Seed Endophytes of *Jasione montana*: Arsenic Detoxification Workers in an Eco-friendly Factory

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Contents

Abstract

Arsenic (As) is a toxic compound for human health and ecosystems. Some organisms have developed different strategies to live in environments contaminated with arsenic (As-tolerant organisms). Some prokaryotes are able to use arsenic as a donor or acceptor of electrons through respiratory processes

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(arsenic oxidizer and reducer bacteria). Certain plants can accumulate it in their tissues (accumulative plants) or imply their uptake and favor their exclusion (exclusion plants). Some fungi and bacteria are able to metabolize organic forms less toxic and volatile it. These mechanisms allow plants, prokaryotes and fungi to develop in environments with high concentrations of As. It is known that microbiota (especially rhizosphere and endosphere) can help plants to survive under arsenic stress conditions. However, little is known about the contribution of seed endophytes in the germination capacity and early development of seedling plants under As conditions. This chapter shows a brief review on the role of endophytic bacteria in the adaptation of plants to As stress conditions. Endophytic bacteria from seeds, obtained from plants that grow in As-contaminated soils, have showed that many of them promote the growth of the plant, have antifungal activity, and are AsV reducer bacteria, with the ability to metabolize arsenic to organic forms. We suggest that they have an important role in germination and early development when the seeds fall into an As-contaminated environment.

Keywords
Endophyte · Arsenic stress · Seed · Plant growth promoting bacteria · Metaorganism

17.1 Arsenic as a Natural and Xenobiotic Pollutant

Arsenic (As) is a toxic and carcinogenic metalloid, which is widely distributed in the earth's crust (Farooq et al. 2016), and contamination by it is caused by natural geological or anthropogenic sources. The anthropogenic sources are very diverse from coal burning, mining, and industrial metal smelting to production of pesticides or fertilizers, wood preservatives, and cotton desiccants (Mukhopadhyay et al. 2002; Ratnaike 2003). However, natural sources are also important, for example, in aquifers microbial reactions such as oxidizing arsenite or respiring arsenate can mobilize arsenic from solid to aqueous phase (Oremland and Stolz 2003). The presence of arsenic in diverse substrates and the persistence of life, even at high concentrations of As, support the hypothesis that arsenic resistance developed early in the evolution of life (Gihring et al. 2003), and as a consequence nearly all organisms possess arsenic detoxification mechanisms (Rosen 2002).

Arsenic exists in the -3, 0, $+3$, and $+5$ valence oxidation states. These molecules are interconvertible depending on the redox status of the environment, and they are highly toxic when found in inorganic soluble forms such as AsIII and AsV. They can be easily incorporated into living organisms and can be accumulated and/or biotransformed (arsenic organic). The As incorporation into plants increases bioavailability levels (Tsai et al. 2009); as a result, As can enter the food chain and pose health risk to humans. Around 60 million people in the world suffer from arsenic toxicity, especially in Asia (Ogra 2009; Byrne and Kappler 2017). Therefore, the

study of detoxification processes and how to prevent As entering the food chain is a global priority both from the human health and ecological points of view.

17.2 Plants as Metaorganisms

Plants can be considered complex multi-genomic organisms (metaorganisms) formed by the plant itself, its microbiome, and the set of interspecific interactions that are established (Fig. 17.1; Bosch and McFall-Ngai 2011; Thijs et al. 2016). The microbiome in turn is complex, made up of the rhizosphere (microorganisms associated with the root and soil), the endosphere (endophytic organisms), and the phyllosphere (organisms that live on the surface of the aerial parts). Next-generation DNA sequencing has shown that each of these fractions can be comprised of certain microorganisms (Akinsanya et al. 2015) and has fundamental roles in the physiology of the plant (Compant et al. 2005; Kembel et al. 2014; Hardoim et al. 2015).

