

Satish Kumar Verma
James Francis White, Jr *Editors*

Seed Endophytes

Biology and Biotechnology



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Prologue

What Are Endophytes?

Endophytes are those bacteria and fungi that exist within the tissues of plants without negative effects on host plants. They have been reported from all the plants examined to date. Some endophytes are vectored on or within seeds of plants. These microbes are acquired by plant seeds and may be important to seedling development. Endophytic microbes support plants through mobilization of nutrients from soils, production of phytohormones, and further protecting plants from biotic (fungal pathogens and insects) and abiotic stresses. Further, seed microbes move into the plant body, protecting the plant from disease and improving the nutritional status of the entire plant. Endophytic bacteria stimulate the expression of host genes relating to nitrogen metabolism and hormone synthesis. The presence of endophytes also induces host defense genes in plants and makes them less susceptible to disease. Endophytic microbes produce a large number of agriculturally important metabolites. Seed-associated microbes are believed to be more adapted to plants and have ecological significance. Application of the seed microbiome in agriculture is needed at this time. We believe that seed and seedling microbes will ultimately replace the use of chemical fertilizers and pesticides that degrade the environment.

A New Definition for Seeds: A Miniature “Noah’s Ark” for Plant Colonization

A seed may be defined as a dormant embryonic plant supplied with nutrients enough to fuel early seedling growth and contained in a hardened protective coating. However, it is becoming increasingly clear that this definition is not complete; seeds also carry a community of symbiotic microbes that provide multiple critical functions for developing seedlings—and without these symbiotic microbes seedlings are less likely to survive. In this respect, a seed is like “Noah’s ark” containing the plant and the microbes that are needed for the growth and survival of the seedling as it colonizes in a new place.

The Intention of This Book

With this book we intend to begin to complete the definition of a seed by revealing the many microbes transmitted in and on seeds—and their numerous functions in cultivated and non-cultivated species. We also intend to show the importance of the microbial components of seeds and the adaptations on/in seeds for vectoring microbes. Finally, we intend to show how seed-vectored microbes can be used in biotechnologies to enhance cultivation of crop plants. This book contains chapters describing biology of endophyte communities, endophyte functions in plants, and applications in agriculture and biotechnology. This book will be useful to students, teachers, and scientist working in the areas of plant–microbe interactions and biocontrol. Since interactions of endophytic microbes with plants are relatively underexplored, people interested to work in this area will find this book useful.

Varanasi, India
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Satish Kumar Verma
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Part I

Seed Endophytes: Introduction, and Methods for Assessment and Management



Seed-Vectored Microbes: Their Roles in Improving Seedling Fitness and Competitor Plant Suppression

1

James Francis White, Jr, Kathryn L. Kingsley, Susan Butterworth, Lara Brindisi, Judy W. Gatei, Matthew T. Elmore, Satish Kumar Verma, Xiang Yao, and Kurt P. Kowalski

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Abstract

This chapter discusses the roles of seed-vectored microbes in modulating seedling development and increasing fitness of plants in terms of increased biotic and

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abiotic stress tolerance. Particular emphasis is placed on microbes that function in the rhizophagy cycle. These microbes have been shown to enter into root cells and stimulate root growth. In some cases microbe entry into root cells results in root growth repression. The term ‘endobiome interference’ has been applied to the phenomenon of plant growth repression due to intracellular microbes. The potential application of endobiome interference to produce bioherbicides that selectively enhance growth of target crops but inhibit competitor weeds is discussed.

Keywords

Bioherbicide · Endophyte · Endobiome interference · Growth promotion · Rhizophagy cycle

1.1 The Seed Microbiome

Plant seeds carry embryonic plants and nutrients for early stages of seedling growth; in some plants seeds also carry small communities of symbiotic microbes (primarily bacteria and fungi) that are needed for defense from pathogens, modulation of plant development, and nutrient acquisition in seedlings (Doty 2017; Gond et al. 2015; Hardoim et al. 2015; Hurek et al. 2002; Clay et al. 2016; Irizarry and White 2017; Johnston-Monje and Raizada 2011; Rodríguez et al. 2017; Sherin et al. 2018; Soares et al. 2015, 2016; Puente et al. 2009; Verma et al. 2017a, b, 2018; White et al. 2012, 2015). Seed-vectored symbiotic microbes are adapted to their host plants and may enable seedlings to survive and thrive (Compant et al. 2010; Kandel et al. 2017). Without symbiotic microbes, many seedlings do not develop properly. They often lack normal root gravitropic response where roots do not grow downward into the soil or other substrates, sometimes growing upward, not producing root hairs, or producing hairs that are sparse or short (Holland 1997; Verma et al. 2017a, b; White et al. 2012). Seedlings without their microbes are more susceptible to abiotic and biotic stress: diseases, herbivory, oxidative stresses, drought, and heavy metals (Rodríguez et al. 2009; Torres et al. 2012; Waller et al. 2005; White and Torres 2010).

1.2 Adaptations of Seeds to Carry Symbiotic Microbes

In many grasses of subfamily Pooideae, fungal *Epichloë* endophytes colonize the ovules of the maternal plant and grow into the embryo inside caryopsis—thus germinating seedlings already contain the fungal endophyte (White and Cole 1986). We have found that some seed-associated tissues appear to function to vector microbes on seeds. Dried paleas and lemmas that adhere closely to grass seed coat (or caryopsis testa) vector bacteria and sometimes fungi that colonize roots and shoots of the germinating seedlings as they emerge from the seeds (White et al. 2012). The characteristically winged seeds of species in the plant family Polygonaceae, where wings are thought to function in dispersal, also carry bacteria that colonize germinating seedlings. In cotton (*Gossypium* spp.; Malvaceae),

elongated trichomes (cotton fibers) carry bacteria that may stimulate seedling growth and protect cotton plants from diseases. Removal of the cotton fibers by acid delinting as is commonly done makes seeds easier to process in mechanical planters but also removes symbiotic bacteria from cotton seeds, leaving the seedlings defenseless from pathogens and insect pests and compromised developmentally (Irizarry and White 2017, 2018). As a consequence, cotton is often considered to be “the world’s dirtiest crop” due to the amount of agrochemicals frequently used in its cultivation (Environmental Justice Foundation 2007).

