution, counted using a phase-contrast microscope and diluted to a solution of 10^7 cells/mL. One mL of the solution was added to 50 mL of 1/2 MS agar medium and poured in square petri dishes that had a final concentration of 10^7 cells/plate. Mock-inoculated plates (without the bacterial inoculum) were prepared by adding an equal volume of physiological buffer. Sterilized *Arabidopsis* seeds were then sown on the plates, that were sealed with Micropore surgical tape (3M, USA) and vernalized for 2 days at 4°C in the dark to synchronize germination. The plates were then placed in vertical position in a growth cabinet for 5 days (22°C, 50% humidity, long day conditions with light intensity of 120-150 µmol/m). After this period, *Arabidopsis* plantlets were transferred onto fresh 1/2 MS plates containing 20 µM PCB-18 or an equal volume of acetone (mock treatment). The plates were incubated for a maximum of 14 days in vertical position in a growth cabinet at the same conditions. Different analyses were then carried out to assess the plant growth conditions and the amount of bacterial root colonization at intermediate time points (see sections 2.9 and 2.10).

2.9. Quantification of bacterial colonization on *Arabidopsis*

Root systems of the growing plantlets (the number varied depending on root dimension) were collected at DAT (Days After Transfer) 0, meaning the moment of the transfer to new plates, 7 and 14. At DAT 14 root colonization was further analyzed on separate root sections: a 0.5 cm long section at the tip of the root (T), a small section near the tip of about 1.5 cm (next to tip, NT), and the remaining section, which ended near the shoot (next to shoot, NS). The roots were placed in pre-weighed Eppendorf tubes, their fresh weight measured, and they were then homogenized with a TissueLyser II (QIAGEN, Germany) using the following protocol: 2 cycles at 20 Hz frequency for 20 seconds and, after adding 900 µL of physiological buffer, 2 cycles at 15 Hz for 1 min. The suspension obtained was used as 10^{-1} solution to prepare serial dilutions up to 10^{-5} for cell counting on LB plates via drop plate count method. After incubation at 30°C, bacterial colonies were counted, and the root colonization efficiency expressed as CFUs/mg by normalizing for the sampled root fresh weight. In the case of bacterial root sections, the resulting CFUs number was expressed in relation to the average length (cm) of the sampled sections.

2.10. Fluorescence microscopy analysis

Fluorescence microscopy analysis was carried out to observe the root colonization profile of the strain through the Unitech NOLIMITS microscopy platform available at the University of Milan. The fluorescence emitted by the JAB1 biosensor strain colonizing *Arabidopsis* plantlets and coding for mScarlet and GFP proteins was observed at the stereomicroscope (Stereo Nikon 310 SMZ) by scanning the root system with 15X magnification. The analysis was performed on *Arabidopsis* roots 9 days after the transfer on acetone or PCB-18-treated plates. For a more detailed microscopic observation of the root tip confocal microscopy was used (Laser Scanning Nikon A1) using 20X magnification and 0.8 numeric aperture. For the red signal of mScarlet, the excitation and emission wavelengths used were, respectively, 561 nm and 570-620 nm and for the green emission of GFP, respectively, 488 nm and 500-550 nm. For confocal microscopy analysis *Arabidopsis* plantlets colonized by the JAB1 biosensor strain were collected at 12 days of growth after the transfer on acetone or PCB-18-treated plates.

3. Results and discussion

3.1. *Arabidopsis* root exudates released under PCB stress sustain and promote *P. alcaliphila* JAB1 growth

The PCB-degrading strain *P. alcaliphila* JAB1 was employed to investigate the ‘cry-for-help’ hypothesis upon PCB contamination stress using the model plant *Arabidopsis thaliana* Col-0. The root exudates released by plants exposed for 7 days to 70 µM PCB-18 were assayed for their ability to recruit and sustain the growth of degrading bacteria, a relevant and essential feature for bioremediation and plant growth promotion (Correa-García et al., 2018; Santoyo et al., 2021). The exposure to PCB stress induced in the experimental setting impacted *Arabidopsis* fitness and growth. During the assay, *Arabidopsis* plantlets presented the typical features connected to the response to abiotic stress like the presence of phytotoxic contaminants (Rolli et al., 2021), as already illustrated in Chapter IV of the present thesis. Strain JAB1 was assayed for its ability to grow by using *Arabidopsis* root exudates collected after PCB exposure or mock treatment. In control media without root exudates, constituted by 1/2 MS supplemented with 70 µM PCB-18 or the same amount of acetone, the strain was not able to grow. Whereas, as depicted in Figure 1, the strain was capable of using compounds present in the root exudates as growth substrates. This is clearly shown by *Pseudomonas* JAB1 growth in the sample labeled as T0, corresponding to the exudates released by *Arabidopsis* plantlets grown for 11 days without PCB stress. This result suggests that the primary and secondary metabolites exuded by *Arabidopsis* are used by JAB1 as nutrient sources and can support its proliferation. Furthermore, significantly higher growth promotion was observed by comparing the bacterial CFUs obtained from the JAB1 culture grown using the root exudates released in presence of PCBs and the CFUs of the culture exposed to only its solvent (acetone). The results of this experiment contribute to support the ‘cry-for-help’ hypothesis in contaminated environments since the root exudates of *Arabidopsis* exposed for 7 days to PCB-18 stress promoted the growth of JAB1 more than mock treatment, suggesting a shift in the composition of the plant root exudates with the potential to sustain a higher proliferation of degrading bacterial strains. This effect can be part of an adaptive strategy that allows the plant to cope with soil contamination by recruiting and sustaining the growth of beneficial bacteria. These microorganisms, in turn, provide useful microbial services to improve the degradation and reduce the phytotoxicity of contaminants, thus potentially restoring the holobiont health status (Rolfe et al., 2019; Rizaludin et al., 2021).

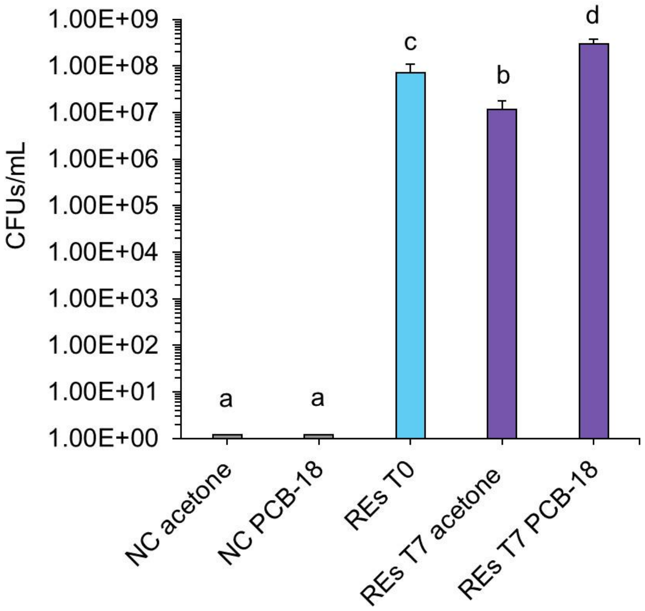


Figure 1. Growth of strain JAB1 on *Arabidopsis* Col-0 root exudates (REs) collected from plants exposed to PCB-18 stress compared to mock inoculation. *NC acetone* and *NC PCB* (grey bars) represent the growth on control media 1/2 MS supplemented only with the same volumes of acetone and PCB-18. *REs T0* (light-blue bar) represents the growth of strain JAB1 in 11 days exudates released by *Arabidopsis* plantlets before the induction of stress. *REs T7 acetone* and *REs T7 PCB* (purple bars) represent, respectively, the growth on REs collected after 7 days exposure of *Arabidopsis* to acetone or to PCB stress. The bars report average data ± standard deviation of 3 replicates. Statistical analysis was performed using the Mann-Whitney test ($p \leq 0.001$).

This evidence was previously observed in legume plants that were exposed to polycyclic aromatic hydrocarbons (PAH) stress. The exposure to contamination induced a shift in the exudation pattern that led to the modification of the associated microbial communities to recruit more efficient degraders (Kawasaki et al., 2012). Similarly, Molina and collaborators proved that clover root exudates, in a soil matrix contaminated by phenantrene, contributed to sustain the growth and ensure the persistence of the beneficial strain *Novosphingobium* sp. HR1a while improving its degradation potential more than non-planted controls (Molina et al., 2021). A recent work demonstrated that the strain JAB1 was able to metabolize several root-exuded compounds comprising flavonoids, phenolic acids and terpenes and that some of these secondary metabolites, including flavanone, quercetin and morin, could induce the expression of the *bphA* gene for PCB degradation *in vitro* (Zubrova et al., 2021). There is still a lack of knowledge regarding the chemical nature of the metabolites specifically released by *Arabidopsis* under PCB stress conditions. In Chapter IV of the present thesis, a first overview on exuded metabolites involved in PCB stress response was presented and their impact on rhizospheric PCB-degrading bacteria was investigated. One of the metabolites that were significantly over-expressed upon PCB stress, hypoxanthine, was used by the JAB1 strain as carbon source and promoted biofilm formation and swimming motility, essential features for the efficient colonization and persistence on plant roots.

