Biotechnological Applications of Bacterial Endophytes

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Abstract: This review starts with a brief introduction on bacterial endophytes. Only small fractions of rhizosphere and phyllosphere bacteria are able to live inside the plant. Endophytes are bacteria and fungi that can be detected at a particular moment within the tissues of apparently healthy plant hosts without producing symptoms in or on the plant. Possible traits required by these bacteria to enter the plant and live inside will be discussed. Furthermore, we will focus on possible biotechnological applications of bacterial endophytes and present case studies as examples. Endophytes can promote plant growth, for example by the production of hormones or by making nutrients (such as nitrogen, phosphate and ferric ions) available to the plant. Endophytes can also promote plant growth indirectly, for example by suppression of plant diseases, by inactivating environmental pollutants, and by alleviating stresses of the plant caused by excess of the hormone ethylene, by heavy metals, by draught and by salinated soil. Some endophytic bacteria can produce nanoparticles which have numerous applications. At the end of the review we will discuss aspects involved in the commercialization of microbes.

Keywords: Biological control, commercial microbial products, disease suppression, endophyte, microbiome, nanoparticles, plant growth-promotion, plant-microbe interaction, rhizoremediation, root hair, stress tolerance.

1. INTRODUCTION

We define the word endophyte as "those bacteria and fungi that can be detected at a particular moment within the tissues of apparently healthy plant hosts without producing symptoms" [1]. This definition excludes pathogens and nodule-producing microbes. Although fungal endophytes also exist, we will focus in this review on bacterial endophytes. The vast majority of bacterial endophytes is (presently) non-culturable or viable but non-culturable (VBNC). Therefore metagenomic approaches are used to uncover the broad diversity of endophytic communities [2-5]. Since endophytes which presently cannot be produced at a large scale are not suitable for biotechnological applications we will focus on culturable endophytes. We will start this review with a brief introduction on bacterial endophytes. Only small fractions of rhizosphere and phyllosphere bacteria are able to live inside the plant. Possible traits required to enter the plant and live endophytically will be discussed. In the rest of this review we will focus on possible biotechnological applications of bacterial endophytes and present case studies as examples.

2. BACTERIAL ENDOPHYTES

Presently, the general belief is that bacterial and fungal endophytes are present in all plants [6]. In order to isolate endophytes, microbes present on the plant surface have to be fully eliminated first. So, requirements for sterilisation protocols are (i) that all microbes present on the plant's surface are killed but also (ii) that these procedures have an as small as possible negative effect on the endophyte population. It is clear that the more stringent the used sterilisation protocol is, the lesser endophytes will be found. So, values reported for cfu's (colony forming units) are minimal values. Criteria for the correct identification of endophytic bacteria have been established elsewhere [6, 7]. These go beyond isolation from surface-disinfected plant tissues and require further support, for instance, by microscopic proof and by the ability of the putative endophyte to re-infect disinfected seedlings [8].

On the one hand, the plant's interior can be considered as a protective environment compared to the highly competitive/predatory environment found outside plant tissues [9]. On the other hand, the interior can be viewed as an hostile environment considering the multiple defence responses that plants deploy against the "invasion" of bacteria that are able to become endophytic [10]. Perhaps, the prevalence of nonculturable and/or VBNC states for most endophytes points towards a survival strategy to overcome stresses operating in plant tissues such as defensive responses [11]. From this point of view, endophytes might have found an evolutionary solution to cope with an extreme environment.

Bacterial endophytes can be found at many sites in the plant, such as root, stem, leave, berry, seed, and xylem sap [6, 8, 12-14] (Fig. **1**). The population density of endophytes is higher in roots than in any other plant organ. In the root the average density is 10^5 cfu per g fresh weight whereas average values of 10^4 and 10^3 are reported for stem and for

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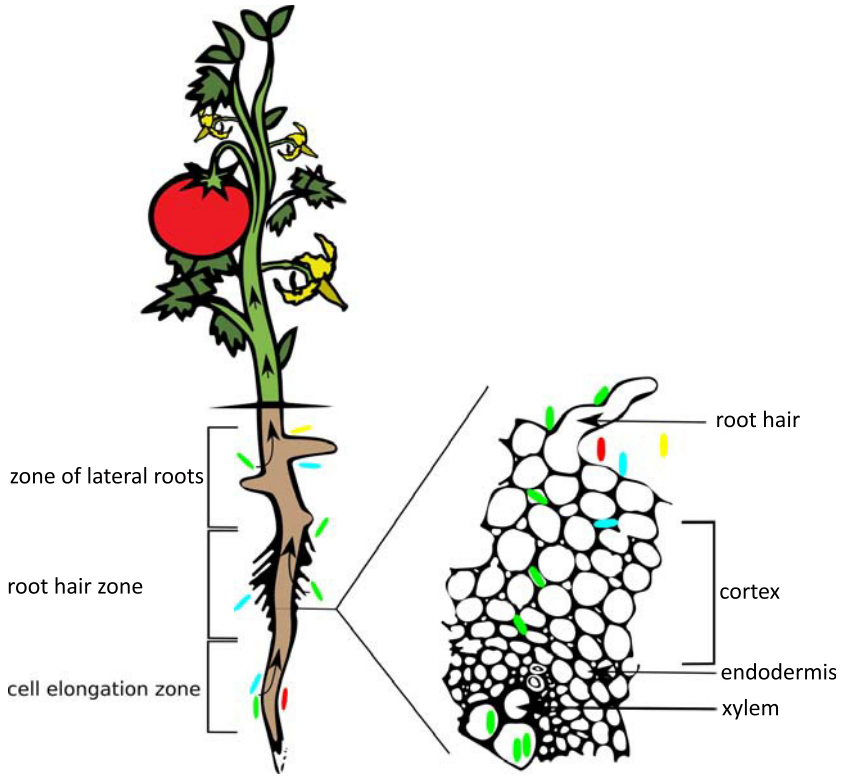

Fig. (1). The main plant colonization routes by endophytic bacteria. Some soil bacteria can enter a plant at several root zones, as explained in the main text. Once endophytically established, they can either remain in the neighbourhood of the site of entry (indicated in blue), or move deeper inside and occupy the intercellular space of the cortex and xylem vessels (indicated in green). Some can even move upwards colonizing above-ground organs/tissues. Red and yellow represent rhizospheric bacteria which are unable to colonize inner plant tissues. From ref. 12; published by Wiley-Blackwell. (Color image available online)

leaf, respectively [15]. A broad spectrum of endophytic bacteria is found in the roots of many plants, comprising hundreds of species (219 in the year 2006) and almost 100 genera (71 in the year 2006), with *Bacillus*, *Burkholderia, Enterobacter,* and *Pseudomonas* being the most common genera [6, 14, 15]. Bacteria of the genus *Bacillus* and *Staphylococcus* dominated in the stems of 1-year-old and 4-year-old ginseng plants, respectively [16]. The dominant endophytic groups of *Sphagnum* mosses belong to the genera *Burkholderia, Pseudomonas, Flavobacterium, Serratia* and *Collimonas* [17]. *Pseudomonas* and *Curtobacterium* were the most abundant genera isolated from the aerial parts of poplar (*Populus* spp.) [18].

A given endophytic microbiome can be modified by factors such as the physicochemical structure of the soil, plant growth phase and plant physiological state, as well as by diverse environmental factors [19-23].