Plants offer carbon sources, nutrients, and an eco-niche for the development of microorganisms (prokaryotes and fungi). Some microorganisms are pathogens, but others collaborate and promote the growth of the host plant. Plant growth-promoting bacteria (PGPB) compete for space and nutrients against phytopathogen organisms, produce hydrolytic enzymes, inhibit pathogen-produced enzymes or toxins, and induce plant defense mechanisms (Cherian et al. 2012). PGPB also are able to produce antibiotics and antimicrobial volatile organic compounds against fungi and bacterial phytopathogens (Sheoran et al. 2015; Verma et al. 2017; White et al. 2018a) and under stress situations (Mallik et al. 2012). Endophytic bacteria are able to synthesize auxins (IAA) that at low concentrations favor growth, induce plant defense systems, and function as a cell-cell signaling molecule. Also, some of them

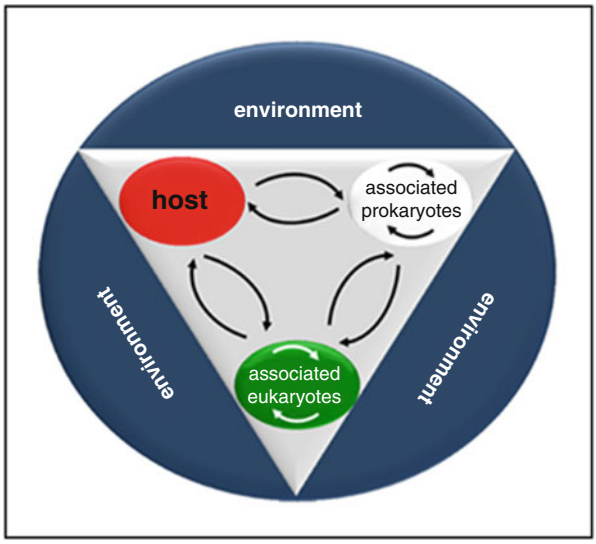

Fig. 17.1 Metaorganism interpretation according to Bosch and McFall-Ngai (2011)

can solubilize precipitated phosphorus helping with phosphorus mobilization and uptake by plants (Ma et al. 2016). The functional group of nitrogen-fixing endophytic bacteria can increase nitrogen fixation rate and assimilable nitrogen accumulation in plants in the long term for nitrogen-poor ecosystems. Another important function that endophytic PGPB may offer is the iron availability to plant roots through the production of chelating agents (e.g., siderophores) under Fe-limiting conditions (Han et al. 2017). It has been widely described (Rajkumar et al. 2009; Sun et al. 2018) how the microbiota, especially that of the rhizosphere, is involved in acclimation of plants to soils contaminated with heavy metals. In plants, stress conditions induce ethylene production, which causes the inhibition of root elongation, lateral root growth, and root hair formation (Ma et al. 2016). But, some endophytic bacteria activate defense mechanisms against this oxidative stress (White and Torres 2010; Torres et al. 2012; Lata et al. 2018; Abbas et al. 2018), such as 1-aminocyclopropane-1-carboxylate (ACC) deaminase production, which hydrolyze ACC, an ethylene precursor. It has also recently been described that during this process ammonia is released and can be up taken by microbes as a nitrogen source (Ma et al. 2016).

In addition to mutualist and pathogenic plant-prokaryote interactions, it has been recently reported that through the rhizophagy cycle, plants may obtain nutrients through oxidative degradation of microbes within roots (White et al. 2018b). Bacteria obtain nutrients in a free-living soil phase, and then, nutrients are extracted from bacteria by oxidation in an intracellular endophytic phase. The recruitment of helper microorganisms (or the nutrients derived from rhizophagy) could be an adaptive advantage in situations of stress such as phytopathogens, nutrient limitation, hydric or oxidative stress, etc. The present chapter focuses on the plant-metaorganism response under arsenic stress conditions. Although the role of fungi in the metaorganism is important, in this chapter we will focus primarily on plant-prokaryotes interactions.

17.3 As-Tolerant Organisms

17.3.1 Arsenic Metabolism in Prokaryotes

Arsenite (AsIII) can be transformed by microbes to less toxic inorganic forms such as arsenate (AsV) by chemical or biological oxidation (Cha et al. 2015). Chemical oxidation is slow and expensive and generates secondary contamination (Tsai et al. 2009), whereas microbial metabolism of arsenic through bioreactors seems to be an efficient (>95%) and is an environmentally friendly technique to remove this metalloid from water (Dastidar and Wang 2009).

