

Chapter 5

Life of Microbes Inside the Plant

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Abstract A hidden microbial world is present in the interior of all plants. Myriads of bacteria and fungi live inside them without causing apparent deleterious effects to their hosts. They are designated as endophytes. Endophytic communities are variable and diverse. Their structure and composition are shaped by a number of (a)biotic factors. Endophytes have found evolutionary solutions to cope with defensive responses deployed by host plants to face colonization by microbes. In return, they live within an ecological niche that provides better protection against a number of stresses and a reliable and constant source of nutrients. Endophytes seem to contribute to plant fitness and development, displaying beneficial traits that can be exploited in agricultural biotechnology. However, many questions related to the endophytic lifestyle remain to be answered. This chapter summarizes present knowledge on how endophytes are able to establish and endure within plants. Potential biotechnological applications are also briefly presented.

5.1 Introduction: Beneficial Endophytes Defined

Plants live in close association with a huge diversity of microorganisms. In fact, the composite genome of these accompanying microbial communities is far larger than that of the host plant, and thus is also referred to as the plant's second genome. Most of the components of the plant-associated 'microbiome' (Chap. 30) are only able to colonize and persist on plant tissue surfaces or in the soil rhizosphere (Chap. 3). However, some can also establish themselves as non-deleterious endophytes. It is likely that all plants carry endophytes, which play an important role in plant fitness and development. Plants can thus be considered as super organisms of which both the plant and its endophytic microbiome work coordinately to shape and sustain an extraordinary ecosystem. It is generally recognized that endophytes represent just a minor fraction of the microbiota inhabiting plant surfaces or living in close

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proximity to them, and comprise mainly microbes originating from the soil region associated with roots (i.e. the rhizosphere, Chap. 3). In fact only small subpopulations of rhizosphere and phyllosphere microorganisms are able to enter and live inside the plant. Relevant reviews suggested are: Bacon and Hinton (2006); Schulz et al. (2006); Rosenblueth and Martínez-Romero (2006); Hardoim et al. (2008); Ryan et al. (2008); Reinhold-Hurek and Hurek (2011); VV.AA. (2013).

The word *endophyte* means ‘in the plant’ and is derived of the Greek words *endon* (within) and *phyton* (plant). Endophytes have been defined by various authors in somewhat different ways (Bacon and Hinton 2006; Rosenblueth and Martínez-Romero 2006; Schulz et al. 2006; Mercado-Blanco and Lugtenberg 2014). It is generally agreed that they are bacteria and fungi that can be detected at any moment within the tissues of healthy plants, and that do not produce disease symptoms. Microbial phytopathogens, nodule-producing microbes, and mycorrhizal fungi may display endophytic lifestyles during part of their lives but they are not considered here as endophytes. Moreover, this chapter is only focused on bacterial endophytes (for endophytic fungi see, for instance, VV.AA. 2013). Mere isolation from surface-disinfected tissues is not enough to claim a true endophyte. Plant surface sterilization protocols must be sufficiently stringent to eradicate the external microbiota without killing bacteria with the tissue. Moreover, the ‘candidate endophyte’ must be shown to be a true endophyte by both its ability to re-infect disinfected seedlings, and by microscopic evidence (Reinhold-Hurek and Hurek 1998).

The vast majority of bacterial endophytes are non-culturable or VBNC. This may represent a survival strategy to persist hostile conditions within the plant. By implementing culture-independent and metagenomics approaches our understanding of endophytes is being steadily enriched. These methodologies will undoubtedly continue to reveal a much wider diversity and abundance of endophytic communities than that uncovered by traditional culture-dependent methods.

5.2 How Do Endophytes Get into the Plant and Spread to Distant Tissues?

Endophytes gain entrance into plants predominantly through the roots but also through leaves, flowers, stems or cotyledons. Indeed, the vast majority of endophytes are soil-inhabitants and plant colonization seems to mainly originate from the rhizosphere. Evidence confirming this possibility has been obtained by combining biotechnological and microscopy tools. Some endophytes have thus been shown to spread systemically from the original penetration site(s), and be found in distant plant tissues and organs. Consequently, the population density of endophytes is usually higher in roots than in any other plant organ. It is important to stress that an amazing variety of endophytic bacteria and fungi (hundreds of different taxa) can be found in diverse organs/tissues of any individual herbaceous, woody or moss species (Hallmann and Berg 2006; Schulz et al. 2006; Mercado-Blanco and Lugtenberg 2014).