The main colonization route used by endophytes seems to be the rhizosphere (Fig. **1**). Experimental support for this notion was provided after isolating three poplar endophytes, labelling them with the *gfp* (green fluorescent protein) gene, and reintroducing them into poplar trees. All strains proved to be efficient rhizosphere colonisers [24]. Bacteria reach the rhizosphere by chemotaxis towards root exudate components followed by attachment [25]. Type IV pili, lipopolysaccharide and exopolysaccharide are bacterial components shown to play roles in attachment of endophytes to plant tissue [12]. The preferred site of attachment and subsequent entry is the apical root zone with a thin-walled surface root layer, such as the cell elongation zone and the root hair zone with small cracks caused by the emergence of lateral roots. Root regions such as the differentiation zone and intercellular spaces in the epidermis have been suggested to be preferential sites for bacterial colonization as well [26]. Root cracks, wounds caused, for instance, by arthropods or nematodes, and emergence sites of lateral roots are generally considered as the main 'doors' for bacterial penetration [27]. At the sites of passive penetration bacteria form biofilms [12, 28-31]. After crossing the exodermal barrier, endophytes can remain at the site of entry [30] or move deeper inside and occupy the intercellular space of the cortex [12, 32, 33]. Bacterial traits putatively involved in endophytic colonization of plant roots have been reviewed elsewhere [27, 31]. For penetration, the bacteria have to produce cellulolytic enzymes required to hydrolyse the exodermal walls, such as endoglucanases and endopolygalacturoni-dases. These enzymes also seem to be important for spreading through the intercellular space of the root cortex and beyond [27, 32, 34]. Endophytes usually do not enter plant cells. Only a few of them can penetrate the endodermal barrier and invade the xylem vessels. Definitive proofs for the identification of sites preferentially used by endophytic bacteria to enter root tissues are scarce.

Recent studies have presented evidence for the notion that root hairs not only play a role in attachment but can also play a major role in root endophytic colonization by bacteria. For example, Prieto *et al*. [35], using fluorescently-tagged bacteria and confocal laser scanning microscopy (CLSM),

have demonstrated that *Pseudomonas* spp. internally colonize olive (*Olea europaea* L.) root hairs, even simultaneously. The strains examined are natural inhabitants of the olive rhizosphere and show biocontrol activity against the soil borne fungus *Verticillium dahliae* Kleb., the causal agent of Verticillium wilt of olive (VWO) [35-37]. These bacteria are able to colonize root hairs and establish within the intercellular spaces of the root cortex under both gnotobiotic and non-gnotobiotic experimental conditions [35, 38, 39] (Fig. **2**). It has been proposed that effective suppression of VWO by strain *P. fluorescens* PICF7 may require the endophytic colonization of olive roots before infection by the pathogen [39]. Moreover, a broad array of defence responses is induced in olive root tissues upon colonization by PICF7 [10]. Another role of internalisation of microbes by root hairs was demonstrated by Paungfoo-Lonhienne *et al*. [40]. These authors reported that root hairs of *Arabidopsis thaliana* and tomato (*Solanum lycopersicum* Mill) can be internally colonized by *Escherichia coli* and *Saccharomyces cerevisae*. They proposed that these non-pathogenic, non symbiotic and non-endophytic microorganisms are taken up by the plant, and that root hairs play an important role in this process. Moreover, they suggested that these plants use the microbes as a nutrient source since they seem to be degraded by the plant [40]. Neither of these two studies has so far presented clues about the exact moment and the exact site used by bacteria to penetrate the root hair cell and how they eventually reach the intercellular spaces of the root cortex. Also, whether these bacteria can enter root hairs *via* active (see, for instance, ref. 26 and citations therein) or passive (i.e. root hair) endocytosis [41, 42] mechanisms remains to be elucidated [43].

Like among rhizosphere bacteria [44, 45], (potential) human-pathogenic bacteria are also found among endophytes [15], which is a possible health concern. It is important to identify in an early stage of the research those endophytes which are potential pathogens. These should not be considered for applications (see also section 5). Based on the results presented by Paungfoo-Lonhienne *et al*. [40] mentioned previously it can be speculated that human pathogenic rhizosphere bacteria can enter the plant and be actively "taken-up". Such a scenario can have serious implications for food security issues [43].

It can be assumed that endophytes take advantage of colonizing and persisting within plant tissues (predominantly the apoplast) because they provide a steady and consistent source of nutrients and, in addition, a niche where competition with other microorganism is relatively low. Regarding to nutritional requirements, available data indicate that (in)organic nutrients are abundant in the intercellular spaces of plant tissues, thereby supporting endophytic communities observed in them. In fact, the concentration of nutrients in both the apoplast and the symplast is interactive with the phloem and it is admitted that the apoplast is not that nutrient-free region as previously thought. The apoplast composition is complex, even controversial, containing a diversity of inorganic nutrients, photosynthesis-derived sugars and related carbohydrates as well as amino acids [reviewed by Bacon and Hinton [9] and references therein].

Using radioactive labelling experiments, it could be concluded that bacterial endophytes feed on plant nutrients [12]. Several groups have analysed the abilities to utilise nutrient sources of endophytes compared to very similar non-endophytic strains. In this way it was observed that endophytic strains of *Paenibacillus polymyxa* from the rhizosphere of wheat (*Triticum* spp.) were able to metabolise sorbitol whereas similar strains from the rhizosphere of wheat were unable to do so [46]. Malfanova *et al*. [47] compared BIOLOG carbon oxidation profiles of seven rhizosphere pseudomonads and seven endophytic pseudomonads of cucumber and observed that the endophytes oxidise L-arabinose significantly more often (7

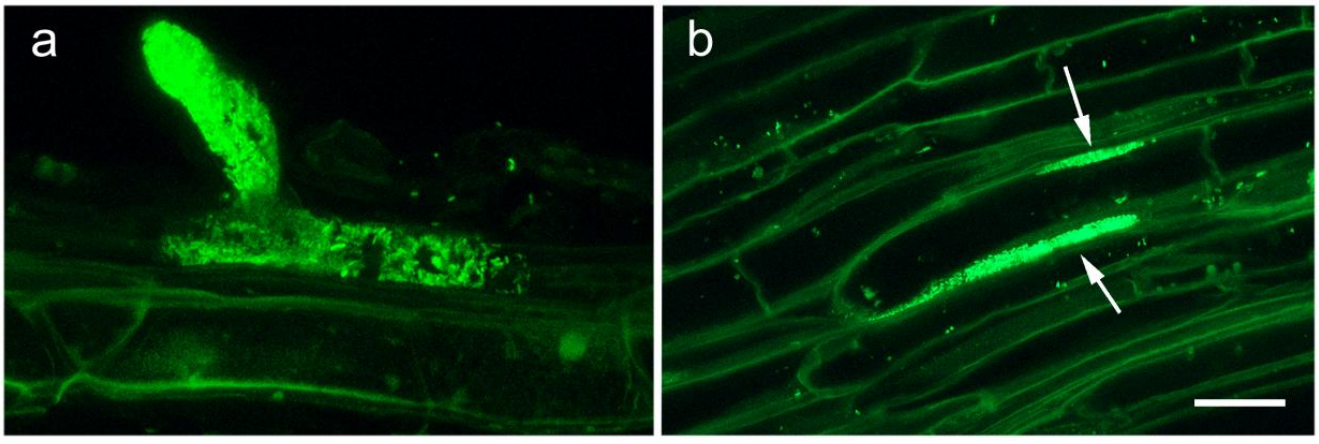

Fig. (2). Confocal laser scanning microscopy (CLSM) images showing the inner colonization by *Pseudomonas fluorescens* PICF7 of: (**a**) an olive (cv. Arbequina) root epidermal cell with a root hair six days after root bacterization under non-gnotobiotic conditions; and (**b**) the intercellular spaces of the root cortex of *in vitro*-propagated olive (cv. Manzanilla) plants in a gnotobiotic system. Images are projections of 20 adjacent confocal optical sections. The focal step size between confocal optical sections was 0.5 µm. Image**s** were taken 6 (**a**) and 8 (**b**) days after dipping the entire root system in a *P. fluorescens* PICF7 cells suspension. PICF7 microcolonies in the cortical intercellular spaces are marked by arrows. Bar represents 10 µm in panel (**a**) and 5 µm in panel (**b**). For details on the endophytic lifestyle of *P. fluorescens* PICF7, on olive roots-PICF7 colonization bioassays, tissue sectioning and CLSM imagery, the reader is referred to refs. 35, 38 and 39. These pictures were taken and kindly provided by Dr. Pilar Prieto (IAS-CSIC).