1.3 Roles of Seed-Vectored Microbes in Plant Seedlings

Considerable experimental evidence has been accumulated that supports the disease-suppressive role of seed-vectored microbes (Verma et al. 2018). These microbes control disease in multiple ways: (1) by direct colonization of potentially pathogenic soilborne fungi and suppression of their growth and virulence, (2) colonization of seedlings resulting in upregulation of defense-related genes that makes plants more resistant to disease (Gond et al. 2015; Irizarry and White 2018), and (3) excluding pathogenic microbes by monopolizing space and/or production of antibiotics or toxins.

Bacteria and fungi associated with seed tissue also influence development of seedlings. This process is not well understood, but microbes colonize the seedlings and increase gravitropic response, root elongation rate, root branching, and root hair elongation (Irizarry and White 2017; Verma et al. 2017a, b; White et al. 2012, 2017). It has been hypothesized that the capacity of microbes to alter hormone levels in plants may account for the capacity of microbes to modulate plant development (Bacon and White 2015). For example, the gravitropic response in seedlings may be suppressed when seedlings contain high levels of ethylene (Buer et al. 2006). Microbes that possess an enzyme (ACC deaminase) to remove a precursor to ethylene may remove ethylene that inhibits the gravitropic response in roots, and this could account for the gravitropic response modulation effect (Bacon and White 2015). Microbes also produce indole acetic acid (IAA); this hormone could explain other aspects of modulation of root growth—including branching and root hair elongation (Bacon and White 2015). In one experiment (White et al. 2012) using grass seedlings with and without seed-vectored bacteria, it was shown that both gravitropism and root hair formation could be restored in axenic seedlings by seed germination and seedling growth on agarose that contained low concentrations of proteins, certain amino acids, or the vitamin thiamine. In this study, it was suggested that microbes modulate plant root development by supplying organic nitrogen or vitamins.

Seed-vectored bacteria and fungi also modify the physiological readiness of the seedling to tolerate oxidative stresses—either biotic or abiotic in origin. Many stresses of biotic and abiotic origin affect plants negatively by increasing internally generated reactive oxygen species (ROS)—which leads to increased internal oxidative damage in plants to membranes, proteins, and nucleic acids—and eventually to cell death (Cabisco et al. 2000; Hamilton et al. 2012; White and Torres 2010). Seed-

vectored microbes colonize seedlings and elicit a reactive oxygen defense response in plants that causes seedlings to upregulate stress resistance and antioxidant genes—resulting in seedlings that are more tolerant to oxidative stresses than seedlings without the microbes (Hamilton et al. 2012; Irizarry and White 2018; Kuldau and Bacon 2008; White and Torres 2010). Some seed-vectored microbes have been found to produce secondary metabolites that directly impact herbivores of the plant and may deter feeding by herbivores (Clay 1988; Clay et al. 2005). Fungal endophytes in genus *Epichloë* (Clavicipitaceae; Ascomycota) produce alkaloids that may intoxicate herbivores and repel them from infected seedlings (Schardl et al. 2013). In the toxic locoweeds (*Oxytropis* and *Astragalus* spp.; Fabaceae) and other plant species, endophytic fungi (e.g., *Undifilum* spp.; Pleosporaceae; Ascomycota) produce the toxic alkaloid swainsonine that intoxicates animals and deters many herbivores from consuming the plant (Cook et al. 2014, 2017). Similarly, seed-vectored fungi of genus *Periglandula* (Clavicipitaceae; Ascomycota) in morning glories (*Ipomoea* spp.; Convolvulaceae) produce ergot alkaloids that render the plant toxic to animals and deter herbivory (Steiner et al. 2011).

1.4 What Happens to Seed-Vectored Microbes?

Some of the microbes that associate with plants are to be found primarily in the rhizosphere and function in the soil. Other microbes colonize surfaces of the plant and function in the rhizoplane (root surface)—or phylloplane (leaf surface). However, some of the seed-vectored microbes show the capacity to colonize seedlings internally and enter into tissues of the plant either intercellularly or intracellularly (Beltrán-García et al. 2014; Stone et al. 2000; White et al. 2014). This internal niche has been referred to as the “endosphere” (Hardoim et al. 2015; Kandel et al. 2017). The microbes that inhabit the endosphere as endophytes form the “endobiome” (Kaul et al. 2017). A diversity of microbes (prokaryotic and eukaryotic) constitute the endobiome. A subset of endobiome microbes internally colonize plant cells—typically locating in the periplasmic spaces between the plasma membrane and plant cell wall (Thomas and Reddy 2013; Thomas and Soly 2009; White et al. 2017, 2018a, b).