Several mechanisms are involved to reduce arsenic toxicity in bacteria (Fig. 17.2):

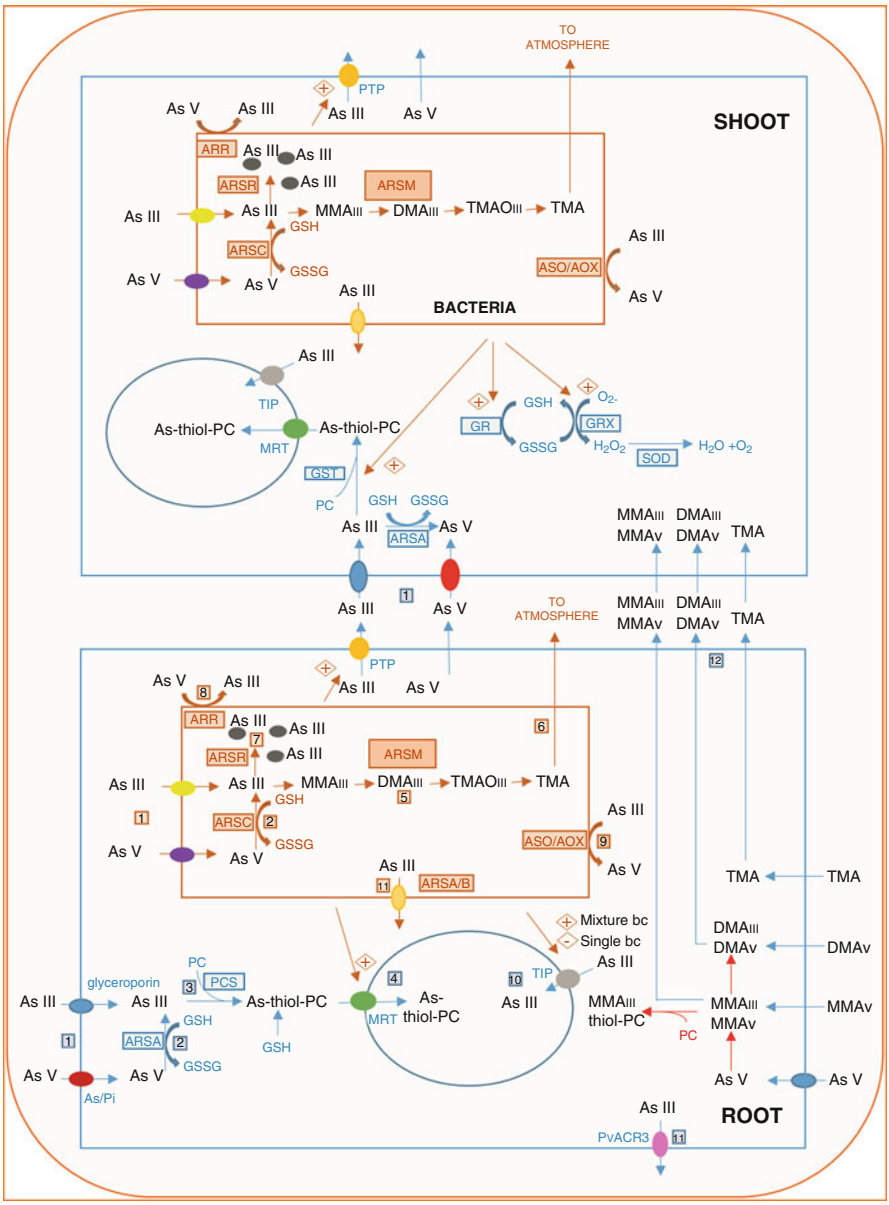

Fig. 17.2 Possible detoxification and elimination processes in endophytic prokaryotes and plants. The brown arrows show some of the processes described in prokaryotes and the blue arrows, those described for plants. The red arrows represent processes that have been described in some plants although it is unknown that bionts are involved. 1. Arsenate and arsenite enter the cell through phosphate (Pi) transporters and aquaglyceroporins, respectively. 2. Reduction of AsV to AsIII using ARSA (plant) and ARSC (prokaryotes). 3 Phytochelatins produced by phytochelatin synthases (PCS). 4. As-Thiol_PC transport into vacuoles. 5. Methyltransferase (ARSM) involves in sequential methylation. 6. Volatile compounds release (TMA). 7. Transcriptional regulator ARSR involve in AsIII detoxification by complexation. 8. Cellular respiration using arsenate reductase membrane (ARR). 9. Arsenite used as electron donor by arsenite membrane oxidases (AOX/ASO). 10.