out of 7) than the rhizosphere strains (2 out of 7). The same result was found when growth on L-arabinose as the sole carbon source was tested. A control experiment showed that all 14 pseudomonads are rhizosphere competent. Since L-arabinose is one of the most abundant sugars in the xylem of cucumber plants [48], and was not detected in the root exudate of cucumber [49] this result suggests that utilisation of L-arabinose might be a trait contributing to the endophytic lifestyle of the isolated endophytes [47]. Evidence that this suggestion possibly applies for more plants comes from the observation that all 51 tested endophytes of rice are able to use L-arabinose (as well as glucose) as their sole carbon source [50]. Although it is likely that L-arabinose utilization contributes to the endophytic lifestyle of pseudomonads in cucumber and perhaps also of diazotrophic endophytes in rice, L-arabinose utilisation may not be a general trait of endophytes. Other carbon sources may play a similar role in other plants and even for other microbes in cucumber and rice [9]. Indeed, Shishido *et al*. [51] provided evidence for roles of D-sorbitol and D-galacturonic acid in endophytic colonization of spruce and work by Krause *et al*. [52] suggested that ethanol produced by water-logged rice is used by *Azoarcus* endophytes. For more information on endophytic bacteria the reader is referred to references [1, 6, 8, 12, 27, 32, 36, 53-56].

3. BIOTECHNOLOGICAL POTENTIAL OF BACTERIAL ENDOPHYTES AND POSSIBLE MECHANISMS INVOLVED

3a. Introduction

A major reason for the increasing interest in endophytes is the potential for biotechnological applications. So far, most microbial products which are produced to promote plant growth and/or health originate from the rhizosphere. The advantage of those endophytic cells, which return to the endophytic stage after application of the product is that they are better protected to biotic and abiotic threats coming from outside the plant. In addition, they are ecologically adapted to the target niche; that is, able to overcome defence reactions.

Bacteria are able to promote plant growth in direct and in indirect ways. Most mechanistic information on plant-beneficial microbes comes from rhizosphere bacteria. Much less is known about endophytes, although they likely deploy the same mechanisms for promoting plant growth and health as rhizosphere bacteria do. Therefore we will discuss the beneficial potential of endophytes based on our knowledge of plant beneficial effects which rhizosphere bacteria can have. For recent reviews on the latter topic, see references [8, 12, 25, 27, 57-62].

Direct plant growth promotion is usually caused by the production of hormones and/or making nutrients available. Indirect plant growth promotion can occur in the presence of pathogens, pollutants or other stress conditions. In the case of pathogens, the beneficial microbe inactivates or kills the pathogen, usually a fungus. In the case of rhizoremediation the beneficial bacterium inactivates a pollutant which prevents germination of the seed or the growth of the plant. Stresses can for example be caused by excess of the hormone ethylene, by heavy metals, by draught and by salinated soil. The bacterial enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase and some other factors can make plants tolerant to several stresses [27, 63, 64].

In the following section the main aspects of our knowledge of mechanisms used by plant-beneficial rhizosphere microbes will be summarized. Subsequently we will describe the current knowledge about beneficial actions of endophytes and discuss the possible mechanisms used by them.

3b. Direct Plant Growth Promotion

Healthy growth conditions promote the robustness of plants and make them less vulnerable for diseases. Microbes can contribute to the health of plants by several mechanisms, including (i) phytostimulation (e. g. by hormone production), (ii) biofertilization (for example by fixation of atmospheric nitrogen, solubilisation of minerals such as phosphorous, and production of siderophores to scavenge Fe^{3+} ions under Fe^{3+} limiting conditions), (iii) induction of stress tolerance (for example by regulation of the level of the stress hormone ethylene by the enzyme ACC deaminase), and by (iv) rhizoremediation (i. e. protection of plants by rhizobacteria against environmental pollutants).

Phytostimulators promote plant growth by the production of hormones. Hormones which can be produced by bacteria are auxins, cytokinins, gibberellins, abscisic acid and ethylene [For detailed info, consult, for instance, references 58, 62, 65-69]. In the context of this review we will restrict ourselves to auxins, gibberellins and ethylene.

Auxins are involved in several plant processes, including lateral root formation, which under certain conditions elevates stress resulting in plant growth-promotion. It has been estimated that as much as 80% of the rhizosphere bacteria can synthesize auxins [62, 68]. Most bacteria use tryptophane as a precursor of auxins [67]. Production of auxins by bacterial endophytes has been reported [16, 17].

Gibberellins are involved in processes such as cell division and cell elongation within the subapical meristem, and also in seed germination. Many rhizosphere bacteria are known to produce gibberellins and secrete them in the rhizosphere [62].

Ethylene is known as the stress hormone. It is synthesized under various stresses. Its precursor, ACC, can be degraded by the enzyme ACC deaminase, which converts ACC into α-ketobutyrate and ammonia, and which is present in many rhizobacteria. Such rhizosphere bacteria can alleviate stress caused by ethylene due to flooding, salination, drought, heavy metals, toxic organic compounds and pathogens [27, 62-64].

Also some volatiles have plant growth-promoting activities, such as acetoin and 2,3-butanediol [70]. Genome sequencing of *Enterobacter* sp. 638, an endophytic plant growth promoting γ-proteobacterium isolated from the stem of poplar (*Populus trichocarpa* x *deltoides* cv. H11-11), a potentially important biofuel feed stock plant, indicated that the genetic determinants required for sucrose metabolism and the synthesis of acetoin and 2,3-butanediol are clustered on a genomic island. These findings point to a close interaction between *Enterobacter* sp. 638 and its poplar host,

in which the availability of sucrose, a major plant sugar, affects the synthesis of plant growth promoting phytohormones by the endophytic bacterium [71].

Biofertilizers can improve plant growth by providing the plant with essential nutrients such as nitrogen, phosphorous and ferric ions when plant growth is limited by low concentrations of these nutrients [For reviews, the reader can consult references 8, 57, 58, 61, 62, 66, 72, and 73].

Some bacteria are able to bind atmospheric nitrogen and convert it into ammonia which can be taken up by the plant. Biological nitrogen fixation can take place in several bacteria of which the nodule-forming bacteria *Rhizobium* and *Bradyrhizobium* are best known. Our definition of endophytes excludes nodule-forming bacteria. However, there are endophytes [16, 17, 31, 74] (as well as free-living bacteria) which do fix atmospheric nitrogen.

Phosphorous can be limiting for plant growth. Some rhizosphere bacteria produce enzymes and/or organic acids which can solubilize bound phosphorous from organic and inorganic molecules, thereby making it available for the plant [66, 75, 76]. One would expect that endophytes are less suited for this function because the insoluble substrate is outside the plant. However, to our surprise, nine out of eighteen endophytes isolated from ginseng stems solubilize mineral phosphate [16]. Moreover, in practice, phosphorus mobilization can be actively performed by endophytes that did not reach the root interior and/or endophytic lifestyle yet; for example, when after application they are still on the rhizoplane or in the rhizosphere soil. Thus, as a biotechnological tool, this trait can be of interest when an endophyte is still living outside the plant tissues.