While microbes may colonize shoots and roots of plant seedlings upon germination, only those in roots play roles in acquiring nutrients from soils (Bowsher et al. 2016). Plants secrete exudates that contain sugars, amino acids, organic acids, and vitamins from roots that stimulate growth of microbes carried on or within seeds—and other microbes may be recruited from soils. It is generally believed that through secretion of exudates, plants alter the numbers and diversity of microbes on root surfaces and in the rhizosphere (Broeckling et al. 2008). Plants are known to increase secretion of exudates in nutrient-limiting soils, likely leading to increased microbial activity around roots and increased “microbial mining” for nutrients (Bowsher et al. 2016). Root exudates attract bacteria in particular that will grow in a biofilm in the root exudates (Funk-Jensen and Hockenhull 1984). In this sense, root exudates act as signal molecules that attract a diverse community of microbes to the exudate zone and biofilm around the root tip meristem (Rudrappa et al. 2008; Badri and Vivanco

2009). Through the continued secretion of root exudates, plants are cultivating microbes, and when nutrients are scarce, plants increase cultivation of microbes by producing more exudates (Bowsher et al. 2016). The response of plants to increase the density and diversity of the microbial community around roots by secreting more root exudates in nutrient-limiting situations is consistent with the hypothesis that the root-associated microbes function in nutrient acquisition. The microbes function to make available to plant roots macronutrients and micronutrients needed by plants. White et al. (2015) conducted an isotope tracking experiment where ^{15}N -labeled protein was incorporated into agarose—then tall fescue (*Lolium arundinaceum*) seedlings, both with and without seed-surface microbes, were grown on the agarose. After analysis of the ^{15}N content of seedling shoots, it was found that seedlings with seed-vectored microbes contained 30% more of the ^{15}N than seedlings without microbes. This 30% increase in nitrogen absorption into plants with seed-vectored microbes may reflect “nutrient mining” by the seed microbes. The superior capacity of microbes to move around in the rhizosphere either by mycelial growth for fungi or by flagellar motility for bacteria enables them to access pools of nutrients that the plants may be unable to obtain alone. The possession of siderophores by bacteria and fungi (Johnstone and Nolan 2015) further enables them to acquire nutrients (like metals iron, zinc, copper, and magnesium) and transport these nutrients back to plants where they may be transferred to plant roots.

A subset of the seed-vectored bacteria and yeasts colonize the exudate zone around the root tip meristem. These microbes penetrate into the outer layers of recently formed root parenchyma cells around the root meristem. These microbes then become situated in the periplasmic spaces of root parenchyma (Verma et al. 2018; White et al. 2019). Initially, intracellular bacteria retain their cell walls and cell shapes—but as root cells differentiate, bacteria are exposed to secreted ROS produced by NADPH oxidases (NOX) present on root cell plasma membranes (White et al. 2014). Intracellular bacteria lose their cell walls to form wall-less L-forms (Errington et al. 2016). Intracellular L-forms proliferate in the periplasmic space by a continuous budding process—referred to as “blebbing” (Errington et al. 2016; Beran et al. 2006). L-forms are exposed to ROS, the most potent of which is likely superoxide produced by NOX enzymes (White et al. 2012, 2014). ROS oxidizes bacterial L-form plasma membranes—and penetrates into the bacterial cytoplasm resulting in oxidative damage internally (Cabiscol et al. 2000; Lamb and Dixon 1997). The net effect of the continuous bombardment of bacterial L-forms by ROS is that bacterial membranes become leaky and cell contents including electrolytes are lost and some of the bacterial cells are entirely degraded (Paungfoo-Lonhienne et al. 2010, 2013; White et al. 2012, 2017). Surviving bacteria trigger elongation of root hairs, and as hairs elongate, bacteria exit the hair at the elongating hair tip where cell walls are incompletely formed (White et al. 2017, 2018a, b); as bacteria exit, they reform cell walls and reenter the rhizosphere where they may acquire additional nutrients. The process of degradation of microbes within roots has been termed “rhizophagy” (meaning “root eating”) (Paungfoo-Lonhienne et al. 2013). The cyclic process where symbiotic bacteria alternate between a free-living soil phase and an intracellular endophytic phase has been termed “rhizophagy cycle” or “rhizophagy

symbiosis” (Verma and White 2018; White et al. 2018a, b). It seems reasonable that the primary function of the rhizophagy cycle is the transport of nutrients via microbes from the rhizosphere to the plant root where nutrients are extracted from microbes (Hill et al. 2011; Beltrán-García et al. 2014; Prieto et al. 2017; White et al. 2018b). It is also logical that microbes that are symbiotic with plants and function in the rhizophagy cycle are adapted to the host plant and show the following features: (1) possess the capacity to enter plant cell walls at the root tip meristem; (2) release electrolytes to plant cells on exposure to ROS secreted by root cell plasma membranes; (3) possess the ability to survive ROS exposure in its host; (4) trigger root hair elongation to exit the hair as it elongates; and (5) are attracted back to the root exudate zone at root tip meristems.