Overall, our knowledge about the specific sites at which endophytic bacteria attach and penetrate into root tissues is scant. Nevertheless, it is generally assumed that bacteria invade roots passively using cracks or wounds located, for instance, at the emergence points of lateral roots. Such root cracks can also be produced by microbial, nematode or arthropod activities. Preferential sites for rhizosphere bacteria attachment and subsequent entry can also be the thin-walled surface layers located in the apical root region, including the root differentiation, elongation and root hair zones as well as the intercellular spaces of the root epidermis. Specific bacterial components that are known to be involved in endophyte attachment to plant tissue include Type IV pili, lipopolysaccharides, and exopolysaccharides (Hardoim et al. 2008; Reinhold-Hurek and Hurek 2011; Mercado-Blanco and Lugtenberg 2014).

Besides being an important attachment structure, root hairs play also a role in root endophytic colonization. Using fluorescently-tagged bacteria and CLSM allowed demonstration that endophytic *Pseudomonas* spp. strains can internally colonize olive root hairs (Prieto et al. 2011) prior to becoming established within the intercellular spaces of the root cortex (Fig. 5.1). Despite root hairs seeming to play a role in bacterial entrance into the roots, several questions remain to be elucidated: (i) the exact timing of and site(s) used for bacterial penetration of the root hair cell; (ii) how the bacteria move to the intercellular spaces of the root cortex; and (iii) whether these bacteria enter root hairs *via* active or passive mechanisms (Mercado-Blanco and Prieto 2012).

Mechanisms used by endophytic bacteria to enter the plant are largely unknown, although several bacterial traits have been proposed to participate in endophytic colonization of plant roots. Once bacteria overcome the exodermal barrier, they may remain either at the site of entry or move towards the intercellular space of the cortex and even to distant parts (Compant et al. 2005; Hardoim et al. 2008; Reinhold-Hurek and Hurek 2011).

5.3 How Do Endophytes Cope with and Adapt to the Inner Plant Environment?

Living inside plant tissues requires adaptation to an environment that provides food and low exposure to (a)biotic stresses. Therein, competition among endophytic microorganisms can be expected, although nothing is yet known about trophic interactions within such microbial communities. However, compared to the highly competitive/predatory environment found outside the plant, its interior is a “safe heaven” for endophytes since it is a reliable and constant source of nutrients. Endophytes have thus evolved to adapt themselves to nutrients available inside plant tissues (Bacon and Hinton 2006; Mercado-Blanco and Lugtenberg 2014).

Data on the ability of endophytes to utilize nutrients found in the plant interior are not abundant. Comparing the abilities of endophytic and highly-related non-endophytic strains to utilize nutrient sources is a strategy to unravel the feeding capabilities of the endophytes. For instance, utilization of L-arabinose has been