- The use of arsenite AsIII as electron donor using arsenite membrane oxidases coded by *aso* or *aox* genes (Silver and Phung 2005), returning to the medium arsenate AsV.
- The capacity to respire AsV using membrane arsenate reductase (like arr gen; Silver and Phung 2005) giving back AsIII (Hamamura et al. 2014).
- Metabolism pathways under control of operons, such as arsRDABC, where arsenate and arsenite enter the cell through phosphate (Pi) transporters and aquaglyceroporins, respectively. ArsC encodes a reductase that reduces AsV to AsIII using glutathione (GSH) as reductant, and ArsR is a regulatory protein acting as a repressor on the arsRDABC operon, when arsenic is not present. ArsA is an ATPase that binds to ArsB and converts the AsIII carrier protein into a primary ATP-driven AsIII extrusion pump, and ArsD exhibits weak AsIII-responsive transcriptional repressor activity (Tripathi et al. 2007).
- Some prokaryotic cells can volatilize AsIII regulated by RM operon (Qin et al. 2006). ArsM gene encodes methyltransferase which carries out the sequential methylation yield mono- (MMA), di- (DMA), and trimethylarsenic (TMA) acid (Bentley and Chasteen 2002), and then trimethylarsine oxide (TMAO) is finally reduced to the volatile trimethylarsine (TMA) (Qin et al. 2006). Although biotransformation in organic arsenic seemed to reduce toxicity (Ratnaike 2003; Mitra et al. 2017), some organic molecules such as MMA could be considered more cytotoxic than AsIII for humans, and it could, also, be an important factor inducing straight head in rice (Mass et al. 2001; Mishra et al. 2017). This apparent contradiction may have biological significance if one considers that the formation of more toxic intermediaries is a transitory stage toward the formation of volatile compounds, which are easily eliminated by facilitated diffusion.
- It seems that AsIII can be detoxified by complexation or chelation with Cys-rich peptides (Páez-Espino et al. 2009).
- Other less frequent genes involved in metabolism of arsenic have been previously described (Chen et al. 2015) such as arsH, an organ arsenical oxidase that detoxifies trivalent methylated MAsIII and aromatic arsenicals by oxidation to the less toxic pentavalent species MAsV.
- Recently, Nadar et al. (2016) and Pawitwar et al. (2017) identified a bacterial gene arsI responsible for aerobic demethylation of methylarsenite (MAsIII) to inorganic form AsIII.

Fig. 17.2 (continued) Transport of free AsIII to the vacuole for its storage. 11. AsIII expulsion by a membrane transporter in prokaryotes (ARSA/B) and plants (AvACR3). 12. Transport of As-methylated molecules from the root to the shoot. Microbial consortium could switch on/of (+/−) modulating different detoxification mechanisms differentially in roots and shoots. These approaches trade off accumulation in roots or mobilization toward shoots

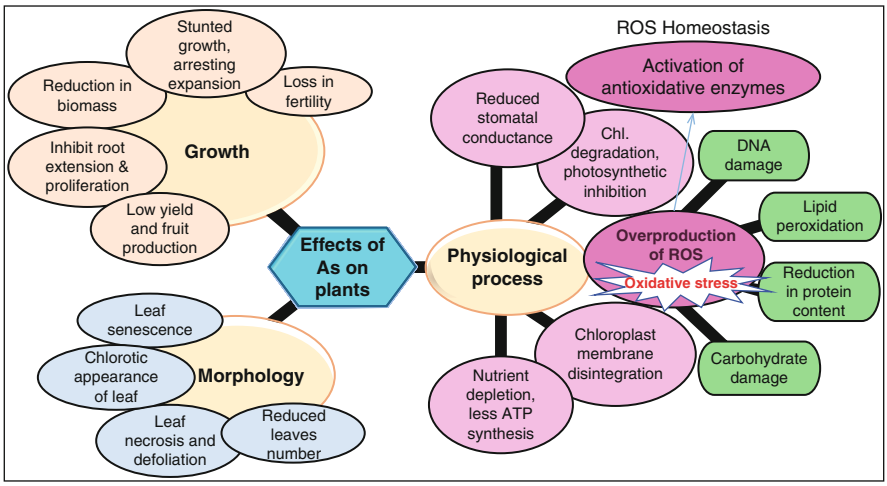

Fig. 17.3 Arsenic toxicity in plants: morphological (reduction in leaf number, chlorosis, leaf senescence, and defoliation), physiological (reduction in shoot and root growth, restricted stomatal conductance and nutrient uptake, chlorophyll degradation, limited biomass, and yield production), and biochemical (overproduction of ROS, leading to carbohydrate, protein, and DNA damage) responses. Reproduced from Abbas et al. (2018)