1.5 Signal Molecules

It has been hypothesized that plants signal microbes to come to the roots by the composition of root exudates (Badri and Vivanco 2009; Clarkson and Marshner 1995); thus the exudates themselves represent signals to symbiotic microbes. We have evidence (White et al. 2018b) that plants detect the presence of some bacteria in the exudate zone by detecting fermentation products of the bacteria—one of which is butyric acid. Butyric acid is an anaerobic fermentation product of carbohydrates by some bacteria. Butyric acid is absorbed in root tip meristems (Lanzagorta et al. 1988; Tramontano and Scanlon 1996)—and its removal from the bacterial biofilm in the exudate zone around the meristem causes some bacteria to upregulate virulence genes (Cox et al. 1994; Sun and O’Riordan 2013) and infect plant root cells around the meristem. A similar mechanism is seen in the intestinal tracts of animals. In animal intestines, bacteria produce butyric acid. As long as butyric acid remains in elevated concentration of the biofilm in which bacteria grow, bacteria remain in the biofilm; however if levels of butyric acid fall due to dysbiosis, gut bacteria like *Salmonella* spp. become virulent and infect gut epithelial tissues (Sun and O’Riordan 2013). In experiments using grass seedlings (*Poa annua*) inoculated with *Pseudomonas* spp. and germinated and grown on agarose amended with 0–10 mM butyric acid, we demonstrated that butyric acid at approximately 5 mM concentration suppressed entry of bacteria into root tip meristem cells. In this experiment (White et al. 2018b), the root tip meristem was unable to remove butyric acid from the bacterial biofilm around the meristem, and as a consequence, bacteria did not penetrate into the root meristem cells. Suppression of bacterial entry into meristem cells resulted in the loss of gravitropic response and root hair formation in seedling roots (White et al. 2018b). Another fermentation product that acts as a signal molecule for plants and bacteria is propionic acid. When propionic acid is present at a sufficient level in the bacterial biofilm around the root tip meristem, bacteria do not penetrate into meristem cells (Unpublished data). Propionic acid is also absorbed by root tip meristems (Lanzagorta et al. 1988; Tramontano and Scanlon 1996), and its removal from bacterial biofilms along with butyrate results in internal colonization of root tip meristem cells.

1.6 Endobiome Interference

We hypothesize that microbes of the endobiome of a particular plant are adapted to the internal conditions of that plant and that the conditions in the endospheres of plants may differ between species of plants. The removal of endobiome microbes from hosts to which they are adapted, and transference to seedling hosts to which they are not adapted, could result in (1) internal colonization, (2) interference with the functioning of other microbes of the endobiome, (3) interference with plant development, or (4) increases in seedling mortality. Perturbations in seedling development or increases in seedling mortality as a result of colonization by nonadapted microbes may result from “endobiome interference.”

To evaluate whether “endobiome interference” occurs, we conducted a series of experiments where we removed microbes (bacteria and yeasts) from seeds of plants, including species rosary pea (*Abrus precatorius*), snakecotton (*Froelichia gracilis*), tomato (*Lycopersicum esculentum*), and annual bluegrass (*Poa annua*), and inoculated them onto axenic seedlings in agarose. We then assessed internal colonization of seedling roots, root growth, and seedling mortality (Table 1.1; Figs. 1.1, 1.2, and 1.3). Test seedlings included dandelion (*Taraxacum officinale*), curly dock (*Rumex crispus*), and clover (*Trifolium repens*). Some experiments were also done where microbes were inoculated onto seedlings of prince’s-feather (*Amaranthus hypochondriacus*) and green amaranth (*Amaranthus viridis*). Microbes included yeasts *Rhodotorula* sp. (strain Abrus#1) and *Aureobasidium pullulans* (strain Froelichia#2) and bacteria *Sphingomonas* sp. (strain Abrus#3), *Rhodococcus* sp. (strain AbrusR), *Micrococcus luteus* (strain Lycopersicon#1), *Curtobacterium* sp. (strain Froelichia#4), and *Paenibacillus* sp. (strain PA-NA-2B1). None of these microbes appeared to be pathogenic or inhibitory of root growth in their original hosts based on growth of seedlings containing microbes on agarose media. In fact, *Micrococcus luteus* was found to be growth promotional in tomato seedlings, resulting in intracellular colonization and increased root hair length. All of the microbes were found to become intracellular in seedling root cells when inoculated onto germinating seeds (Figs. 1.1c, e, f, 1.2, and 1.3). The occurrence of effects that we consider to constitute endobiome interference depended on the microbe, and the host seedling the microbe was inoculated into. The two most potent microbes in terms of increased mortality in seedlings after 3 weeks included the yeast *Aureobasidium pullulans* (Froelichia#2) and bacterium *Micrococcus luteus* (Lycopersicum#1). In terms of inhibition of root growth, *Rhodotorula* sp. (strain Abrus#1), *Sphingomonas* sp. (strain Abrus#3), and *Micrococcus luteus* (Lycopersicum#1) were more inhibitory. *Curtobacterium* (strain Froelichia#4) was growth promotional in all three species, increasing root growth and reducing seedling mortality in test seedlings. The mechanisms of inhibition of root growth or increase in seedling root growth are not clear.

However, two factors seem relevant to the longevity of microbes, including (1) entry of microbes into root cells and (2) resistance of microbes to ROS secreted by the host. Microbes that are highly resistant to ROS secreted by root cells may be difficult to control once they are in the endosphere—and especially when they become intracellular. Microbes that enter root cells and situate in close contact