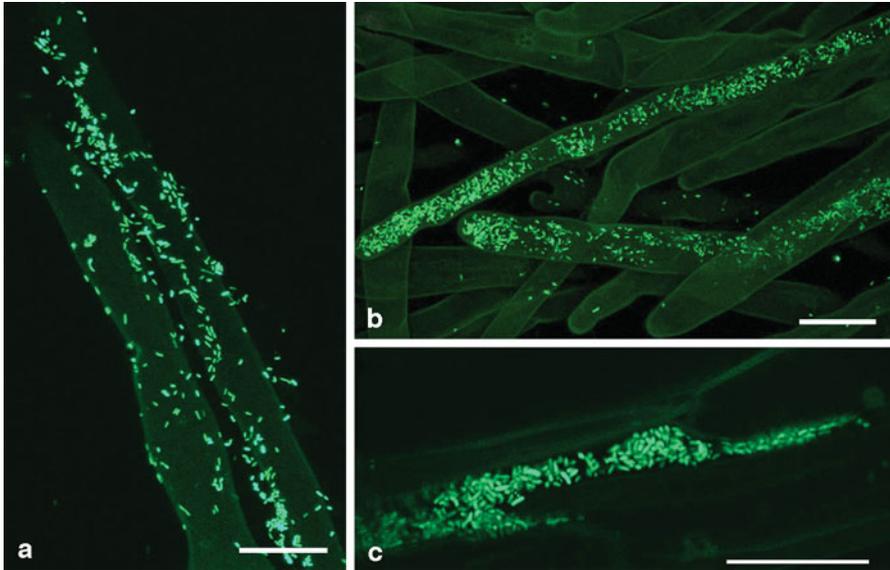


Fig. 5.1 CLSM images of *in vitro* micropropagated olive roots (cv. Manzanilla) colonized by enhanced green fluorescent protein (EGFP)-tagged *Pseudomonas fluorescens* PICF7. **a** Surface colonization of root hairs by PICF7 cells; **b** Detection of EGFP-tagged PICF7 cells inside two root hairs; **c** Intercellular colonization of the root cortical tissue by PICF7 cells. Scale bar represents 50 μm in panel **a** and 20 μm in panels **b** and **c**. For details on olive roots-PICF7 colonization bioassays, tissue sectioning and CLSM imagery, see Prieto et al (2011). These CLSM microphotographs are reproduced from Prieto et al. (2011), doi:10.1007/s00248-011-9827-6

suggested to be an important trait contributing to the endophytic lifestyle of several *Pseudomonas* spp. strains in cucumber (Malfanova et al. 2013). It is likely that other carbon sources may play similar roles in other plant-endophyte associations, and evidence/suggestions for such a role are available (Mercado-Blanco and Lugtenberg 2014). Because of the ability of endophytes to preferentially metabolize specific carbon sources, competition for these nutrients can be a determining factor in shaping the endophytic community in a given host plant or tissue. Indeed, adaptation to specific nutrients' availability may limit the number of endophytic microbes that can be taken up and survive inside plant tissues either under natural conditions or upon artificial inoculation. Comparative genomics approaches aimed to unravel specific traits linked to endophytic lifestyles can provide relevant information on this issue (Mitter et al. 2013; Brader et al. 2014; Ali et al. 2014).

As a toll to be paid for their *in planta* protection, endophytes must develop strategies to silence or evade the various responses that plants use to confront 'non-hostile' endophyte colonization, or attacks by phytopathogens. The strategies that endophytes have evolved to elude such responses and be recognized as innocuous invaders occur by an as yet poorly-understood modulation of the plant-deployed immune response (Zamioudis and Pieterse 2012). It is conceivable that the prevalence of non-culturable

and/or VBNC states encountered among endophytes could be a survival strategy to prevail over stresses operating within plant tissues.

With respect to how endophytes cope with plant defense responses our knowledge is also scant (Reinhold-Hurek and Hurek 2011). Inner colonization and persistence of endophytes within plant tissues likely entail broad changes at the transcriptomic level for both partners. However, little information is available about the genetics changes that an endophyte provokes in the host plant and how colonization modifies the behavior of the new resident. Likewise, the effects that introduced endophytes may provoke on a pre-existing endophytic microbiome and *vice versa* are largely unknown. However, some studies are available and results indicate that colonization of plant tissues by an endophyte triggers, among other changes, a broad range of defensive responses (Conn et al. 2008; Schilirò et al. 2012).

Finally, it is worth mentioning that the composition, abundance, distribution and functionality of the endophytic microbiome found in a given host plant, plant tissue or organ is not static. On the contrary, an endophytic microbiome can be modified over time by the plant growth phase and physiological state, and/or diverse environmental, biological and physical-chemical factors (see, for instance, Van Overbeek and van Elsas 2008; Ardanov et al. 2012).

5.4 The Search for Endophytic-Specific Traits

An important question still to be solved is whether endophytes have specific traits that define their lifestyle. An environment providing specific nutrients, but hostile because of active defense responses, should be a driving force selecting adapted phenotypes (see previous section). Similarly, the presence of microbe-specific machineries for plant tissue penetration could be necessary for establishment as beneficial endophytes, although it is likely that some of the mechanisms could be shared by pathogens. It is also possible that, in contrast to facultative endophytes, obligate endophytes might carry genetic and metabolic determinants more essential to the endophytic lifestyle. Comparative genomics and bioinformatics approaches could definitively help in unraveling specific traits linked to endophytism. Even though studies like these show that some characteristics seem to be shared by different endophytes, the emerging picture is of a broad genetic diversity (Mitter et al. 2013; Ali et al. 2014).

Quorum-sensing (QS) (Chap. 8) and the ability to overcome plant defenses seem to be commonly found in the genomes of the endophytic bacteria analyzed so far. QS systems allow bacteria to 'sense' their own concentration/abundance, thereby triggering the expression of specific target genes only at a high cell density. Considering that bacterial endophytes can reach high populations densities in defined sites, production of QS signals within plant tissues and how those signals operate in endophytic-mediated processes deserve investigation. In contrast, Type III (Chap. 7) secretion systems, which mediate, in gram-negative pathogens and rhizobial symbionts, the delivery to the host plant of effector proteins that suppress

the host defense response, seem rare among endophytes. Instead, other types of secretion systems such as Type VI seem to be more frequent. Such findings can thus shed light on the potential differences between pathogenic and endophytic lifestyles displayed by plant-associated bacteria. Currently, however, the number of genomes and genomic information available from claimed endophytes is still too low to draw sound conclusions (Reinhold-Hurek and Hurek 2011; Mitter et al. 2013; Ali et al. 2014).