Iron ions are needed by all organisms. Microorganisms have developed a strategy enabling them, usually only under iron limiting conditions, to scavenge ferric ions. Iron is an element that despite its abundance is largely unavailable because it is poorly soluble [8]. This is the case in most soils except the acid ones. This bacterial strategy implies the biosynthesis and secretion of low-molecular weight molecules, designated as siderophores, which display a high-affinity for ferric ions and have a wide structural diversity [77]. Synthesis of siderophores is concomitant with the production of protein receptors for the recognition and uptake of Fe^{3+}-siderophores complexes. Production of siderophores by beneficial plant-associated bacteria has been studied in some detail for a long time because of their involvement in plant disease suppression [78-80]. Siderophores are commonly produced by endophytic bacteria *in vitro* [81]. Since production of siderophores is a response to overcome an adverse situation (i.e. iron limiting conditions), it can be hypothesized that bacteria able to develop an endophytic lifestyle may synthesize these metabolites to cope with microenvironments such as the root interior which is highly depleted of bioavailable iron [56].

Several reports indicate that production of bacterial siderophores may affect iron plant nutrition. For example, iron uptake in pea (*Pisum sativum* L.) and maize (*Zea mays* L.) is inhibited when purified pseudobactin is applied to plants under gnotobiotic conditions [82]. Also, amendment of Fe^{3+}-pyoverdines (pyoverdine is also called pseudobactin) to peanuts (*Arachis hypogaea* L.) produced lime-induced chlorosis amelioration [83, 84]. Furthermore, barley (*Hordeum vulgare* L.) seedlings grown under hydroponic conditions used Fe^{3+}-pseudobactin-358 efficiently as an iron source and chlorophyll synthesis was stimulated [85]. Pot-grown mung bean plants (*Vigna radiata* L. Wilzeck) show significant increases in total and physiological available iron, enhance chlorophyll levels, and reduce chlorosis upon treatment with a siderophore-producing *Pseudomonas* sp. strain [86]. Whether these and/or other effects can be induced in the host plant by siderophores synthesized by endophytically established bacteria remains to be investigated. As far as we know, no report has demonstrated yet true production/detection of a siderophore when a bacterium has established endophytically. For instance, the olive root endophyte *P. fluorescens* PICF7 produces pyoverdine under iron-limiting conditions *in vitro* [36], but evidence of its production in/on olive roots is not (yet) available.

3c. Indirect Plant Growth Promotion

3c.1. Disease Suppression

Antagonists are naturally occurring organisms with the potential to interfere with pathogen infection, growth, and survival. The mechanisms used by rhizosphere bacteria to protect plants against pathogenic fungi have been well studied [8, 25, 57, 59, 61, 62, 87-92]. Presumably, endophytic bacteria use similar mechanisms for the control of fungal plant pathogens. However, their hidden life within the plant tissue makes it much more difficult to uncover effective mechanism(s) involved in biocontrol by endophytes. This is due to the facts that (i) it is not possible to discriminate between effects caused by applied bacteria which are inside the plant and applied bacteria which are outside, and (ii) studies with endophytes often lack the genetic approaches of mutation and complementation required to draw hard conclusions. Antibiosis and induced systemic resistance are the best studied mechanisms studied in endophytic biocontrol microbes. Examples of biocontrol exerted by, for instance, endophytic *Pseudomonas* spp. strains against different phytopathogens in diverse host plants are available [39, 93-96].

A significant fraction of the indigenous endophytic bacteria in plant roots is able to produce antibiotics towards fungal pathogens *in vitro* and therefore might use the mechanism of antibiosis for disease control [13]. This percentage varies from 0 to more than 50 percent, depending on the pathosystem, plant species, pathogen infestation, habitat, and vegetation period [17, 97, 98]. The major endophytic antagonists found in potato are *Pseudomonas*, *Streptomyces* and *Bacillus* [13]. In another study [99] it was found that the rhizosphere and endosphere of wild olive trees (*O. europaea* L. subsp. *europaea* var. *sylvestris*) contain bacteria, of which some showed *in vitro* antagonism against *V. dahliae.*

Some rhizosphere bacteria can induce resistance towards pathogenic fungi, bacteria and viruses at sites in the plant where the applied beneficial bacterium is not present. Apparently these bacteria trigger a reaction in the plant that gives rise to a signal that spreads systemically through the plant and enhances the defensive capacity of distant tissues

to subsequent infection by some pathogens. The mechanism used by these bacteria is designated as Induced Systemic Resistance (ISR) [27, 62, 100-103]. ISR is different from Systemic Acquired Resistance (SAR) in several physiological and biochemical phenotypes [22]. ISR can be induced by many different bacterial surface molecules, secreted metabolites, and volatiles [59, 62, 89]. Examples of bacterial endophytes which have been suggested or claimed to induce ISR are *Bacillus amyloliquifasciens, Bacillus pumilus, Bacillus subtilis, Pseudomonas fluorescens, Pseudomonas syringae,* and *Serratia marcescens* [100].

Genomics approaches are providing relevant information on the changes that endophytic colonization may provoke in the host plant, including those related to systemic defense responses [10, 104]. Similarly, *In Vivo* Expression Technology (IVET) [105] used for rhizosphere bacteria [106] may assist in unraveling mechanisms underlying plant-bacterial endophyte interactions, helping to understand consequences such as plant growth promotion and biocontrol.

Some rhizosphere bacteria suppress plant diseases by the mechanism Competition for Nutrients and Niches (CNN) [107]. Since endophytes seem to use specific plant carbon sources (see section 2) it is likely that competition for these nutrients limits the number of endophytic bacteria that can be taken up and survive after inoculation. In this way, competition for nutrients could play an indirect role in disease suppression. It is also likely that after inoculation competition for niches by indigenous rhizosphere bacteria, for example during entry, can be limiting disease suppression. So, CNN may be important in disease suppression by endophytes, although in a somewhat different way than in the case of rhizosphere bacteria.

The role of siderophore-mediated competition for Fe^{3+} in disease suppression has been explained by means of sequestering iron from the environment, making it unavailable for the pathogen [80]. Thus, siderophore-mediated disease suppression has been reported in pathosystems such as Fusarium wilt of carnation (*Dianthus caryophyllus* L.) [108], Fusarium wilt of radish (*Raphanus sativus* L.) [109], and Pythium damping-off of tomato [110]. In contrast, in pathosystems like *Pythium* damping off of cucumber (*Cucumis sativus* L.) [111], *Pythium aphanidermatum* root rot of cucumber [112], Take-all of wheat caused by *Gaeumannomyces graminis* var. *tritici* [113] and bacterial speck caused by *Pseudomonas syringae* pv. tomato on Arabidopsis [114], none or just a minor role for bacterially-produced siderophores could be demonstrated.

Some bacteria produce fungal cell wall lytic enzymes [115]. They can lyse fungi and use the dead material as food. This mechanism is called Predation and Parasitism (P&P). Endophytic bacteria from potato roots express high levels of hydrolytic enzymes such as cellulose, chitinase, and glucanase [81] which can degrade fungal cell wall components. Chitinase is considered as the major responsible enzyme for fungal cell wall degradation and several examples of chitinolytic endophytes which protect against plant diseases are known [13]. A chitinase of *Bacillus cereus* appeared to be involved in the protection of cotton seedlings from root rot disease caused by *Rhizoctonia solani* [116]. Moreover, chitinolytic *B. subtilis* strains are able to reduce symptoms caused by *Verticillium dahliae* in several host plants [117]. Therefore, P&P could be a mechanism contributing to the control of fungal diseases by endophytic microbes [13].