3.2. JAB1 biosensor strain design and construction

With the aim to evaluate if an increase in bacterial abundance triggered by plant root exudation was also flanked by the activation of the degrading function, a bacterial biosensor for the detection of the activation of the aerobic PCB degradation pathway in presence of putative inducing molecules was constructed. The strain *Pseudomonas alcaliphila* JAB1 was successfully transformed via electroporation using a plasmid construct harboring an inducible *gfp* gene which expression is regulated by the same putative mechanism that regulates *bph* operon transcription in JAB1 (Figure 2). The *bph* operon is the chromosomal gene cluster that encodes for the enzymes necessary for aerobic biphenyl/PCB catabolism (Vergani et al., 2019). The genetic organization of the *bph* operon in JAB1 was demonstrated as almost identical to the one of *Pseudomonas furukawaii* KF707 and *bph* transcription regulation was postulated to be mediated by a GntR-type regulator that shares its structure and mechanism with the KF707 BphR1 transcriptional activator (Zubrova et al., 2021).

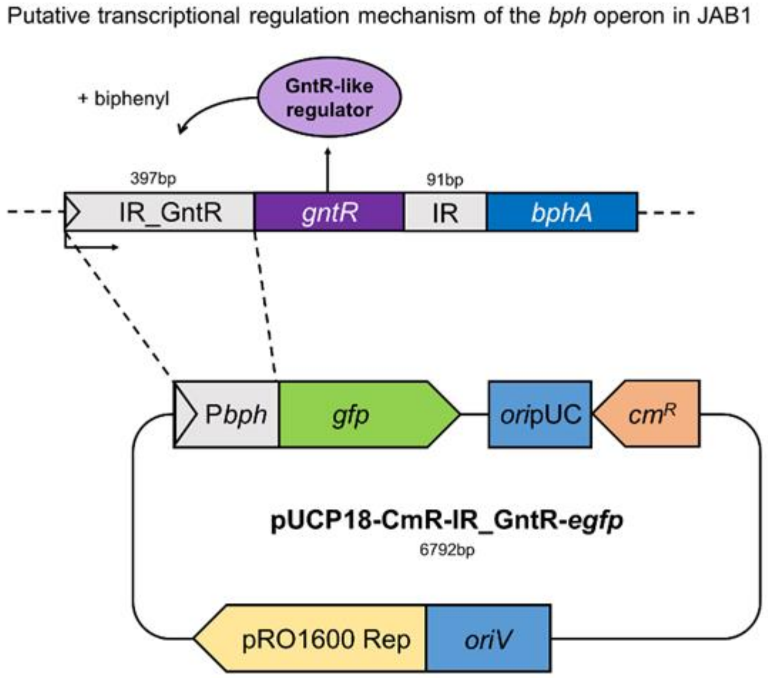


Figure 2. Schematic representation of the plasmid pUCP18-CmR-IR_GntR-egfp. The plasmid was constructed for the transformation of *P. alcaliphila* JAB1 to express the eGFP fluorescent protein under the control of the same putative transcriptional mechanism as the *bphA* gene for biphenyl degradation. The pUCP18 plasmid backbone harbors a high-copy-number replication origin (*ori*pUC) and *oriV* from the *P. aeruginosa* plasmid pRO1600 that needs the pRO1600 Rep protein for replication. The plasmid also harbors the chloramphenicol resistance gene *cm*^{R}. In pUCP18-CmR-IR_GntR-egfp, the *gfp* gene is expressed under the control of the putative promoter regulating *bph* transcription (P*bph*) which in JAB1 is encoded into a 379 bp upstream region (IR_GntR) that controls the transcription of a GntR-like regulator (encoded by *gntR*), of a 91 bp intergenic region (IR) and of the *bphA* gene. GntR positively regulates its own transcription together with the *bph* operon.

Employing the JAB1 biosensor strain could allow to rapidly monitor the induction of the PCB oxidative pathway through the appearance of an inducible green fluorescence. The biosensor strain was further tagged chromosomally with a constitutively expressed mScarlet protein via conjugation to allow the visualization of the colonization profile and its specific pattern of localization on *Arabidopsis* roots. A schematic representation of the workflow adopted in this work is depicted in Figure 3.

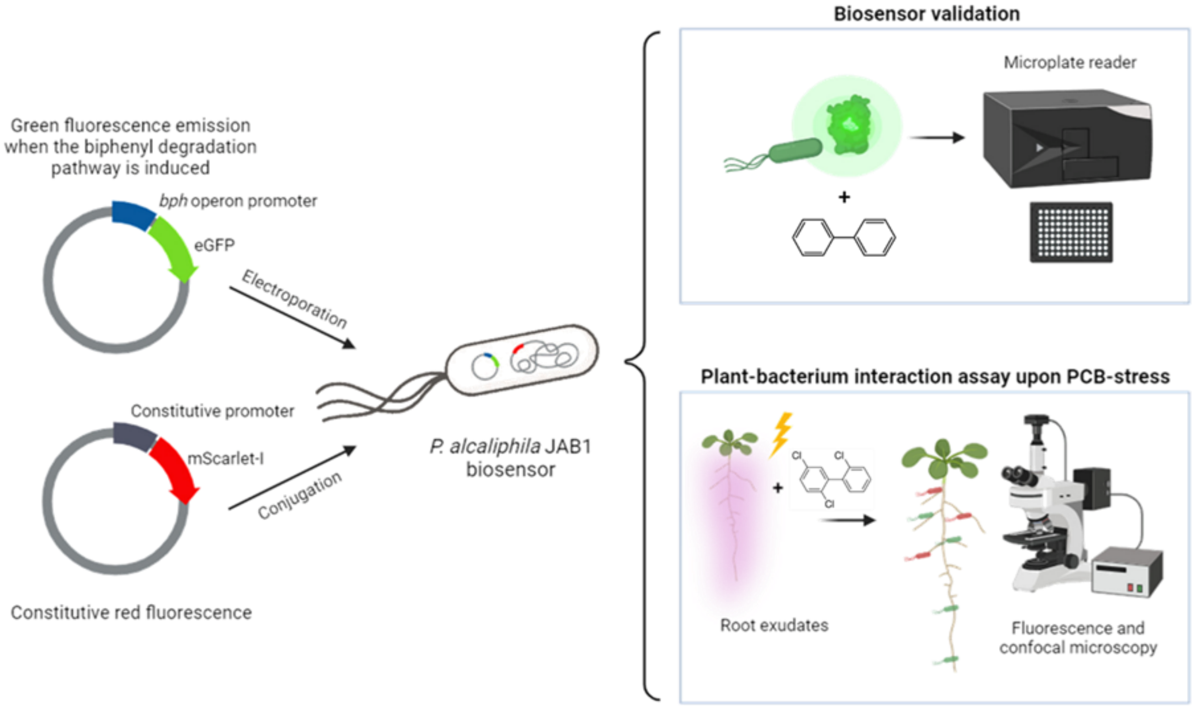


Figure 3. Workflow followed in the present study to generate the JAB1 biosensor strain and analyse its potential in plant-bacteria interaction studies performed upon PCB stress. The JAB1 biosensor was tagged via conjugation with a chromosomal constitutive mScarlet protein to identify the root colonization pattern and transformed with a plasmid carrying an inducible eGFP protein, regulated by the putative *bph* operon promoter, to monitor the activation of the PCB catabolic pathway. The strain was then validated for its sensitivity using biphenyl (upper box) and employed for plant-bacterium interaction assays in presence of PCB-18 (lower box).

3.3. Sensitivity of the JAB1 biosensor strain to biphenyl

The sensitivity and linear response of the JAB1 biosensor strain to biphenyl was tested *in vitro* through a dose-response assay in presence of increasing concentrations of biphenyl (0, 1, 10, 100 and 250 µM). In the induction assay the GFP fluorescence intensity (RFU) was measured and normalized for the OD_{600}. Biphenyl was chosen for the validation of the bioreporter since it represents the main inducer of the PCB oxidative pathway (Jha et al., 2015). As reported in Figure 4A, the JAB1 biosensor strain showed a detection threshold for biphenyl of 100 µM after a 2-hour exposure to the inductor. The RFU/OD_{600} values registered at 100 µM biphenyl exposure were in fact significantly different from the values observed in the non-induced control exposed to the biphenyl solvent only (acetone). Figure 4B shows a linear response of the induction ratio given by biphenyl within the concentration range tested (r^2 = 0.996). After the 2-hour exposure, the highest concentrations of biphenyl tested did not alter the growth of the strain (data not shown).