Table 1.1 Endobiome interference experimental result summary

Microbe	Host origin	Target host	Intracellular	Δ Root length (%) ^a	Δ Mortality (%)
<i>Rhodotorula</i>	<i>A. precatorius</i>	Dandelion	Yes	-40	+18
<i>Rhodotorula</i>	<i>A. precatorius</i>	Curly dock	Yes	-45	-27
<i>Rhodotorula</i>	<i>A. precatorius</i>	Clover	Yes	-64	+24
<i>Sphingomonas</i>	<i>A. precatorius</i>	Dandelion	Yes	-28	+39
<i>Sphingomonas</i>	<i>A. precatorius</i>	Curly dock	Yes	-19	0
<i>Sphingomonas</i>	<i>A. precatorius</i>	Clover	Yes	-40	-6
<i>Rhodococcus</i>	<i>A. precatorius</i>	Dandelion	Yes	-7	+50
<i>Rhodococcus</i>	<i>A. precatorius</i>	Curly dock	Yes	-23	-3
<i>Rhodococcus</i>	<i>A. precatorius</i>	Clover	Yes	-13	0
<i>Aureobasidium</i>	<i>F. gracilis</i>	Dandelion	Yes	+46	+44
<i>Aureobasidium</i>	<i>F. gracilis</i>	Curly dock	Yes	-17	+57
<i>Aureobasidium</i>	<i>F. gracilis</i>	Clover	Yes	+2	+10
<i>Curtobacterium</i>	<i>F. gracilis</i>	Dandelion	Yes	+15	-13
<i>Curtobacterium</i>	<i>F. gracilis</i>	Curly dock	Yes	+10	-13
<i>Curtobacterium</i>	<i>F. gracilis</i>	Clover	Yes	+7	-7
<i>Micrococcus</i>	<i>L. esculentum</i>	Dandelion	Yes	-60	+80
<i>Micrococcus</i>	<i>L. esculentum</i>	Curly dock	Yes	-30	-14
<i>Micrococcus</i>	<i>L. esculentum</i>	Clover	Yes	-27	+31
<i>Paenibacillus</i>	<i>P. annua</i>	Dandelion	Yes	-12	+51
<i>Paenibacillus</i>	<i>P. annua</i>	Curly dock	Yes	-30	-14
<i>Paenibacillus</i>	<i>P. annua</i>	Clover	Yes	-	-

^aPercentages are from means of 40 seeds/seedlings; mortality includes germination suppression and seedling death after 3 weeks on agarose; Δ designates change (Δ Root length = change in root length compared to control, and Δ Mortality = change in mortality compared to control)

with root cell plasma membranes may be able to extract more nutrients from plant cells and may more frequently trigger cell death. *Micrococcus luteus* and *Aureobasidium pullulans* are good examples where this may be occurring. Both microbes are resistant to ROS due to production of antioxidants (White et al. 2018b). *Micrococcus luteus* produces antioxidant carotenoids, catalases, peroxidases, and other antioxidant enzymes that reduce the negative effects of host-secreted ROS (Mohanna et al. 2013). Similarly, *Aureobasidium pullulans* possesses antioxidant cell wall components mannans and glucans (Machova and Bystricky 2013), and because they are eukaryotic, their plasma membranes are reinforced with ergosterol to stabilize the membrane and prevent passage of ROS into the cytoplasm (White et al. 2018b). This oxidative resistance may enable *Micrococcus luteus* and

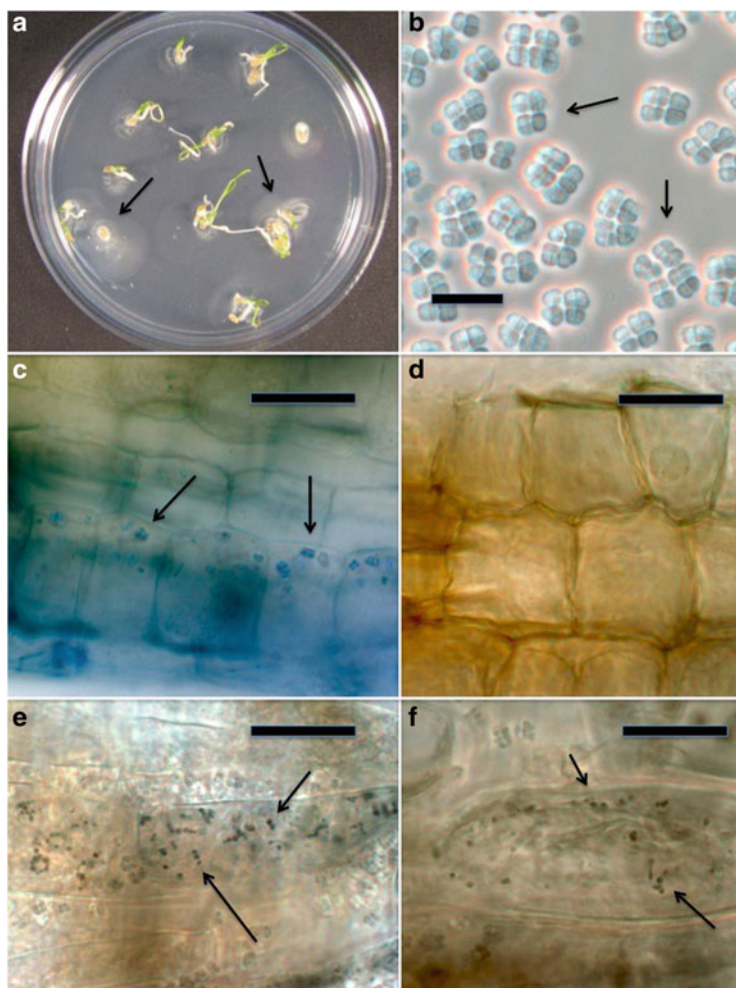


Fig. 1.1 *Micrococcus luteus*. (a) *Micrococcus luteus* in the seedling of carrots (arrows) show white accumulations of bacteria around seedlings on agarose after 2 weeks. (b) Tetrads of *Micrococcus luteus* (arrows) from colonies on yeast extract-sucrose agar (bar = 10 μ m). (c) Cells around root tip meristem of a seedling of *Rumex crispus* showing tetrads of *Micrococcus luteus* (arrows) in the periplasmic space of cells (bar = 25 μ m). (d) Cells around the root tip meristem of a seedling of *Rumex crispus* that had not been inoculated with *Micrococcus luteus*, showing that bacteria are not visible in cells (bar = 25 μ m). (e) and (f) Parenchyma cells of *Rumex crispus* showing spherical bacterial L-forms of *Micrococcus luteus* (arrows) in the periplasmic space of cells (bar = 25 μ m)

Aureobasidium pullulans to proliferate within root cells in an unregulated manner. Overgrowth of these microbes within root cells and tissues results in diversion of seedling nutrients from support of seedling growth to microbe replication—resulting in seedling growth suppression. This is especially evident in the case of *Micrococcus luteus* where inoculated seedlings on agarose were found to have repressed root growth with bacteria accumulating en masse around seedling roots.