Finally, and taking into account that bacterial endophytes can reach high populations densities in defined spots, both the production of quorum-sensing (QS) signals inside plant tissues and how they operate in endophytic-mediated processes are matters deserving investigation. Indeed, Gram-negative bacteria contain a communication system based mostly on diffusible *N*-acyl homoserine lactone signal molecules. This QS system allows bacteria to sense the density of cells of their own kind and to express target genes only at a high cell density. In this way, a variety of physiological processes, including the production of many antibiotics and pathogenicity/virulence factors, and rhizosphere colonization are regulated [118, 119].

3c.2. Stress Tolerance

The stress hormone ethylene is responsible for many stress reactions in the plant [62, 63, 64, 120]. Biosynthesis of ethylene and its levels in plants are tightly regulated by many environmental cues, including (a) biotic stresses [121]. Ethylene is biosynthesized by the conversion of S-adenosylmethionine (S-AdoMet) to ACC, the immediate precursor of ethylene. This step is catalyzed by the enzyme 1-aminocyclopropane-1-carboxylate synthase (ACS), which has a central controlling role in ethylene biosynthesis [27, 122]. Application of bacteria producing the enzyme ACC deaminase reduces ethylene levels in the plant, and can result in the decrease of many forms of plant stresses. Especially the group of Glick has shown that ACC deaminase producing bacteria can allow plants to become tolerant to stresses caused by flooding, salination, drought, heavy metals, toxic organic compounds and pathogens [62, 63, 64, 120, 123].

Bacteria can modulate ethylene levels by inhibiting the ethylene biosynthetic enzymes ACS and/or β-cystathionase [124]. Modulation of ethylene levels by bacteria through any of these two mechanisms requires that plant cells synthesizing ethylene and bacteria able to degrade/inhibit are in close proximity/contact [63, 64], a scenario found in plant-endophyte interactions. It is plausible to think that plants might have favored colonization by bacteria with high ACC-deaminase activity which eventually become endophytic and contributed to ameliorate stresses caused by high ethylene levels. It was suggested that bacteria selected by the plant were then able to establish in a more favorable niche where nutrients supply is constant [27].

3c.3. Rhizoremediation

Pollution of soil and water by components such as herbicides, explosives, polyaromatic hydrocarbons, and heavy metals is a huge problem. In the USA alone, the costs of restoration of all contaminated sites are estimated at 1.7 trillion US$. The costs of remediation are highly variable and depend on soil properties, on site condition, and on the volume of material to be remediated. In 1995 the costs were estimated to vary from US$ 10 to US$ 3000 per m^3 [123].

The term rhizoremediation [125] is used for the degradation of environmental pollutants by microbes residing in the rhizosphere. It is sometimes also called

phytoremediation [126]; in this case it is suggested that the degradation is carried out by the plant but the activity of the microbes on the root is then usually ignored. For most pollutants, bacterial strains can be selected which degrade the pollutant. However, when applied in soil, the selected bacterium is often inactive and dies unless they co-metabolise the pollutant together with a regular carbon source [127]. To solve this problem, Kuiper *et al*. [128] selected a (non-endophytic) bacterium which not only degrades the pollutant (in this case naphthalene) but also efficiently uses grass root exudate as their carbon source [126, 128]. This strain, *P. putida* PCL1444, stably degrades naphthalene, uses grass root exudate efficiently, protects PLL1444-coated grass seeds from poisoning by naphtalene and allows them to grow as healthy plants [128, 129]. Grass was chosen since varieties exist which root several meters deep into the soil. It is conceivable that this strategy, to combine a relatively rich carbon source with the ability to inactivate pollutants, can be used for endophytes. Indeed, the yellow lupine (*Lupinus luteus* L.) endophyte *Burkholderia cepacia* has been genetically engineered to improve remediation of organic pollutants [130].

4. CURRENT STATUS OF BIOTECHNOLOGICAL APPLICATIONS OF BACTERIAL ENDOPHYTES

4a. Plant Growth Promotion by Endophytes

4a.1. Endophytes which Promote Plant Growth and Also Suppress Diseases

Promotion of plant growth can be a consequence of providing (micro)nutrients and phytohormones to the plant. Thus, production of the hormone IAA (indole-3-acetic acid) as well as phosphate solubilization were associated to growth promotion in soybean by endophytic bacteria [111].

Benefits for direct plant growth promotion due to the presence of endophytic bacteria have been demonstrated. For instance, plant growth promotion exerted by consortia of bacterial genera has been reported for oilseed rape (*Brassica napus* L.) and tomato (*Solanum lycopersicum* Mill) [131], rice (*Oryza sativa* L.) [95], soybean (*Glycine max* L.) [111] or spontaneous legumes [132]. The actual contribution of each partner in the consortium to plant growth promotion has not been established.

Bacillus subtilis strain HC8 significantly promotes growth of radish. No *in vitro* production of ACC deaminase or auxin was observed. The strain does produce gibberellins which could be responsible for the growth promotion. The strain is also a very good biocontrol strain of tomato foot and root rot (TFRR) [133] (see section 4b).

4a.2. Endophytes which Fix Atmospheric Nitrogen

Production of nitrogen fertilisers is expensive and their application increase the amount of nitrate in ground water. Biological nitrogen fixation in plants is known to occur by (Brady)rhizobia but this symbiosis is practically limited to legumes, such as pea and soybean [134]. A discovery by Döbereiner and co-workers in Brazil has shown the potential of endophytic bacteria to enhance cereal biomass in the absence of nitrogen fertilizer. They found diazotrophic endophytes, such as *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae*, in lines of sugar cane (*Saccharum officinarum* L.) that were bred in the absence of nitrogen fertilizer. They demonstrated that sugar cane plants infected with these diazotrophic strains are capable of deriving all of their nitrogen needs from N_2. For a more extensive review on this topic the reader is referred to ref [135].

More recently, the ability of the endophyte *A. diazotrophicus* strain PAI5 to enhance the growth of sugar cane SP70-1143 was evaluated using studies which include the non-nitrogen fixing Nif mutant Mad3A. Wild type and mutant colonized the sugar cane plants equally well and persisted in mature plants. Sugar cane plants inoculated with *A. diazotrophicus* generally grew also better in the field and had a higher total nitrogen content 60 days after planting than control plants inoculated with the Nif mutant or uninoculated. Also, the cane weight and the dry weight of green leaves were considerably and significantly higher [136]. It was concluded that fixed nitrogen was transferred from the bacterium to the plant and that this may be a significant mechanism of plant growth promotion. Interestingly, when N was not limiting, growth enhancement also occurred in plants either inoculated with the wild type or with the mutant, suggesting the activity of an additional plant growth promoting factor in *A. diazotrophicus*. It is important to stress that only those sugar cane lines which were developed in the absence of high nitrogen fertilization appear able to develop the nitrogen-fixing symbiosis [137]. This implies that searching for nitrogen-fixing symbioses in maize and other monocots might only be successful if early lines of these plants are examined [138].

Some endophytic diazotrophs have been shown to be able to fix atmospheric nitrogen *in planta*. For example, $^{15}N_2$ incorporation studies showed that sugar cane plants inoculated with the endophyte *A. diazotrophicus* Pal5 took up up to 0.6% of their total N over a 24 hr period [136]. Similar studies with *Herbaspirillum* sp. B501 showed that rice plants inoculated with this endophyte were able to take up 0.14 % of their total N over a 24 hr period [139].

Diazotrophic bacterial endophytes have also been found in maize [140-142]. Examples of diazotrophic bacterial endophytes found in corn stems and roots are *Bacillus, Enterobacter, Erwinia, Klebsiella*, and *Xanthobacter* [143]. According to McInroy and Kloepper [4, 137] *Pseudomonas* and *Bacillus* are becoming dominant in maize stems as the plant matures.