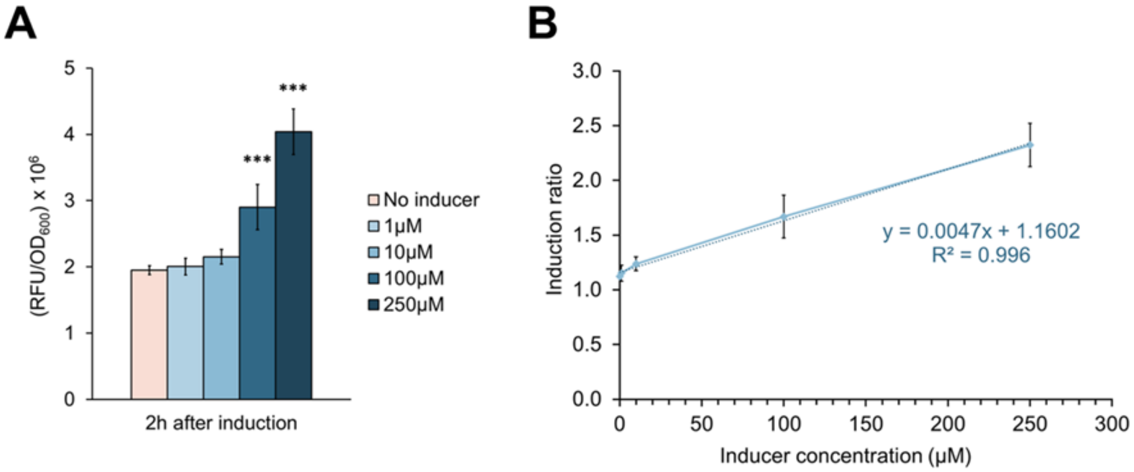


Figure 4. Dose-response assay to test the biosensor sensitivity to increasing biphenyl concentrations. (A) Detection threshold of the JAB1 biosensor strain when exposed to biphenyl. The biosensor was incubated for 2 h in presence of the indicated concentrations of biphenyl and the GFP fluorescence intensity (RFU) emitted was measured using a Tecan reader and normalized to the OD_{600} value. The *No inducer* column represents the RFU/OD_{600} values of the biosensor strain exposed to the biphenyl solvent only (acetone). Statistical analysis was performed using the Mann-Whitney test by comparing the values obtained in presence of biphenyl with the control in absence of inducer (***$p \leq 0.001$). **(B)** Linear response of the induction ratio values to increasing concentrations of biphenyl. The induction ratio was calculated the RFU/OD_{600} of the inducer-exposed samples divided by the RFU/OD_{600} at time 0, before biphenyl addition. The linear regression equation and the R-squared value are reported in the graph. For both figures, average ± standard deviation values of two independent replicates are represented.

As reported in a previous study involving biosensors to detect organic and lipophilic compounds, testing higher effector concentrations might prevent a linear correlation and lead to a saturation response due to the scarce water solubility of the inducer molecule (Sevilla et al., 2015). Depending on the experimental conditions, the reporter protein and the effector compounds, bacterial bioreporters showed wide ranges of detection sensitivity limits, comprehensively reviewed by Van der Meer and Belkin (2010) and Shemer and Belkin (2019). Strains belonging to a set of Lux-based induction biosensors constructed with *R. leguminosarum*, for instance, could detect flavonoid hesperidin concentrations ≥ 1 μM as well as salicylic acid concentrations ≥ 1mM (Pini et al., 2017). Sun and colleagues developed a biosensor for the detection of naphthalene in contaminated water and soil that showed a specific and significant response at 10 nM concentration after a 4-hour exposure to the contaminant (Sun et al., 2017). A PCB-responsive biosensor strain was also developed: Liu and collaborators chromosomally engineered *P. fluorescens* F113 strain with an inducible GFP protein obtaining the biosensor F113L::1180gfp. When incubated in presence of sodium citrate and different concentrations of 3-chlorobenzoic acid (3-CBA), the biosensor showed a growth-dependent response to different concentrations of 3-CBA and a detection sensitivity of ~10 μM (extrapolated from Liu et al., 2007). The concentration range used in our work was adequate for the purpose of our study. Indeed, the *Arabidopsis* root exudates concentrations in *in vitro* experimental settings most likely correspond to hundreds µM (Toussaint et al., 2012; Schütz et al., 2021). Nevertheless, it must be considered that for the applicability of the biosensor settings that resemble natural conditions (e.g. utilization in experiments in soil), a more sensitive detection range capable of detecting lower concentrations, might be more effective for an exhaustive detection of root exudates, which might present lower bioavailability in soil (Shaw et al., 2006; Cesco et al., 2012; Del Valle et al., 2020; Sugiyama, 2021).

3.4. The expression of biphenyl degradation pathway in *P. alcaliphila* JAB1 is influenced by root exudates released upon PCB stress

To assess if the JAB1 strain was involved in a ‘cry-for-help’ response to PCB contamination and if specific root exudates induced and stimulated its biodegradative pathway, a plant-bacteria interaction assay was set up. *Arabidopsis* plants were exposed to 20 µM PCB-18 as already described in Chapter III of the present thesis. The concentration used induced visible stress signs on *Arabidopsis* plantlets, like decreased fresh weight and leaf chlorosis caused by the phytotoxic effect of PCB-18, without totally inhibiting plant growth. Plants were grown in 1/2 MS solid medium inoculated with the JAB1 biosensor strain or in sterility (mock). By adopting the developed biosensor strain in this assay, it was possible to combine the observation by fluorescence microscopy of the red fluorescence to observe the pattern of colonization of metabolically active bacterial cells on the roots, and the green fluorescence to visualize the stimulation of their PCB degradation activity induced by root exudation. As illustrated in Figure 5, fluorescence microscopy analysis of wild type *Arabidopsis* L*er* plantlets inoculated with the biosensor for 9 days showed that, upon mock treatment (acetone), the mScarlet signal was well visible along the entire length of the root, indicating that the strain colonized *Arabidopsis* roots efficiently and homogeneously. No GFP signal was visible in mock treatment, indicating that the root exudates released without PCB stress did not trigger the degradative pathway in JAB1 and also that the basal GFP signal without induction resulted to be very low. Low background fluorescence is an essential feature in biosensor analyses to ensure specificity of the response (Sevilla et al., 2015). Interestingly, in presence of PCB stress, the red fluorescent signal was less marked along the root, nonetheless showing high intensity on the root tip. This intense signal was also mirrored by the green fluorescent signal, indicating that the JAB1 biosensor strain was expressing the PCB catabolic genes while colonizing this specific root area.

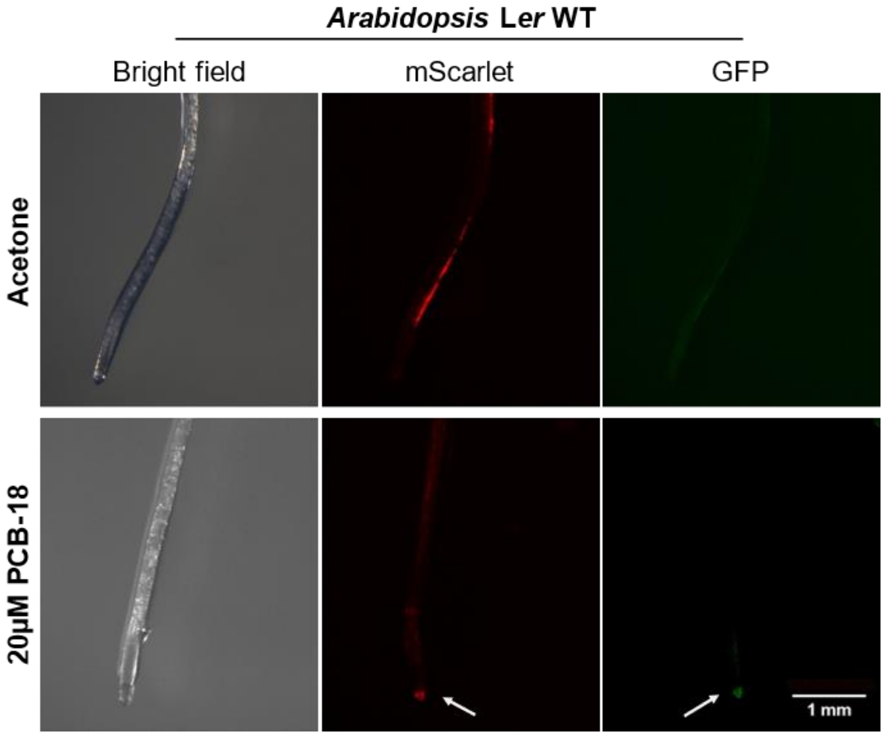


Figure 5. Microscopy analysis of WT *Arabidopsis* L*er* roots colonized by the JAB1 biosensor obtained at the stereomicroscope after 9 days exposure to mock treatment (acetone) or PCB stress (20 µM PCB-18). Arrows highlight the intense fluorescent signal present on the root tip of PCB-treated plants.