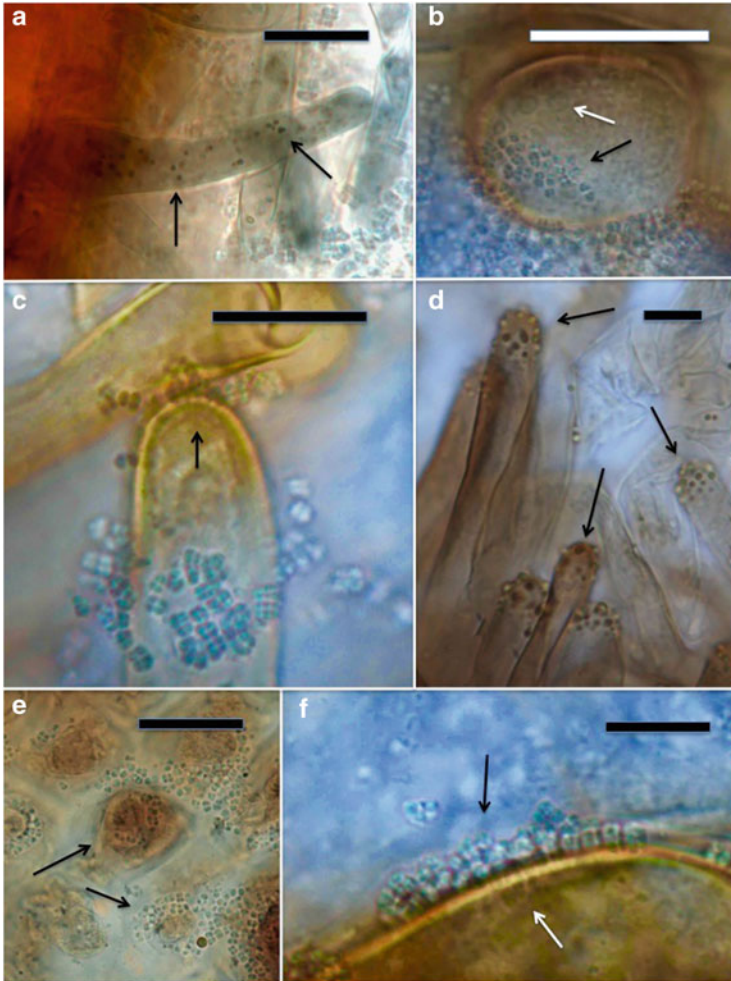


Fig. 1.2 *Micrococcus luteus* in host tissues (stained with 3,3-diaminobenzidine for 15 h followed by aniline blue). (a) L-forms of *Micrococcus luteus* (arrows) in root hair of *Rumex crispus* (bar = 25 μ m). (b) Root hair initial of *Rumex crispus* seedling showing spherical L-forms in the periplasmic space (white arrow) and blue tetrads of *Micrococcus luteus* reforming as bacteria (black arrow) exit through the cell wall and spill off the side of the root hair initial (bar = 25 μ m). (c) Root hair of *Rumex crispus* seedling showing *Micrococcus luteus* exiting the root hair at the hair tip and reforming tetrads (arrows; bar = 25 μ m). (d) Root hairs of carrot (*Daucus carota*) seedling showing exiting of *Micrococcus luteus* from the tips of hairs (arrows; bar = 20 μ m). (e) Root hair initial of carrot seedling showing *Micrococcus luteus* emerging from the hair initial (arrows; bar = 20 μ m). (f) Root hair tip of *Rumex crispus* seedling showing spherical L-forms (white arrow) in the periplasmic space and tetrads (black arrow) of *Micrococcus luteus* just outside the cell wall; exit channels are visible passing through the cell wall (bar = 5 μ m)