The genus *Azoarcus* includes members that are diazotrophic under microaerobic conditions. *Azoarcus* predominates in the inside the roots of Kallar grass. *Azoarcus* sp. strain BH72 resides in the xylem and not in living plant cells [144]. The presence of *Azoarcus* cells in rice seedlings promotes the growth of the plants. Supply of nitrogen derived from fixation of atmospheric nitrogen by the grass endophyte *Azoarcus* sp. strain BH72 has been shown in Kallar grass [74, 145].

Doty *et al*. [146] isolated bacteria from within surface-sterilized stems of native poplar (*Populus trichocarpa*) and willow (*Salix sitchensis*) in a riparian system in western Washington state. Several of the isolates grew well in nitrogen-limited medium. The presence of *nifH* was

confirmed in several of the isolates including species of *Burkholderia, Rahnella, Sphingomonas,* and *Acinetobacter*. Nitrogenase activity was also confirmed in some of the isolates. The presence of these diazotrophic microorganisms may help explain the ability of these pioneering tree species to grow under nitrogen limitation.

4a.3. Hormone-Producing Endophytes which Stimulate Plant Growth

The IAA-producing endophytic bacteria *P. putida CR3 and Rahnella aquaqtilis* HC2 are able to stimulate the growth of some cereals and of radish [147]. Moreover, *B. subtilis* HC-8, which produces gibberellins, also can promote plant growth [133].

4b. Disease Suppression by Endophytes

Malfanova *et al.* [133] isolated *B. subtilis* strain HC-8 from the stem of giant hogweed (*Heracleum sosnowskyi* Manden). *In vitro,* this endophyte exerts a number of promising traits for plant protection against diseases such as antagonistic activity against the pathogenic fungi *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Forl) and *Fusarium solanum,* and against the plant-pathogenic oomycete *Pythium ultimum*. Further tests showed indeed that it protects tomato seedlings against TFRR. Cyclic lipopeptides produced by this strain were suggested to be responsible for disease suppression [133]. Further research [148] showed that HC8 produces at least twenty one different lipopeptides, derivatives of all three families of cyclic lipopeptides namely fengicins, iturins, and surfactins. The different derivatives differ in fatty acid chain length and saturation. This is the first time that an endophytic *B. subtilis* is described which is able to produce all three families of cyclic lipopeptides. *In vitro*, the fengicins are the most active ones against Forl. Production of fengicins was also described for the endophytic bacteria *B. amyloliquefaciens* ES-2 [149] and *B. subtilis* BFS01 [150]. However, in contrast to HC8, neither of these strains co-produces significant amounts of all three cyclic lipopeptides. It should be noted that *B. subtilis* strain HC8 also significantly promotes growth of radish [133] (see section 4a).

Traditionally some fungi and especially mycorrhizae were considered as the only microbes which could exert a positive effect on growth and survival of forest trees [28]. However, recent results with endophytes are promising for suppressing diseases of trees and other woody plants.

Interesting results have been obtained in the suppression of oak wilt disease caused by the pathogen *Ceratocystis fagacearum*. About 20% of the bacterial endophyte isolates from surviving live oak (*Quercus fusiformis* Small) inhibited the pathogen *in vitro*. In some experiments preinoculation with isolates of *Pseudomonas denitrificans* of containerised live oak inoculated with the oak wilt pathogen, the number of diseased trees was reduced by 50 percent and the percentage of crown loss by 17 percent [93].

O'Neill *et al*. [151] showed that isolates from surface-sterilised roots of white x Engelman hybrid spruce seedlings caused reproducible spruce seedling biomass increases up to 36 percent 2 months after sawing in greenhouse trials. The active strains were identified as *Paenibacillus*, actinomycetes (most likely *Streptomyces*) and *Phyllobacterium*. Interestingly, the addition of small amounts of forest soil, known to contain seedling growth inhibiting organisms (so-called minor pathogens), had an effect on the outcome of the experiment. One actinomycete strain and a *Bacillu*s isolate stimulated seedling growth only in the presence of forest soil, suggesting that they act as biocontrol agents (BCAs). Interestingly, another actinomycete and the *Phyllobacterium* strain stimulated spruce seedling growth only in the absence of forest soil. In their presence, seedling growth was inhibited. So, under these conditions the added microbe had no effect. This suggests that in this case growth promotion caused by the added microbes was unrelated to biocontrol of minor pathogens and could be the result of direct plant growth promotion, e. g. by production of plant growth regulators [28].

Experiments in which the effect of inoculation of conifer seedlings on relative growth rates was measured suggested that a period of growth in a controlled environment facilitates establishment of endophytic populations and therefore may be important for the successful application of plant growth-promoting bacterial endophytes in forestry [28].

Strain *P. fluorescens* PICF7 is a natural inhabitant of the olive rhizosphere and has been shown to display *in vitro* antagonism against two different pathotypes of the soil-borne pathogen *Verticillium dahliae* Kleb [36]. This fungus is the causal agent of Verticillium wilt of olive (VWO), considered one of the most important biotic constraints in many areas where this woody crop is grown [37, 136]. Since currently available disease control actions are ineffective when applied individually, an integrated management strategy with emphasis on preventive (i.e. before-planting) measures has been proposed [37, 152]. For instance, appropriate choice of planting sites with no or low inoculum levels of *V. dahliae*, use of pathogen-free planting material, and protection of this material during plant propagation at the nursery with BCAs constitute excellent starting points within this integrated management framework. An interesting option for protecting olive planting material from *V. dahliae* is the use of bacterial endophytes as BCAs [36, 153].

Besides its effective *in vitro* antagonism against the pathogen, strain PICF7 was demonstrated to be an effective BCA of VWO [36, 39]. Interestingly, PICF7 was not the best olive root colonizer among several strains tested, but behaved as one of the best BCAs against the highly virulent, defoliating pathotype of *V. dahliae*. Microscopic demonstration that PICF7 can internally colonize olive root tissues has also been provided [38], as well as the evidence that root hairs can play an important role in endophytic colonization of olive root tissues [35, 39] (Fig. **2**). Internal colonization of root tissues by strain PICF7 seems to be restricted to defined zones and spread of PICF7 to distant areas through the vascular vessels has not been observed. An important conclusion from these studies was that early colonization of intact olive roots by PICF7, at both superficial and endophytic levels, seemed to be needed to control *V. dahliae* effectively [39]. Nothing is yet known about which PICF7 traits are involved in biocontrol and

endophytic colonization. However, recent functional genomics analysis has revealed that a broad range of defense responses are induced in olive root tissues upon colonization by PICF7, including up-regulation of diverse transcription factors known to be involved in systemic defense responses [10]. This offers the opportunity to explore whether PICF7 could be an effective BCA against different pathogens infecting olive tissues other than roots. However, while PICF7 is able to trigger multiple defensive responses in olive roots, including genes involved in ISR and SAR responses, control of olive knot disease caused by *P. savastanoi* pv. savastanoi (Psv) [154] in stems of olive plants whose roots were colonized by PICF7 was not achieved [155]. However, strain PICF7 was able to *in vitro* antagonize Psv. Moreover, artificial introduction of PICF7 in olive stems induced a transient decrease of Psv population on/in inoculated stem tissues, altered the appearance of knots produced by the pathogen at the macroscopic level (*in vitro*-propagated plants), decreased the maturation process of Psv-induced tumors (woody olive plants), and modified the localization of Psv on/in tumors [155]. It remains to be elucidated whether additional biocontrol mechanisms (i.e. antibiosis) may operate *in planta* upon PICF7 introduction. Likewise, to what extent endophytic colonization by PICF7 may influence the rhizosphere and endosphere microbiomes, which have been revealed as diverse and containing taxa displaying antagonist activity against *V. dahliae* [99], are matters that need further investigation.