It is important to underline that the GFP signal was not directly induced by the mere presence of PCB-18 in *Arabidopsis* growth medium: an *in vitro* assay demonstrated that, after 2-hour exposure, 20 µM PCB-18 was not a sufficient concentration to trigger the GFP synthesis significantly more than control conditions (Figure 6A) and to induce the catabolic pathway in the biosensor (Figure 6B and 6C). Therefore, in this work we previously demonstrated that *Arabidopsis* Col-0 root exudates released under PCB stress supported the growth of *P. alcaliphila* JAB1 more than the exudates released in control conditions. During this assay, the metabolites exuded by *Arabidopsis* L*er* WT roots when exposed to PCB-18 were additionally involved in the activation of the PCB/biphenyl oxidative pathway. Given these results we can infer that the plant might modify its exudation pattern upon PCB stress to sustain the growth and activate the metabolism of contaminant-degrading bacteria.

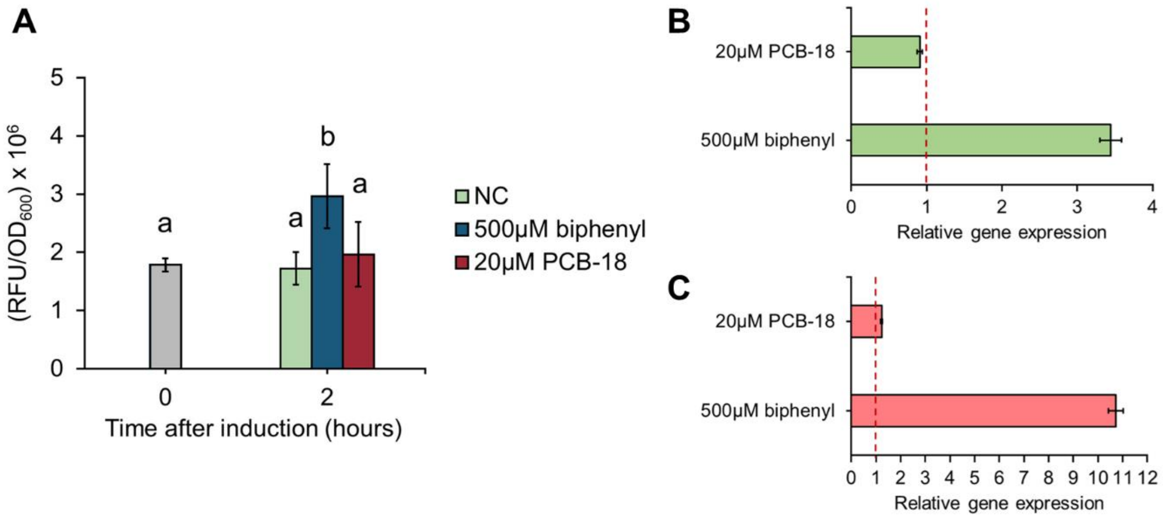


Figure 6. Induction assay to test whether the 20 µM PCB-18 concentration used in the plant-biosensor interaction assay induced the *bph* oxidative pathway and the expression of GFP in the JAB1 biosensor strain. **(A)** Quantification of the fluorescence emitted by the inducible GFP protein expressed as RFU/OD$_{600}$. The biosensor was incubated for 2 h in presence of the indicated concentrations of PCB-18, biphenyl (as positive control for induction) or of an equal volume of acetone (NC) and the GFP fluorescence intensity emitted measured using a Tecan reader and normalized to the OD$_{600}$ value. The time 0 column represents the fluorescence intensity registered before supplementing the effector molecules. Statistical analysis was performed using ANOVA followed by Tukey-Kramer *post-hoc* test on 3 independent experiments using a confidence interval of 95%. **(B)** and **(C)** Relative gene expression of the *gfp* **(B)** and the *bphA* **(C)** genes in strain LB400 exposed to the indicated concentrations of PCB-18 and biphenyl (positive control for the induction). The relative gene expression for both genes was quantified via RT-qPCR using *infB* as housekeeping gene. The results of the relative expression of *gfp* and *bphA* transcripts are expressed as $2^{-\Delta\Delta Ct}$, indicating the average fold change over the non-induced control (acetone). The red dotted line (relative gene expression value = 1) represents the baseline for gene expression in the non-induced control with acetone only. Error bars represent the standard deviation of 3 independent experiments.

3.5. Flavonoid overexpression in the *tt8* Arabidopsis mutant line triggered the expression of PCB catabolic genes mirrored by an intense GFP signal

The precise chemical exudation pattern of *Arabidopsis* plants exposed to the contamination to recalcitrant pollutants like PCBs is still to be fully clarified. However, previous studies demonstrated that specific secondary metabolites found in plant root exudates, often flavonoids, can enhance *in vitro* the oxidative pathway of PCB-degrading bacteria by acting as inducers or co-metabolites due to their molecular similarity with these xenobiotics (Pham et al., 2012, 2015; Toussaint et al., 2012; Pino et al., 2016). Zubrova and collaborators also conducted these *in vitro* experiments on *P. alcaliphila* JAB1, elucidating that, besides being able to catabolize some secondary metabolites including flavonoids, organic acids and monoterpenes, the strain was also biostimulated in the biosynthesis of the degrading machinery necessary for PCB degradation by flavonoids like flavanone, quercetin, morin and catechin (Zubrova et al., 2021). Therefore, we established an interaction assay as previously described between the JAB1 biosensor strain and mutants of *Arabidopsis* displaying distinct flavonoid exudation profiles. For the assay WT *Arabidopsis* (ecotype L*er*), the null-flavonoid producer *tt4* and the over-producing mutants *tt8* (accumulates mainly flavonoid aglycones) and *ttg* (synthesizing flavonoid conjugated forms) were used (Narasimhan et al., 2003). As visible in Figure 7A and 7B, respectively, *tt4* and *ttg* mutant lines were homogeneously colonized by the JAB1 biosensor strain as visible by the red fluorescent signal that did not significantly differ between stressed and mock-treated plants. Furthermore, the GFP signal in both the mutant lines was not detectable in both control conditions and upon PCB-18 stress, indicating that the strain was capable of colonizing the roots of the mutant lines, but that the root exudates released with or without PCB stress did not activate the PCB degradation pathway of JAB1.

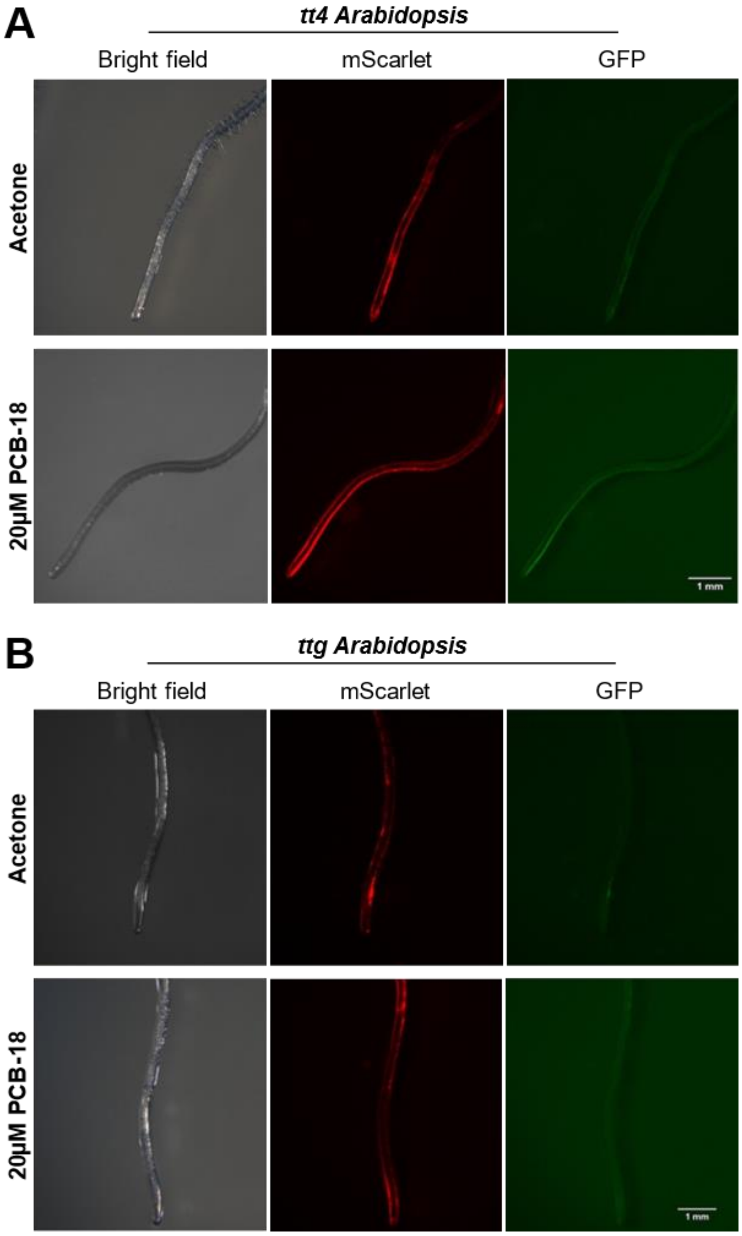


Figure 7. Images of the roots of *tt4* **(A)** and *ttg* **(B)** *Arabidopsis* mutant lines colonized by the JAB1 biosensor obtained at the stereomicroscope after 9 days exposure to mock treatment (acetone) or PCB stress (20 µM PCB-18).