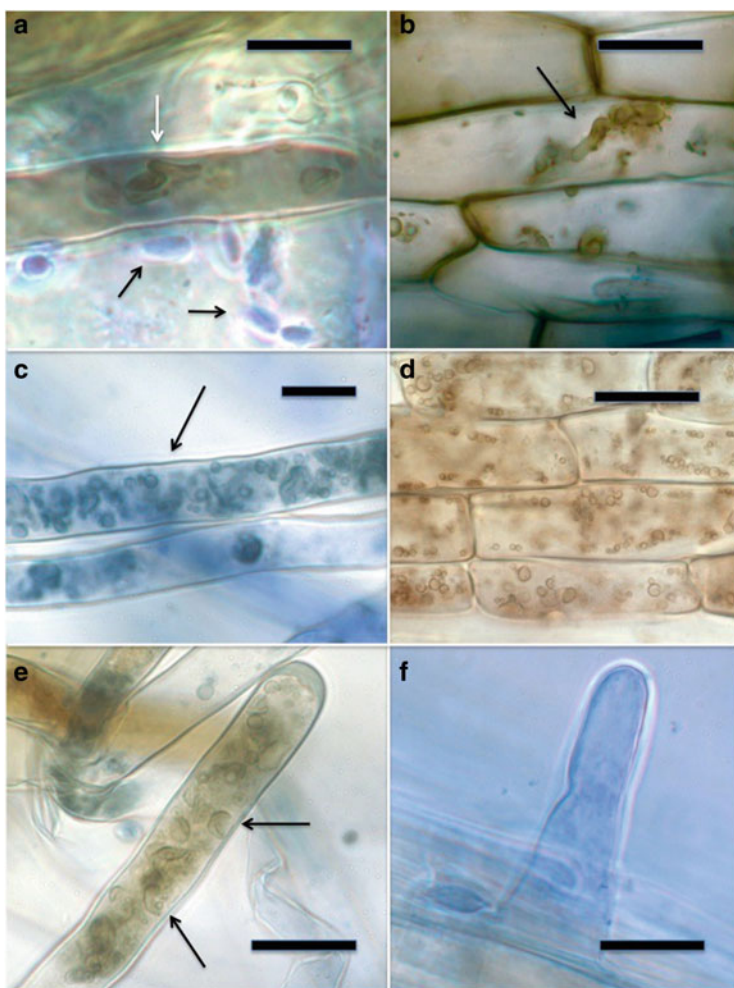


Fig. 1.3 *Aureobasidium pullulans* in seedling cells (stained with 3,3-diaminobenzidine for 15 h, followed by aniline blue). (a) Root hair of *Amaranthus hypochondriacus* seedling inoculated with *Aureobasidium pullulans*, showing intracellular, brown-staining, and collapsed walled yeast cells (white arrow) and extracellular, blue-staining yeast cells (black arrows; bar = 20 μ m). (b) *Amaranthus viridis* seedling root parenchyma cell showing intracellular walled hypha (arrow) of *Aureobasidium pullulans* (bar = 25 μ m). (c) *Froelichia gracilis* seedling root hair showing intracellular yeast mycosomes (arrows; bar = 20 μ m). (d) *Amaranthus viridis* root parenchyma cells showing abundance of intracellular brown-staining yeast mycosomes (bar = 25 μ m). (e) *Froelichia gracilis* seedling root hair with intracellular yeast mycosomes (arrows; bar = 20 μ m). (f) *Amaranthus viridis* root hair without intracellular yeasts (bar = 20 μ m)

1.7 Mode of Entry of *Micrococcus luteus* into Root Cells

We tracked *Micrococcus luteus* through seedling tissues and cells in the previously described “endobiome interference” experiments. *Micrococcus luteus* initially infected root meristem cells—entering periplasmic spaces of outer layers of root tip meristem cells as walled tetrads (Fig. 1.1b, c). As root cells matured, the tetrads converted to unicellular cells—likely L-forms. Spherical cells (wall-less L-forms) were visible in periplasmic spaces of root epidermal cells and root hairs when they formed (Figs. 1.1e, f and 1.2a, b, f). The spherical cells did not swell or lose capacity to stain with aniline blue, suggesting that plant ROS was not degrading the intracellular bacterial cells. This is an indication that *Micrococcus luteus* is resistant to ROS produced by NOX on the root cell plasma membranes. When less oxidatively resistant pseudomonads are used, L-forms in periplasmic spaces swell and lose interior staining with aniline blue due to loss of cell contents by the L-forms (White et al. 2014, 2017). The spherical bacteria of *Micrococcus luteus* in primordial root hairs were seen to exit root hair tips through channels in the plant cell walls. Once outside, bacteria reformed walls and tetrad shapes (Fig. 1.2b–f). This route of bacteria through root tissues is what has been observed for bacteria in the rhizophagy cycle. It seems evident that microbes that show endobiome interference are partially compatible with host plants but they are not adapted to the host like native symbiotic microbes—and rather than increase the growth and survival of seedlings, they reduce growth and/or increase seedling mortality.

1.8 Intracellular Phases of *Aureobasidium pullulans* and *Rhodotorula* sp.

Atsatt and Whiteside (2014) demonstrated that *Aureobasidium pullulans* and *Rhodotorula pinicola* develop an intracellular phase in plants. The intracellular phase includes cells that retain cell walls and those forms that lack cell walls termed “mycosomes.” Our experiments with various Amaranthaceae suggest that *Aureobasidium pullulans* may be a frequent endophyte in this family of plants—although much more work is needed to evaluate its functional role in these plants. In *Abrus precatorius*, *Rhodotorula* sp. may be a common seed-vectored endophyte. Mycosomes appear to behave like bacterial L-forms in that they bud or “bleb” sequentially to form chains (Atsatt and Whiteside 2014). The wall-less mycosome phase may also be a response to plant-produced reactive oxygen or to particular nutrients to which fungi are exposed to in plant tissues. Mycosomes have also been reported to spontaneously revert to the walled cell phase (Atsatt and Whiteside 2014). Intracellular walled *Aureobasidium* cells are visible in Fig. 1.3a, b, while mycosomes are seen in Fig. 1.3c–e. It is unclear whether these fungal endophytes may be degraded in plant cells or whether they can function in the rhizophagy cycle to provide nutrients to the host plant. Yeasts may be entering root cells at the root tip meristems just as do bacteria based on the presence of mycosomes

in meristematic cells in our inoculation experiments. Much additional work is needed to evaluate the function of the intracellular phases of fungi in plant tissues.