5.5 What Endophytes Do for the Plant and Their Potential to be Exploited in Agro-Ecosystems

The presence of non-deleterious endophytes can benefit the host plant in different ways, a scenario of utmost interest once these microbial communities and their multitrophic interactions are properly characterized, understood and harnessed. Endophytes are of increasing interest because of their potential biotechnological applications (see, for instance, Ryan et al. 2008; VV.AA. 2013; Brader et al. 2014; Mercado-Blanco and Lugtenberg 2014). Among the beneficial traits with potential to be exploited in agro-ecosystems, promotion of plant growth, and control of plant diseases are of particular significance. In most cases the mechanism(s) involved, particularly those related to biocontrol, remain to be elucidated. Nevertheless, it has been suggested that beneficial effects deployed by bacterial endophytes might operate through mechanisms similar to those described for rhizosphere bacteria (Kloepper and Ryu 2006). Additional applications of endophytes beyond agricultural biotechnology rely on the ability of the organisms to produce a broad range of bioactive metabolites which are relevant for other purposes, including human health (i.e. antibiotics, antitumor compounds, anti-inflammatory agents, etc.) (see, for instance, Christina et al. 2013; Brader et al. 2014).

Plant growth promotion can be achieved either directly or indirectly. There is little knowledge available about mechanisms of growth promotion exerted by endophytes, and operating *in planta* (Hardoim et al. 2008). Nevertheless, considering that many endophytes are also free-living rhizosphere microorganisms, it is plausible to assume that mechanisms to stimulate plant growth deployed by the latter may also operate once endophytic growth is established. However, this assumption still needs to be confirmed.

Direct promotion of plant growth by endophytic bacteria and fungi can be achieved by the microbe providing (micro)nutrients (biofertilization) and/or phytohormones (phytostimulation) to the plant. Indirect plant growth promotion is a consequence of the suppression of plant diseases exerted by pathogenic microorganisms, and can be mediated by direct antagonism/antibiosis against the pathogens, advantageous out-competing for nutrients and/or space, or by triggering in the host plant enhanced defense capacities against pathogen attack. Growth can also be stimulated indirectly by alleviation of stress caused by environmental pollutants (rhizoremediation, see

Chap. 29) or other stressful abiotic (heavy metals, drought, salinated soils) conditions. For instance, synthesis of the enzyme ACC deaminase reduces the level of the stress hormone ethylene by converting ACC into α -ketobutyrate and ammonia. Production of this enzyme by plant-growth promoting bacteria, including those displaying endophytic lifestyles, can make host plants tolerant to a number of stresses (Chap. 27; Hardoim et al. 2008).

5.6 Concluding Remarks

Many questions on how, why and when any given endophyte(s)-plant consortium is established remain to be elucidated. A list of questions to be answered on this topic has been recently outlined by Mercado-Blanco and Lugtenberg (2014). For instance, little is known on how an ‘endophytic candidate’ is able to overcome or modulate defensive barriers/responses to successfully penetrate and establish within plant tissues. The identification of traits involved in the colonization and persistence within the plant is still incomplete. Similarly, understanding the influence of environmental, physiological, developmental stages and/or genetic factors on endophytes is essential if these organisms are expected to be further developed as biocontrol or (phyto)rhizoremediation tools. In summary, two main questions that should now be put forth are: (i) which driving forces are operating to build up an endophytic community, and (ii) what does the endophytic microbiome do for the plant. The implementation of currently available and powerful ‘-omics’ and microscopy technologies (see Chap. 31) will undoubtedly provide some answer to these questions, as well as those aimed at unraveling the molecular processes that define endophytes (Mitter et al. 2013; Ali et al. 2014).

From a practical perspective, more studies are needed to understand whether the performance of any artificially-introduced endophyte can be affected by the native microbiome of the host plant; and *vice versa*, i.e. how the indigenous endophytic microbiota can be influenced by the introduction of a ‘newcomer’ into this delicately balanced microenvironment, and whether such introductions may alter the plant’s development and fitness.

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