The flavonoid hyper-accumulating line *tt8*, instead, showed an almost non-detectable red signal indicating root colonization in mock-treated plants, accompanied with an absent GFP emission (Figure 8). On the other hand, in presence of PCB-18 both the fluorescence signals emitted by the biosensor were intense and distributed along the root, again with a detectable and sharp focus on the root tip. This observation indicated that *tt8* roots were more intensely colonized in presence of PCB stress, and that the catabolic pathway was activated as well. The cause of this effect, in contrast with the WT and the other two *Arabidopsis* mutants, may reside in the differential exudation and accumulation of secondary metabolites in *tt8*, in particular flavonoids aglycones. Indeed, flavonoids like flavanone, apigenin, quercetin and naringenin are all unsubstituted flavonoids that were used by JAB1 as carbon sources and that induced *bphA* expression (Zubrova et al., 2021).

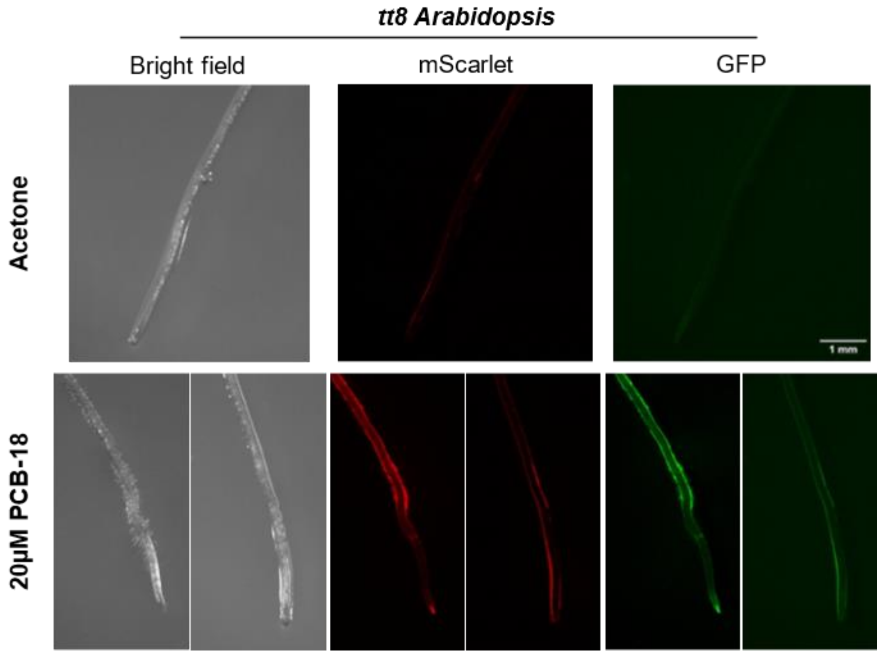


Figure 8. Stereomicroscope images of *tt8 Arabidopsis* mutant line roots colonized by the JAB1 biosensor strain obtained at the stereomicroscope after 9 days exposure to mock treatment (acetone) or PCB stress (20 µM PCB-18).

3.6. Re-isolation of bacterial cells from *Arabidopsis* roots confirms the evidence observed through fluorescence microscopy

Fluorescence microscopy images highlighted the efficient colonization of strain JAB1 of *Arabidopsis* roots (Figure 5, 7 and 8) and, in particular, the higher cell density in *tt8* mutant plants in presence of PCB-18 compared to mock-treated plants (Figure 8). Re-isolation of the strain from the roots of *Arabidopsis* and of the mutant lines for flavonoid biosynthesis was performed to corroborate the results previously observed. For this purpose, *Arabidopsis* plants were grown in 1/2 MS medium containing 20 µM PCB-18 or acetone for 14 days in presence of strain JAB1. Colonized roots were sampled at day 7 and 14 after transfer in the PCB-contaminated medium. As illustrated in Figure 9A, when comparing mock treatment and PCB stress, at DAT (Day After Transfer) 7 only the flavonoid over-producing line *tt8* showed an increase in bacterial colonization rate given by the presence of the contaminant (6.54 x 10^5 CFUs/mg and 2.59 x 10^6 CFUs/mg in acetone and PCB-18, respectively), while the WT and *tt4* null-flavonoid producing line showed a decrease in bacterial colonization under PCB stress. Instead, at DAT 14 PCB stress induced an enhancement in bacterial colonization rate in all mutant lines, with the exception of WT *Arabidopsis* (Figure 9B). Although involving substantially different experimental set-ups, this re-isolation result appears to be in contrast with the evidence previously reported about JAB1 growth in liquid medium composed by *Arabidopsis* root exudates (Figure 1). However, it must be considered that the experiments involved two different plant accessions and that WT L*er* and Col-0 *Arabidopsis* metabolic profile of exudation differs significantly and clustered separately when undergoing a metabolomics analysis (Mönchgesang et al., 2016). We can therefore infer that root exudates released in L*er* and Col-0 plants challenged by PCB stress must as well include distinct metabolites with altered abundancies with the potential to influence bacterial growth, recruitment, and adhesion to the root.

If comparing the growth of strain JAB1 in all mock-treated (acetone) *Arabidopsis* lines, WT roots had an overall positive impact on the colonization rate of the bacterium, showing a higher number of CFUs/mg compared to *tt4*, *tt8* and *ttg* at DAT 7, and only compared to *tt4* and *ttg* at DAT 14 (WT: 5.90 x 10^6 CFUs/mg and 5.73 x 10^5 CFUs/mg at DAT 7 and 14, respectively). Conversely, in presence of PCB-18 stress, at DAT 7 *tt8* mutant line induced an increase in the colonization rate of strain JAB1 if compared to *tt4* and *ttg* and if compared to all other *Arabidopsis* lines (WT, *tt4* and *ttg*) at DAT 14 (*tt8*: 9.80 x 10^5 CFUs/mg and 9.38 x 10^5 CFUs/mg at DAT 7 and 14, respectively).