1.9 Does Endobiome Interference Affect Plant-Plant Interactions?

It is possible that some plants may maintain microbial symbionts and nourish them within tissues and cells as defensive or offensive weapons that may be employed against competitor plant species. The plants from which we obtained seed-vectoring microbes in the endobiome interference experiment are generally aggressive weedy species. However tomato plants are known to have allelopathic properties—where tomato plants may suppress growth of some other plant species. *Abrus precatorius*, *Froelichia gracilis*, and *Poa annua* are competitive and may be invasive. It is entirely possible that these species use their endobiomes against competitor plant species—where microbes may colonize competitor seedlings and reduce their growth and persistence. This possibility seems likely when it is considered that microbes in the rhizophagy cycle alternate between an endophytic/intracellular phase and a free-living soil phase (Prieto et al. 2017; Verma et al. 2018; White et al. 2018b). These microbes may move out from the plant, forming a zone around plants where certain vulnerable competitor species cannot grow. Seedlings of competitor species that begin to grow in that zone could be colonized and their nutrients used by the microbes for reproduction; return of bacteria to the original host plant may deliver nutrients extracted from the competitor plant species. In a previous study (White et al. 2017) of seed-vectoring pseudomonads from invasive *Phragmites australis*, pseudomonads were seen to promote the growth of grass seedlings but were seen to inhibit growth of competitor dicot species (*Taraxacum officinale* and *Rumex crispus*). It is conceivable that plant species that share endobiome microbes, where those microbes are growth promotional in both plant species, may grow together, while endobiome interference may force plant species apart. Additional experiments are needed to determine whether endobiome interference is a factor in plant-plant interactions in natural plant communities.

1.10 Potential Applications of Endobiome Interference to Control Invasive or Weedy Plant Species

Invasive and weedy plant control generally employs the use of chemical herbicides or mechanical removal of plants (Kowalski et al. 2015). Endobiome interference could offer an alternative means whereby particular weeds could be controlled without herbicides. It may be possible to enhance growth of crop species and simultaneously repress growth of weedy competitor species through applications of microbes that are growth promotional in crops—but produce endobiome interference in competitor plants. Such an approach could reduce applications of agrochemicals in crops with economic and environmental benefits.

1.11 Conclusions

Seed-vectored microbes play roles in modulation of seedling development, defense from abiotic and biotic stresses, defense from pathogens and herbivores, and nutrient acquisition. It is also possible that through endobiome interference, symbiotic microbes of one plant may suppress growth of competitor plant species by reducing seedling growth and increasing seedling mortality. The mechanisms of endobiome interference are not well understood; however, oxidative resistance of the microbe may reduce the capacity of host cells to control intracellular microbes using ROS produced by NOX enzymes on root cell plasma membranes. We hypothesize that endobiome interference is a factor in plant-plant interactions in natural plant communities. If the hypotheses expressed in this chapter are proven, endobiome interference could be a strategy that may be developed to control invasive or weedy plant species.

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Thinking About PPFM Bacteria as a Model of Seed Endophytes: Who Are They? Where Did They Come from? What Are They Doing for the Plant? What Can They Do for Us?

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Abstract

Bacteria in the genus *Methylobacterium* (PPFM bacteria) are distributed globally and are associated with algae, mosses, ferns, liverworts, gymnosperms and angiosperms as co-evolved symbionts. As such, they share significantly in plant metabolism, stimulating plant growth and development through the production of plant hormones and vitamins. This chapter discusses the PPFMs as model plant symbionts and considers how their symbiotic relationship with plants and be exploited to our benefit.

Keywords

Methylobacterium spp. · PPFM bacteria · Plant/microbe symbiosis

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2.1 Fooled by a Bacterium

My interest in seed endophytes comes from a time when I was fooled by them. In the summer of 1988, I was a postdoctoral fellow studying the urease enzymes of soybean in Joe Polacco's lab at the University of Missouri. The lab had identified two distinct urease isozymes by genetic means, and crosses were made to produce urease knockouts. Surprisingly, the knockouts retained a urease activity. Was this a third isozyme? The possibility was unlikely given the comprehensive screening for urease mutants that the lab had just carried out.

We were still wondering about how we could have missed the third soy urease when we heard a talk by plant pathologist, John Dunleavy (1988). He described finding a methylotrophic bacterium on the leaf surfaces of corn and soybean plants distributed ubiquitously across Iowa. Of significance to us, he mentioned that the bacterium produced a urease enzyme. This was a true "aha" moment that left us wondering whether this bacterium could be the source of the third urease. The bacterium was *Methylobacterium*, known in the literature as a PPFM (pink-pigmented facultative methylotroph: Corpe and Basile 1982; Corpe and Rheem 1989). Returning to the lab, I ground up some soybeans and leaves and plated them out on a selective medium to look for the PPFMs. Of course, they were abundant on all of our plates and easily recognized by their bright pink color. It took a while for us to convince ourselves that the urease produced by these bacteria was the mysterious third urease in soy, not because the two activities were not biochemically identical; they were. What took time was convincing ourselves that this enzyme activity produced by a bacterium associated with the plant could be expressed at a level that allowed it to masquerade as a plant enzyme. We had been fooled by a bacterial endophyte (Holland and Polacco 1992).

2.2 Recognizing the Significant Role of Bacteria in Plant Metabolism

I grew up with the idea that seeds, fruits, and the internal tissues of plants were sterile. The notion that internal tissues of plants are sterile traces back to the experiments of Louis Pasteur, who demonstrated that grape juice drawn from the fruit with a sterile needle did not ferment like juice from crushed grapes. His conclusion was that there were no bacteria or fungi under the skin of the fruit. This idea ran counter to results obtained by Galippe, whose experiments concluded that bacteria from the soil were a source of bacteria on all fruits and vegetables (reviewed in Compant et al. 2012), but such was the reputation of Pasteur that his results influenced generations of botanists and botany texts, while Galippe's results were buried. One of Pasteur's students, Fernbach, even carried out experiments on a variety of fruits and vegetables to confirm his mentor's results. Although he routinely found bacteria associated with the plants he examined, he passed them off as contaminants of what must otherwise have been sterile tissue (Fernbach 1888). Given the idea that bacteria are no more than insignificant contaminants on plants,