Specific strains of endophytic bacteria can inhibit pathogens affecting grapevine (*Vitis vinifera* L.) (reviewed by [156]. For instance, an endophytic strain of *B. subtilis* has been shown to reduce Eutypa dieback as well as infection and colonization by the pathogen (*Eutypa lata* [Pers.] Tul. & C. Tul., which is the major aetiological agent, although other fungi are associated with the disease as well). Direct antibiosis seemed to be a potential mechanism involved since inhibition of mycelial growth, pathogen hyphae malformation and reduction of ascospores were observed in *in vitro* tests. Moreover, spraying vine wood with a suspension of this strain reduced the percentage of infection by the pathogen [157]. *Botrytis cinerea* Pers. is known to cause grey mould and Botrytis bunch rot affecting young grape fruit during the ripening process [156]. The endophytic plant growth-promoting bacterium *Burkholderia phytofirmans* strain PsJN [158] has been demonstrated to reduce *B. cinerea* infections in grapevine plants [159, 160], to promote plant growth, to establish rhizosphere and endophytic subpopulations in different organs and to systemically spread within grapevines [32, 161, 162]. Crown gall in grapevines, caused by the phytopathogenic bacterium *Agrobacterium vitis*, has been reported to be prevented by endophytes of the xylem sap of vine plants, including *Enterobacter agglomerans*, *R. aquatilis*, and *Pseudomonas* spp. strains [163]. Finally, symptom development of Pierce's disease caused by *Xylella fastidiosa* can be reduced by avirulent, endophytic *X. fastidiosa* strains [164]. However, while biological control of susceptible grapevines cultivars - by treating them with non pathogenic strains of *X. fastidiosa* - appears to be a disease management strategy, their use could pose certain risks since the likelihood of mutation or transfer of virulence genes among closely related strains cannot be completely ruled out and evaluation of risks should therefore be considered [164].

4c. Phytoremediation by Endophytes

The use of endophytes for remediation of soils and water has been studied and reviewed [14, 126, 165]. For example, endophytes have been isolated which degrade explosives [102], herbicides [166], and hydrocarbons [167, 168]. Examples of successful remediation of hydrocarbons by plant/endophyte combinations have been summarised in ref. 165. The plant species include birdsfoot trefoil (*Lotus corniculatus* L.), Italian ryegrass (*Lolium multiflorum* Lam.), maize, pea, poplar, wheat and yellow lupine. The endophytic bacteria include *Burkholderia cepacia*, *Enterobacter*, *Pantoea* and *Pseudomonas* [165].

An interesting endophyte-plant partnership is shown in the degradation of organic contaminants. The lipophilicity of these compounds appeared to be the determining factor for root entry and translocation. Very water-soluble organic compounds such as 2-butanone are taken up by the plant roots into the transpiration stream by passive influx. Less water-soluble organic contaminants and weak electrolytes (such as weak acids and bases and some herbicides) are readily taken up by the plant. After uptake, plants depend on their associated microbes, especially endophytes, for efficient degradation of organic compounds [14].

The use of bacterial endophytes for reducing the level of toxic herbicide residues in soil was successfully demonstrated after inoculation of pea plants with the poplar endophyte *P. putida* which is naturally able to degrade 2,4 dichlorophenoxyacetic acid (2,4-D). Whereas the 2,4-D level in the soil decreased, the plants neither accumulated 2,4-D in their aerial tissues nor showed toxic effects. This result indicates that endophytes are promising organisms to help decrease the levels of toxic herbicide residues in crop plants [166].

The presence in soil of the volatile organic compound toluene causes phytotoxicity and results in volatilisation of toluene through the leaves. Barac *et al*. [130] used a *B. cepacia* natural endophyte VM1468 of lupine, to construct the derivative plasmid pTOM-Bu61, carrying a toluene degradation plasmid. After lupine seeds were surface-sterilised and inoculated with the engineered strain, phytotoxicity of toluene was decreased and 50 to 70 percent less toluene was evapotranspired through the leaves [130]. Inoculation of poplar with VM1468 had a similar effect. The strain did not become established in the endophytic community but there was horizontal gene transfer of pTOM-Bu61 to different members of the endogenous endophytic community, demonstrating *in planta* horizontal gene transfer among plant-associated endophytic bacteria. This transfer could be used to change natural endophytic microbial communities to improve the remediation of environmental insults [169]. Recently, the use of transgenic plants and endophytic microorganisms for phytoremediation was nicely reviewed [170].

Poplar trees growing on a TCE (trichloroethylene)-contaminated site, were *in situ* inoculated with the TCE-degrading strain *P. putida* W619-TCE. This reduced TCE evapotranspiration by 90% under field conditions. This result

was achieved after the establishment and enrichment of *P. putida* W619-TCE as a poplar root endophyte and by further horizontal gene transfer of TCE metabolic activity to members of the poplars endogenous endophytic population. Since *P. putida* W619-TCE was engineered *via* horizontal gene transfer, its deliberate release is not restricted under European genetically modified organisms (GMO) regulations [171].

In 1999, 275 poplar trees were planted on a field site near a car factory in order to install a bioscreen. The aim was to combine the biodegradation activities of poplar and its associated rhizosphere and endophytic microorganisms for containing a BTEX (benzene, toluene, ethylbenzine, and xylenes) contaminated groundwater plume. This BTEX plume occurred as the result of leaking solvents and fuel storage tanks. Monitoring, conducted over a 6-year period (1999-2005) after the planting of the trees suggested that the poplar trees and their associated microorganisms had, once the tree roots reached the contaminated groundwater zone, an active role in the remediation of the BTEX plume, resulting in full containment of the contamination. Analysis of the microbial communities associated with poplar demonstrated that, once the poplar roots got in contact with the BTEX contaminated groundwater, enrichment occurred of both rhizosphere and endophytic bacteria that were able to degrade toluene. Interestingly, once the BTEX plume was remediated, the numbers of toluene degrading rhizosphere and endophytic bacteria decreased below the detection limit, indicating that their population resulted from selective enrichment by the presence of the contaminants [172].

5. ROLE OF ENDOPHYTES IN PLANTS GROWING IN SALINATED SOIL AND IN SOIL CONTAMINATED WITH HEAVY METALS

Wheat growth in saline soils is stimulated by seed inoculation with enhanced root colonizing bacteria [173]. Similarly, the endophytes *P. fluorescens* CR2, *B. subtilis* HC8, *P. putida* CR3 and *R. aquatilis* HC2 appeared to be able to promote growth of wheat in soil to which one percent NaCl was added [147]. *Rahnella aquatilis* HC2 also promoted growth of oak in soil to which 0.25 percent NaCl was added [147]. At the same time, all four strains promoted growth of wheat in the presence of 0.8 mM Cd^{2+}. Although ACC deaminase was expected to be involved in stress tolerance, none of these four strains grew on ACC as the sole N-source. Only *P. putida* CR3 and *R. aquatilis* HC2 produced auxin [147]. For the phytoremediation of toxic metals, endophytes possessing a metal-resistance/sequestration system can lower metal phytotoxicity and affect metal translocation to the above-ground parts. Endophytes which can degrade organic contaminants or improve extraction of metals offer promising ways to improve phytoremediation of mixed pollution [14].