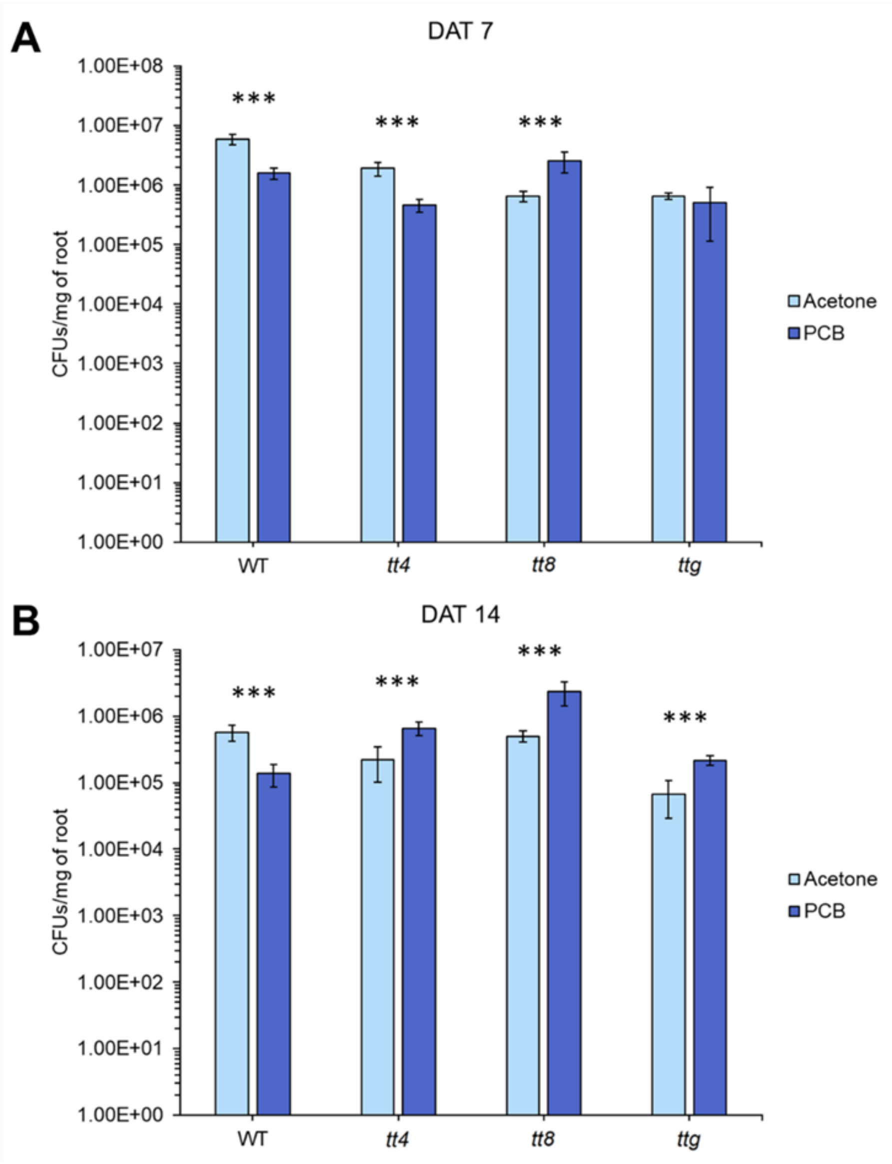


Figure 9. *P. alcaliphila* JAB1 colonization efficiency in *Arabidopsis* plantlets at day 7 **(A)** and day 14 **(B)** after transfer on plates supplemented with 20 µM PCB-18 or an equal amount of acetone as mock treatment in the *in vitro* assay to evaluate plant-bacteria interactions upon PCB stress. The barplots represent average values of CFUs normalized to the root fresh weight (CFUs/mg) ± standard deviation. Statistical analysis was performed using Mann-Whitney test by comparing the colonization efficiency PCB-18 with the control (***$p \leq 0.001$).

The results confirmed the evidence observed via fluorescence microscopy: strain JAB1 efficiently colonizes *Arabidopsis* roots, and in particular *tt8* mutant line improves the colonization rate of the strain in presence of PCB stress. These observations suggest that *Arabidopsis* actively changes its root exudation profile and the pattern of metabolites released upon stress perception to recruit and sustain the growth of a beneficial PCB-degrading strain. Interestingly, *Arabidopsis tt8* mutant line is an over-producer of flavonoid aglycones. Despite the gap of knowledge about *Arabidopsis* WT and mutant lines root exudation upon PCB stress, we can infer that flavonoids might play a role in the higher colonization rate of strain JAB1 in these plantlets and be players in the plant ‘cry-for-help’ response. As previously discussed, *P.*

alcaliphila JAB1 is capable of using specific flavonoids as growth substrates (Zubrova et al., 2021).

3.7. *P. alcaliphila* JAB1 colonizes *Arabidopsis* root tip at high efficacy upon PCB stress while expressing PCB degradation genes

To further investigate and visualize the root tip colonization observed in WT and *tt8 Arabidopsis* plantlets exposed to PCB stress, this specific region was analyzed via confocal microscopy as depicted in Figure 10. Confocal microscopy showed that, in mock treatment without PCB, the JAB1 strain was localized on the root tip in both WT and *tt8*, in particular on the root cap as visible by an intense red fluorescent signal given by the constitutive mScarlet protein. Conversely, no GFP signal was visible in absence of PCB-18. As already visible from Figure 5 and 8, upon PCB stress the GFP signal co-localized with the red fluorescence, indicating both bacterial colonization of metabolically active cells and activation of the biphenyl degradation pathway occurring on the root cap (Figure 10).

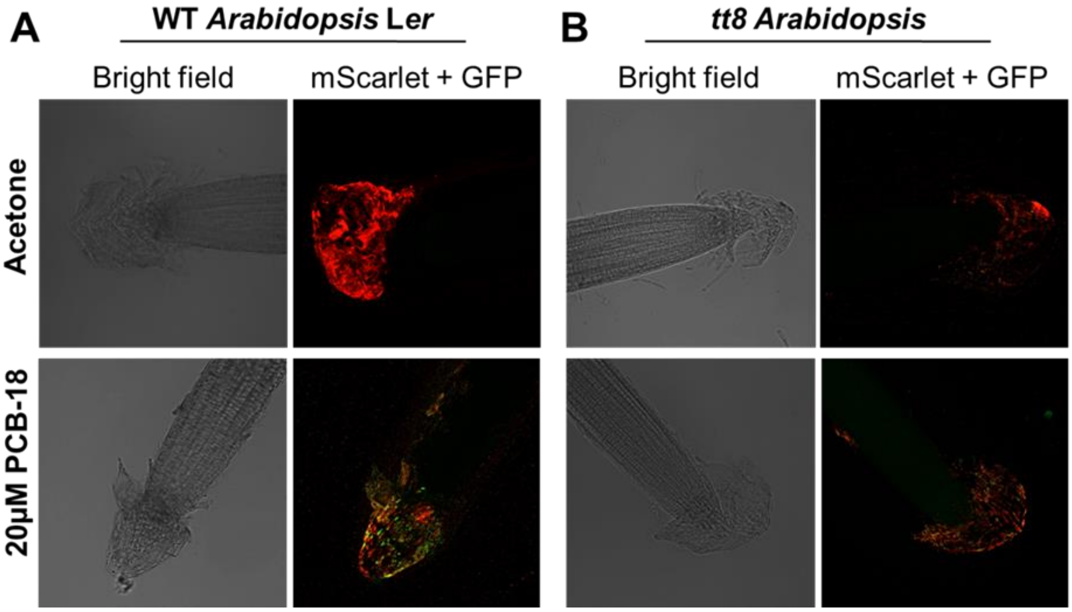


Figure 10. Confocal microscopy images of the root tip of WT *Arabidopsis* **(A)** and *tt8 Arabidopsis* mutant line **(B)** colonized by the JAB1 biosensor after 12 days exposure to mock treatment (acetone) or PCB stress (20 µM PCB-18). As visible in the bright field picture, all the roots present an intact root cap. The mScarlet and GFP fluorescent signals expressed by the biosensor cells are visible in the same maximum intensity projection image.

To confirm this evidence, a supplementary control analysis was performed on root tips in which the root cap was mechanically damaged, detached or removed. Indeed, as illustrated in Figure 11, in this case the fluorescence signal intensity visible in Figure 10 is lost while some residual fluorescence is still visible on the shreds of tissue still attached after the cap removal. Furthermore, a part of the bacterial cells that were weakly adherent to the root cap were released and scattered around the root tip. The distribution of *P. alcaliphila* JAB1 on the root tip was thoroughly investigated because of its peculiarity.

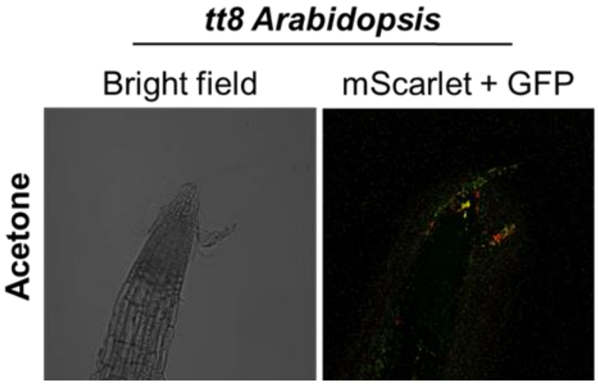


Figure 11. Confocal microscopy images of the root tip of *tt8 Arabidopsis* mutant line colonized by the JAB1 biosensor after 12 days exposure to mock treatment (acetone). As visible in the bright field picture, *tt8* presents a damaged root cap. The mScarlet and GFP fluorescent signals expressed by the biosensor cells are visible in the same maximum intensity projection image.