17.3.2 Arsenic Metabolism in Plants

Arsenic is responsible for some important changes in plants (Fig. 17.3): morphological (reduction in leaf numbers, chlorosis, leaf necrosis, senescence, and defoliation), physiological (restricted stomatal conductance and nutrient uptake, chlorophyll degradation, and limited biomass), and biochemical (overproduction of reactive oxygen species, ROS). ROS are quite dangerous for plant metabolism and can cause un-repairable damage to important macromolecules, including lipids, proteins, carbohydrate, and DNA. It has been noticed that the generation of ROS in plants is linked to the conversion of As(V) to As(III), and this increases ROS-mediated damage (Abbas et al. 2018). However, arsenic concentration in plants seems to be modulated by abiotic factors such as availability and concentration of various mineral nutrients (iron, phosphorus, sulfur, and silicon) in soil solution, soil oxidation/reduction status, or interchange between organic and inorganic As compounds (Bakhat et al. 2017). Despite arsenic toxicity, some tolerant plants can accumulate and translocate into their tissues high concentrations of arsenic (accumulating or hyper-accumulating) or prevent their entry (excluder plants).

From a metabolic point of view, plants detoxify AsV and AsIII using mechanisms that are like those discovered in microbes and others exclusive of eukaryotes (Abbas et al. 2018). There are several tolerance mechanisms (Fig. 17.2):

- Plants (and bacteria) take up arsenate AsV and arsenite AsIII through phosphate transporters and aquaglyceroporins, respectively (Asher and Reay 1979; Meharg and Jardine 2003; Han et al. 2017).
- Synthesis of GSH to produce phytochelatins (polymers of GSH, PCn) to bind AsIII (Srivastava 2016), which is obtained from AsV reduction by arsenate reductase (ARSA) using GSH (Ellis et al. 2006) or H_2O_2 as reducers (Abbas et al. 2018).
- AsV, AsIII, and PCn-AsIII could be translocated, from root to shoot and vice versa through the xylem and apoplast. Two critical aquaporins Lsi1 (influx transporter) and Lsi2 (efflux transporter) for AsIII uptake and As transport from epidermis to root xylem have been recently described (Chen et al. 2017a, b).
- PCn-AsIII to form a variety of complexes (Raab et al. 2005). These complexes can be sequestered in the vacuole by transporters (Mukherjee et al. 2018). Mishra et al. (2017) showed that As-thiol complexation restricts the As translocation to shoots.
- Inorganic AsIII is accumulated in high amounts in vacuoles (hyper-accumulating species). This suggests that the majority of As stored is not complexed and that plants might have vacuolar AsIII transporters, possibly with similarities to bacterial AsIII extrusion pumps. AsIII transporters such as PvACR3 have been shown to be responsible for AsIII efflux into vacuoles for sequestration and to external environment efflux (Chen et al. 2013; Indriolo et al. 2010).
- It is common in excluder plants to restrict the influx of As inside the cell by constitutive suppression of high-affinity phosphate/AsV transport (Bleeker et al. 2003) or activating As efflux (Mosa et al. 2012).
- Different molecules such as proline (by reducing As uptake, enhancing pigment concentration), nitric oxide (causing vacuole sequestration, decreasing chlorosis), salicylic acid (reducing As uptake by regulation of transporters, limiting As translocation to shoots, maintaining redox balance), etc. modulate As-induced toxicities in plants (Abbas et al. 2018).

Arsenic is predominantly found as inorganic species in most terrestrial plants with organic forms being rare. Zhao et al. (2010) and Mishra et al. (2017) noted that most of the methylated arsenic detected in plants (MMA and DMA) comes from their direct absorption from the soil, since these arsenic species are frequently used in pesticides (Fig. 17.2). Studies suggest that uptake of the methylated As species by roots takes place through the aquaporin (Li et al. 2009; Zhao et al. 2010). Zhao et al. (2006) and Geiszinger et al. (2002) describe some exceptions where moso bamboo and *Trifolium pratense* are capable of methylation of the inorganic forms. However, these results do not mean that this conversion is carried out by the enzymatic machinery of the plants, keeping the door open to the possible intervention of the microbiome for this task.

In marine organisms, most arsenic appears in organic forms like arsenobetaine and arsenosugars, which are nontoxic. These molecules are not common in terrestrial plants (Li et al. 2003), and in this case, it seems that prokaryotes may be directly involved in this biotransformation (Ritchie et al. 2004).