6. ENDOPHYTES AND NANO(BIO)TECHNOLOGY

The biogenetic production of nanoparticles, which are of great interest in nanotechnology and medicine, has been reported for different microorganisms [174-177] including endophytic fungi [178, 179]. Application of nanoparticles are numerous, including delivery of drugs and genes [180], detection of pathogens [181] and proteins [182], tissue engineering [183] and tumor destruction [184]. Endophytic *Bacillus* sp. strains have been reported as producers of nanoparticles. Endophytic strains of *Bacillus* spp. isolated from the medicinal plants *Adhatoda beddomei* C.B. Clarke (Malabar nut) and of *Garcinia xanthochymus* Hook. f. ex T. Anderson (Egg tree) were used to synthesize silver nanoparticles (AgNPs) by reduction of a silver nitrate ($AgNO_3$) solution after a few days of incubation at room temperature [185, 186]. The synthesized AgNPs showed antibacterial activity against pathogenic bacteria such as *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella typhi* and *Klebsiella pneumoniae*. The use of geranium (*Pelargonium graveolens* L'Hér.) leaves and an endophytic fungus (*Colletotrichum* sp.) was reported for the extracellular synthesis of gold nanoparticles from aqueous chloroaurate ions [187]. Differences were observed depending on the presence or absence of the fungus. Thus, gold nanoparticles synthesized from geranium leaves alone (highly variable in shape) seemed to use terpenoids as reducing and capping agents while nanoparticles produced from *Colletotrichum* sp. used polypeptides/enzymes and were essentially spherical in shape. The reason for shape variability was unclear but the authors proposed the possibility to control nanoparticle shape using host leaf-fungus systems [187]. The possibility to use endophytic bacteria with/without their host plants has not been explored so far and represents an exciting new field of research.

7. COMMERCIAL MICROBIAL PRODUCTS

If the major plant growth promotion effect of a microbe to be marketed is biocontrol of a plant disease, the product should be registered as a plant protection product (PPP). This applies in all countries of the world. For the application as a biofertiliser, plant strengthener, microbial biostimulant or as a soil improver, the regulations are less strict, differ from country to country, and are under discussion [188] and therefore will not be discussed here. Since the rules for a PPP are more stringent and clearer, we will discuss these briefly and focus on the situation in the EU. Registration of a PPP in the EU basically requires proof of safety as well as proof of efficacy. In fact, a company should already from the beginning of the research process on a potential PPP realise which facts are important for registration and collect the relevant data.

The commercialisation of a marketable product is a challenge. Excellent reviews on this topic have been written (see refs. 62, 87, 189], and more recently ref. [188]. The process includes the following steps. For which plant/pathovar system does the market require a biological product? How to position this product to successfully compete with possible competitors? How profitable is the product expected to be? After initial screening in small scale greenhouse tests, it is important to do preliminary safety tests to protect researchers and production personnel. As a first approach for safety, one can identify as potential pathogens those strains which (i) based on their identity are on the list of (potential) pathogens [190], (ii) grow at 37 °C and therefore may multiply in the human body, and (iii) kill or inhibit the growth of the model animal *Caenorhabditis elegans* [191].

What is the best way to ferment and formulate the strain? One has to make choices such as between solid or liquid fermentation, the most suitable growth conditions for a long shelf life, whether the product should contain only cells or also the secreted metabolites, whether it should be applied as a liquid, a powder or as granules, how the product should be marketed and labelled, and whether it is compatible with agricultural practice [188]. The most promising strain for introduction into the market is subsequently selected. This strain as well as the product in its final formulated form are subsequently tested for toxicity for the environment and for human beings. Efficacy in the field is tested in the form of the final product [87, 188].

Based on the results, a registration dossier is prepared. In the EU, pre-registration is first done in one country, the rapporteur member state. Subsequently the approved file can be used as a basis for pre-registration in other member states. Pre-registration is for a limited period of time. Due to attempts to harmonise national laws, pesticide regulation 1107/2009/EC demands that the evaluation of proposed commercial PPPs has to be performed at the EU level before being included in Annex I. The average total costs of registration of a microbial biocontrol agent in a rapporteur member state in Europe have been estimated as 860,000 *EUR* for a biological product (and as 1,410,000 *EUR* for a chemical product) [188]. For inclusion of a PPP in EU Annex 1, the mean registration time is 75 months and the overall registration costs is estimated to be approximately 1,890,000 *EUR*, of which 66% is spent on toxicological tests and 21% on efficacy tests [188].

As far as we know, no endophytic PPP has been registered yet. An endophyte would have the advantage for biocontrol that is well protected from threats from the outside environment and is also ecologically adapted to the targeted niche. A drawback might be that registration becomes more complicated because of the risk that the endophyte is present in edible plant parts, such as fruits.

8. CONCLUSIONS AND FUTURE PERSPECTIVES

The advantage of the application of microbes in comparison with chemicals is that microbes are much more efficient in their application of active compounds. They produce their secondary metabolites practically only at the plant surface or inside the plant (in a quorum-sensing-dependant way; see section 3c.1) whereas a major fraction of externally applied chemicals will not even come into contact with the plant and will therefore only pollute the environment. A disadvantage of the application of microbes in comparison with chemicals is that the performance of microbes is more often inconsistent. It is conceivable that the application of endophytes could be an advantage since they are present in a much more protected environment than rhizosphere bacteria and therefore likely to be less vulnerable to changing environmental conditions. It might well be that being inside is an excellent option to be 'warm and well-fed'. Endophytes might have found an evolutionary solution to live within a 'safe heaven'. Or alternatively, plants have found a way to improve their fitness either in a passive way (allowing beneficial bacteria to enter and be established for long) or in an active way ("mixotrophy", degrading them after a while).

Many basic questions remain to be solved before we understand how endophytes live. Examples of questions to be answered are the following. Some questions clearly overlap. We tried to group the questions in a logical way.

What is the role of endophytes for the plant? In other words: why and how do plants select specific rhizosphere bacteria to become endophytic? What does the endophytic microbiome do for the plant? Can we approach this question by changing the microbiome using antibiotics and/or other growth conditions?

Which bacterial and plant traits are involved in colonization and persistence within plant tissues? How do plants tolerate the 'invasion' of beneficial endophytes? How do endophytic bacteria modulate plant defence responses in order to be considered as non harmful?

How have both partners evolved to shape the endophytic microbiomes? What are the driving forces operating to build up endophytic communities? How do environmental, physiological, developmental stages and/or genetic factors influence indigenous microbiomes?

How do biotechnological applications (introduction of bioformulations of BCAs, either endophytic or not) influence indigenous microbiomes?

The answers to these and other questions will contribute to optimally applying endophytes in biotechnology.

LIST OF ABBREVIATIONS

ACC = 1-aminocyclopropane-1-carboxylate

ACS = 1-aminocyclopropane-1-carboxylate synthase

AgNPs = Silver nanoparticles

BCAs = Biocontrol agents

BTEX = Benzene, toluene, ethylbenzine, and xylenes

Cfu = Colony forming unit

CLSM = Confocal laser scanning microscopy

CNN = Competition for Nutrients and Niches

2,4-D = 2,4 dichlorophenoxyacetic acid

EHEC = Enterohaemorrhagic *Escherichia coli*

Forl = *Fusarium oxysporum* f. sp. *radicis-lycopersici*

GMO = Genetically modified organism

gfp = Green fluorescent protein

IAA = Indole-3-acetic acid

IVET = *In Vivo* Expression Technology

ISR = Induced Systemic Resistance

P&P = Predation and Parasitism

PPP = Plant Protection Product

Psv = *Pseudomonas savastanoi* pv. savastanoi

QS = Quorum sensing

S-AdoMet = S-adenosylmethionine

SAR = Systemic Acquired Resistance

TCE = Trichloroethylene

TFRR = Tomato foot and root rot

VBNC = Viable but non-culturable

VWO = Verticillium wilt of olive

CONFLICT OF INTEREST

The authors report no conflict of interest.

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