Indeed, the root cap is a layer of cells forming an interface between the root tip meristematic tissues and the soil, protecting the root during its growth and facilitating soil penetration (Badri and Vivanco, 2009; de la Fuente Cantó et al., 2020). During this process, the meristematic tissue in the root tip renews itself while border cells gradually shed from the root cap (Arnaud et al., 2010). The root cap secretes a mucilage matrix constituted by polysaccharides and proteins that provides a carbon source to facilitate the recruitment of microorganisms, allows lubrication and often permits phytopathogen control (Qu et al., 2020; Tian et al., 2020). Border cells of the root cap are also known to produce and exude secondary metabolites with low molecular weight like flavonoids that can play a role in the crosstalk with soil microorganisms (Sasse et al., 2018). Even though the root cap is considered a hotspot for rhizosphere interactions and a focal point of rhizodeposition and exudation, some studies report a scarce amount of bacterial colonization in this section. For instance, *Pseudomonas fluorescens* SBW25 colonized maize roots less abundantly when in the vicinity of the root tip probably due to the presence of mucilage. In this study, when testing decapped roots, a higher density of root-colonizing bacteria was found near the root tip (Humphris et al., 2005). Also, in *Pisum sativum* the colonization rate of a *Rhizobium* species was found lower near the root tip (Pini et al., 2017). It has to be considered that the presence of an intact root cap is fundamental for root tip colonization because of its potential carrying capacity: it has been hypothesized that plants with rapid decay of border cells and high mucilage biosynthesis (e.g. maize) can repel bacteria, in contrast with species with a more stable root cap structure, like *Arabidopsis*, where the root cap collects rhizospheric bacteria and constitute a reservoir to allow further root colonization (Dupuy and Silk, 2016). Indeed, evidence regarding root cap colonization by bacteria is reported in literature: a live-imaging experiment proved that motile cells of *B. subtilis* migrate and initiate root colonization in lettuce starting from the root cap, where soil particles form pores on the root surface (Liu et al., 2021). Furthermore, *B. cereus* YL6, a plant growth promoting strain with phosphate solubilization activity isolated from Chinese cabbage plants, showed the ability to colonize this peculiar root zone in the first days of contact with the growing root (Wang et al., 2017). Strain JAB1 that was used in the present work showed to be particularly adapted to the root cap ecological niche in *Arabidopsis*, as well as being stimulated in the expression of catabolic genes necessary for PCB degradation by the metabolites exuded in this region, as showed by the strong GFP signal observed in this area. To quantitatively verify the results obtained via confocal microscopy regarding the preferential colonization of the root tip, re-isolation of the strain from different compartments of *Arabidopsis* roots at DAT 14 was performed. This assay was carried out to comprehend the distribution of the JAB1 strain on the entirety of the root. In particular, the roots of WT and *tt8 Arabidopsis* were divided in 3 sections as reported in paragraph 2.9: the tip of the root (T), the small section near the tip of about 1,5 cm (next to tip, NT), and the remaining section, which ended near the shoot (next to shoot, NS). The re-isolated CFUs from each section were normalized for the average section length to obtain bacterial colonization density. The graphs reported in Figure 12 show an enhancement in bacterial density upon PCB-18 stress in both WT and *tt8* plantlets even though only in T and NT sections, while in the NS section the bacterial density decreases in presence of PCB-18.

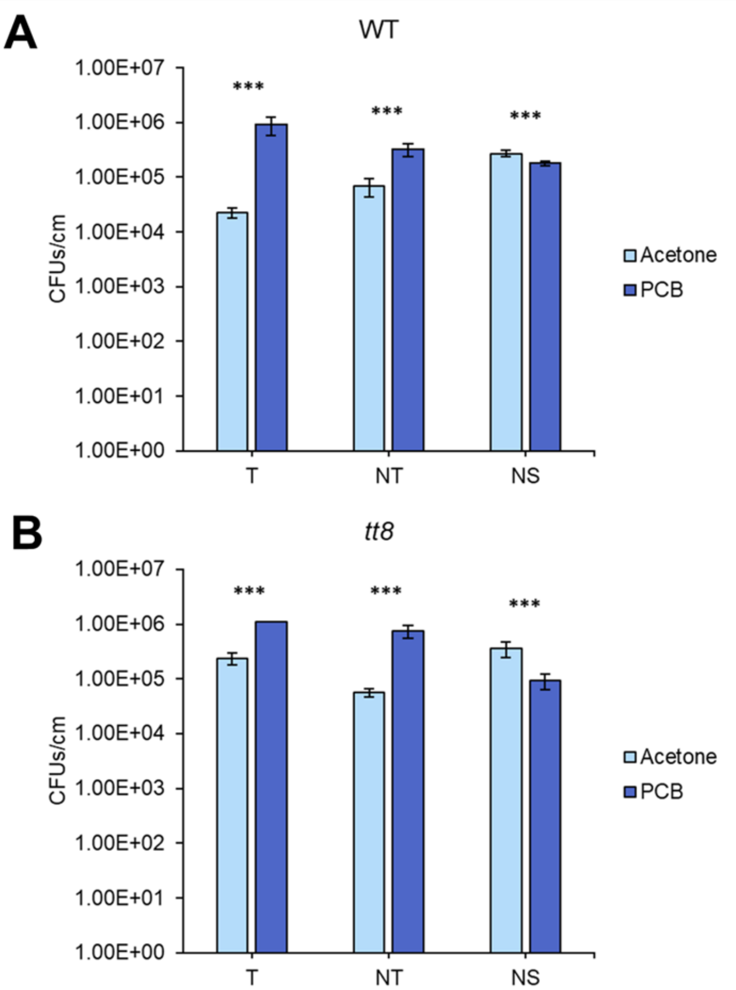


Figure 12. *P. alcaliphila* JAB1 colonization efficiency in WT **(A)** and *tt8* **(B)** *Arabidopsis* plantlets at day 14 after transfer on plates supplemented with PCB-18 or an equal amount of acetone as mock treatment in the *in vitro* assay to evaluate plant-bacteria interactions upon PCB stress. Re-isolation was performed on different root sections: tip of the root (T), a small section near the tip of about 1.5 cm (next to tip, NT), and the remaining section, which ended near the shoot (next to shoot, NS). The barplots represent average values of CFUs normalized to the section length (CFUs/cm) ± standard deviation. Statistical analysis was performed using Mann-Whitney test by comparing the colonization efficiency PCB-18 with the control (****p* $\leq$ 0.001).

This result apparently diverges from the evidence reported in Figure 9 concerning WT *Arabidopsis* colonization with or without PCB stress. In the previous re-isolation experiment, the entirety of the root was considered, and the average colonization density was calculated including therefore also lesser populated regions. By separating root sections, it was evident how the location on different segments variably influenced the interaction with JAB1 strain and its recruitment, putatively through a different root exudation pattern. Indeed, the results here obtained confirm fluorescence microscopy observations (Figure 5 and 8) for which in both WT and *tt8*, root tip colonization by the strain increases in presence of PCB stress compared to the mock treatment (acetone), indicating the root tip is a densely populated root portion. Importantly, it must be noted that in the present re-isolation experiment the higher increase in colonization density in the WT was registered in the root tip (T), while for *tt8* in the section near the tip (NT), as also clearly visible from fluorescence microscopy images (Figure 8). As previously described, the root tip localization of the strain results atypical, even though it might be ascribable to the nature of *Arabidopsis* root cap structure, that acts as a collection point for bacteria to subsequently start whole root colonization (Dupuy and Silk, 2016). However, the results show how this peculiar colonization hotspot is occupied mainly when the plant is exposed to PCB stress, indicating that there might be an unknown function of this region involved in the response to the contaminant, supposedly a specific and modified exudation pattern to facilitate the recruitment of beneficial bacteria.

4. Conclusions and future perspectives

Bacterial biosensors can be considered useful synthetic biology tools to develop tailor-made remediation approaches and to unfold the dynamics of plant-bacteria interactions mediated by root exudation (van der Meer and Belkin, 2010; Pini et al., 2017). This crosstalk is particularly relevant in rhizoremediation strategies, that combine the activity of plants and their root-associated bacteria for the removal of soil contamination (Vergani et al., 2017). This approach can be strategic to monitor the biodegradation of PCB congeners which removal, mediated by degrading bacteria activation, is hard to be monitored by chemical analyses due to the nature of these pollutants. In this work, we designed a functional bacterial biosensor based on the PCB degrading strain *P. alcaliphila* JAB1 for the direct detection and observation of metabolically active cells triggered in the expression of the oxidative pathway for PCB degradation. By applying the strain *in vitro* on *Arabidopsis* plants exposed to PCB stress, it was possible to shed light on the involvement of naturally exuded PSMs in the activation of the pathway during a more realistic interaction set-up. The findings of our work substantiate the conclusions of a series of *in vitro* study previously reported using pure compounds (Pham et al., 2012, 2015; Zubrova et al., 2021) and support the occurrence of a hypothetical 'cry-for-help' strategy employed by plants upon contamination stress. Indeed, the root exudates released by WT *Arabidopsis* Col-0 plants exposed to PCB-18 supported the growth of strain JAB1 better than the root exudates of untreated plants. In our hypothesis, the root exudates of stressed plants might be enriched in primary or secondary metabolites crucial for recruiting and sustaining the growth of PCB-degrading strains, to benefit from their useful microbial services and counteract the phytotoxic effects caused by the stress. While further investigating this aspect using the newly developed bioreporter strain, our results highlighted a supposed involvement of flavonoids in triggering the expression of the *bph* operon in presence of PCB-18. The root system of *Arabidopsis* mutant line *tt8*, characterized by the over-accumulation and biosynthesis of flavonoid aglycones (Narasimhan et al., 2003), enhanced the colonization efficiency of the strain upon PCB stress compared to the WT at to the other *Arabidopsis* metabolic mutants. The specific root exudation pattern of *tt8* also enhanced the expression of the *bph* gene cluster involved in PCB oxidation, observable as widespread expression of the inducible GFP. Only in presence of PCB stress, a peculiar colonization pattern was observed in WT and *tt8* plants: strain JAB1 was found with high abundance in *Arabidopsis* root tip and colonized in particular the root cap, showing also PCB-degrading activity. This region might have a role in the response to the contaminant, being thus particularly relevant for rhizoremediation: indeed, previous studies showed that root tip colonization can be considered an initial reservoir for beneficial bacteria to accomplish efficient root colonization of the entirety of the root (Dupuy and Silk, 2016). The results of this work contribute to increase the knowledge on the holobiont functioning upon stress caused by xenobiotics and decipher the mechanisms underlying the crosstalk between plants and bacteria mediated by root exudation. Furthermore, they support the hypothesis that the ‘cry-for-help’ is part of the strategy adopted by the holobiont to cope with the phytotoxic effects PCBs. In this scenario, the range of application of bacterial biosensors should not be limited to model plants and laboratory studies. Indeed, the biosensor developed in this work by engineering the JAB1 strain can be a valuable tool in future studies with non-model plants, facilitating the selection of candidate plant species exuding a specific array of secondary metabolites to induce and boost the expression of contaminant-degrading enzymes in bacteria, therefore improving the efficacy of rhizoremediation approaches.

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Rhizoremediation is a sustainable clean-up strategy for soils contaminated by recalcitrant xenobiotics, like polychlorinated biphenyls (PCBs). It can be applied *in situ* even in large contaminated areas and preserves soil functionality, in alternative to conventional remediation techniques that require soil excavation and treatment (Rohrbacher and St-Arnaud, 2016; Aparicio et al., 2022). This biotechnology approach takes advantage of the natural resources and services provided by the plant holobiont, resulting from the crosstalk and the beneficial interactions between plants and their associated microorganisms (Thijs et al., 2017). Indeed, bacterial detoxification alone often lacks efficacy in the clean-up of PCBs due to the high persistence and poor bioavailability of the xenobiotic mixtures in soil (Vergani et al., 2017b). Plant root exudation chemistry influences the composition of root-associated microbiota, potentially attracting and sustaining the proliferation of degrading bacteria and eventually stimulating the expression of their degradative metabolism (Jha et al., 2015; Hoang et al., 2021; Mishra et al., 2021). This adaptation strategy was described as a hypothetical ‘cry-for-help’ of the plant to counteract the phytotoxic effects caused by xenobiotics. Nevertheless, the molecular mechanisms underlying plant-bacteria interplay upon contaminant stress, as well as the precise role played by root exudates in influencing recruitment and metabolism of bacterial PCB degraders, are yet to be fully elucidated.

Currently, numerous methods are available to study the interplay between plants and bacteria and its implications: -omics approaches as metabolomics and exometabolomics could target plant root exudates to describe the specific set of metabolites synthesized and released by the roots upon different growth or stress conditions (Zhalnina et al., 2018). In this work, for the first time, metabolomics was used to describe the changes in root exudation induced by the ‘cry-for-help’ occurring in *Arabidopsis* upon PCB stress. The identification of specific metabolites secreted with differential abundancies in control and PCB-18 stressed plants, allowed the investigation of the role of these compounds in influencing the recruitment, growth and activity of model PCB degrading strains. Following common experimental approaches, the analysis was performed on root exudates collected in an *in vitro* set up using a model plant, therefore representing an approximation of the natural exudation process occurring in soil. Our results allowed the identification of five metabolites which exudation was either upregulated or downregulated significantly upon PCB-18 stress. Each metabolite was subsequently tested for their effect on metabolic activities and rhizocompetence traits of a selection of PCB degrading strains. Interestingly, the presence of the secondary metabolite scopoletin was inhibited in presence of PCB stress: we hypothesized that its reduction in *Arabidopsis* root exudates may have the potential to alter the rhizosphere ecological niche, creating more suitable conditions for the recruitment and colonization of PCB degraders.

Although *Arabidopsis* represents the ideal model plant for pioneering studies considering the vast availability of biosynthetic mutants of this plant, future research should address the identification of the root exudation pattern of plants previously identified as promising for phyto-rhizoremediation of PCB contaminated soils like *Festuca arundinacea* or *Medicago sativa* (Terzaghi et al., 2019). The differential exudation of plant metabolites could then be linked to possible shifts in the rhizosphere microbial community structure induced by PCB stress. These analyses could be carried out using metagenomics and potentially scaled up by using real contaminated soil matrices.

This PhD work helped to broaden the knowledge about plant-bacteria crosstalk mediated by root exudates and, specifically, unveiled the contribution of plant flavonoids in establishing functional interactions with the beneficial PCB degrading strain *P. xenovorans* LB400. Indeed, to get deeper insights on the mechanisms of action we applied *in vitro* assays using flavonoid mixtures that could represent exudation conditions progressively resembling natural ones, starting from pure molecules and then employing mixtures of root exudates naturally released by different *Arabidopsis* genotypes. Thereby, micromolar concentrations of flavonoids demonstrated their biostimulant action on numerous rhizocompetence traits of strain LB400 (e.g. swimming motility, chemotaxis and biofilm formation), on bacterial proliferation and on PCB degradation potential, important features for efficient root colonization in the context of rhizoremediation. These observations were made possible through devising a specific plant-bacteria interaction plate assay that simulated PCB-induced phytotoxicity in *Arabidopsis*.

The information collected about the influence of root exudates on degrading bacteria metabolism and activity is essential to set up ideal conditions for *in situ* rhizoremediation, that would potentially be enhanced and accelerated through biostimulation by two proposed strategies. Firstly, it would be possible to select or genetically modify appropriate host plants, with a specific pattern of exudation and able to release optimal amounts of selected metabolites that could be used as growth substrates or as inducers of PCB degradation, in the prospect of a rhizoengineering approach (Thijs et al., 2017). Secondly, the addition of exogenous plant metabolites, such as flavonoids, directly to the soil as amendments, ideally by utilizing food waste matrices rich in flavonoids to pursue a circular economy view, could biostimulate rhizoremediation efficiency (Uhlik et al., 2013; Koprivova and Kopriva, 2022).

Our analyses focused in particular on single model PCB degrading strains or on strains previously isolated from contaminated soils (Vergani et al., 2017a; Ridl et al., 2018): a promising follow-up would be the exploitation of culturomics approaches to select consortia of efficient PCB degraders and analyze their collective behavior by developing function-specific synthetic communities.

Finally, the fluorescent-tagged PCB degrading strains here developed, namely the mScarlet-tagged LB400 strain and the JAB1 biosensor strain, can be considered valuable tools to readily localize the effect of exudation on bacterial colonization of the root through microscopy. Moreover, the application of JAB1 biosensor allowed to observe the specific areas where root exuded compounds triggered the expression of the GFP protein, encoded under the control of the *bph* operon promoter for PCB degradation, therefore providing meaningful knowledge about plant root exudation chemistry and, overall, about the holobiont functioning upon xenobiotic stress.

As a general conclusion, this thesis contributed to set the bases for the investigations of plant-bacteria association upon PCB stress and pointed out the importance of developing tailor-made *in vitro* assays and experimental set ups using model plants and PCB degrading bacteria to implement new insights in large-scale rhizoremediation interventions. The studies conducted in this work and the results obtained confirmed the importance of root exudation in assembling a root-associated microbiota able to provide the plant with useful services to improve contaminant degradation and restore the holobiont fitness under xenobiotic stress. In particular, the role of secondary metabolites like flavonoids was elucidated, revealing them as key mediators of the process of root colonization and of the biostimulation of aerobic biphenyl degradation, both necessary features to achieve an effective rhizoremediation of low chlorinated PCBs.

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PUBLICATIONS

Ghitti, E., Rolli, E., Vergani, L. and Borin, S. **(2024).** Flavonoids influence key rhizocompetence traits for early root colonization and PCB degradation potential of *Paraburkholderia xenovorans* LB400. *Frontiers in Plant Science,* 15:1325048.
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CONTRIBUTIONS IN NATIONAL AND INTERNATIONAL CONFERENCES

Oral presentations

Ghitti, E. "The role of root exudates in promoting beneficial interactions and rhizoremediation potential of PCBs-degrading bacteria". 27th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology. Portici (NA), Italy, 13-15 September 2023, pp. 510-514.

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Poster presentations

Ghitti, E., Rolli, E., Borin, S. "The influence of plant flavonoids on rhizocompetence traits of the polychlorinated biphenyls-degrading bacterium *Paraburkholderia xenovorans* LB400: a promising resource for rhizoremediation". 7th International Conference on Microbial Diversity. Parma, Italy, 26-29 September 2023, P126.

Ghitti, E. "Role of root exudated secondary metabolites in beneficial interactions between plant and polychlorinated biphenyls degrading bacteria". 26th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology. Asti, Italy, 19-21 September 2022, p. 258.

Ghitti, E., Rolli, E., Vergani, L., Borin, S. "Influence of Plant Secondary Metabolites on Functional Traits of the Polychlorinated Biphenyl (PCB)-Degrading Strain *Paraburkholderia xenovorans* LB400". Harnessing the Plant Microbiome (Nature Conferences, University of California, Davis, USA). Online, 22-24 October 2021, p. 62.

Ghitti, E. "Exploiting Beneficial Interactions Between Plants and Bacteria: PCB Biodegradation by Soil Microbiome Upon Stimulation by Root Exudates". First Virtual (25th) Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology. Online, 14-15 September 2021, pp. 140-141.

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