17.3.3 Is There Evidence that Plant Microbiota May Confer Tolerance of Plants to Arsenic?

As-resistant endophytic bacteria (AEB) have effective mechanisms for detoxification of As, not only for themselves, but potentially also for organisms associated with them (Páez-Espino et al. 2009). For that reason, when plants grow in contaminated soil, they naturally recruit endophytes with the necessary contaminant-degrading genes (Siciliano et al. 2001) and with a potential use for phytoremediation (Titah et al. 2011). Therefore, it is expected that the microbiome that accompanies As-tolerant plants will have genes involved in their elimination and detoxification. Rathinasabapathi et al. (2006) described, for the first time, the phyllosphere importance as a potential habitat for arsenic-resistant microorganisms, identifying an arsenic-resistant epiphytic bacterium (genetically related to *Variovorax paradoxus*). Therefore, rhizosphere will be a potential microbial source (*Azospirillum brasilense*) for speciation, bioavailability, and plant uptake of As (Lyubun et al. 2006). Recently, As-resistant rhizobacteria have been successfully used in the elimination of arsenic from soil by inoculating in plants (Lampis et al. 2015). In summary, rhizosphere- and/or metalloid-contaminated soils are a good reservoir of bacteria to be inoculated in plants and conferring them better development and acclimatization under stress conditions (Franchi et al. 2016, Lyubun et al. 2006).

While rhizobacteria associated with As-tolerant plants have been well reported (Zecchin et al. 2017; Mallick et al. 2018), information about endophytes was less known. The first characterizations and identifications of endophytic bacteria isolated from As-tolerant plants have shown that many of them behave like PGPB and are AEB with ability of both AsV reduction and AsIII oxidation (Xu et al. 2016; Zhu et al. 2014; Tiwari et al. 2016; Selvankumar et al. 2017), while others are exclusively As-reducers or As-oxidizers (Zhu et al. 2014; Han et al. 2016).

Indigenous As-resistant bacteria have been used in bioaugmentation experiments and re-inoculated increasing As and P uptake and growth and increasing the capacity to accumulate As in fronds (Han et al. 2016); activating detoxification mechanisms through phytochelatin complexation (Mesa et al. 2017); increasing siderophore and IAA production; enhancing arsenic accumulation in leaves, phosphorus assimilation, and photosynthetic performance; and regulating the formation of PCs and the storage in vacuoles of both As-thiol-PC complexes and As (III) free (Mukherjee et al. 2018). In this way, AsIII accumulates in the vacuoles of the root and would not (or in lesser amounts) reach the shoots. Besides this, microbial consortia could activate antioxidant defense mechanisms in the shoot and root (Mukherjee et al. 2018; Fig. 17.2). Therefore, bacterial consortium or single bacterium from As-resistant plants could be potentially useful for inoculation in nonresistant plants (Ryan et al. 2008; Ma et al. 2011).

The use of engineered bacteria overexpressing As detoxification or elimination genes is also an interesting and plausible option (Ryan et al. 2008) although, as far as we know, this has not been done yet for the elimination of this contaminant. We propose, whenever possible, the use of indigenous bacteria as more appropriate than engineered bacteria, both from an economic and ecological point of view. According

to Hayat et al. (2017), those indigenous bacteria capable of eliminating contaminant compounds without environmental damage could be considered in biotechnology as "eco-friendly nano-factories."

17.4 Endophytic Bacteria in Seeds

In the conceptual framework of "metaorganism," some authors have proposed that microbiota in seeds would have evolved by co-selection with the plant species, providing important traits for plant survival. The genes of the microbiota could complement those codified by the chromosomes of the plants, and therefore, both would determine the phenotype of the plant and microbial genomes (see Mitter et al. 2017).

Endophytes find the way to reach into host plants through different ways: (1) colonization of root epidermis and cortex, and then, using the vascular system or apoplast, endophytes colonize shoot and leaves; (2) colonization of tissue wounds, stomata, and lenticels (Cherian et al. 2012) (these mechanisms are considered as horizontal transmission); and (3) from one generation to the next one through the seed (vertical transmission, Ferreira et al. 2008). Depending on the life strategy, endophytic bacteria can be obligate or facultative. Obligate endophytes are strictly dependent on the host plant for their growth and survival, and facultative endophytes have a stage in their life cycle in which they can exist outside their host plants (Rajkumar et al. 2009). Traditionally, it has been considered that horizontal transmission could be the main way to incorporate bacteria into the host plant. However, seeds are an important source of endophytic bacteria (Coombs and Franco 2003; Kaga et al. 2009; Barret et al. 2015). From the seed, and during the process of plant germination and development, bacteria can be distributed to the root and the shoot. Some of them can be found, later, as epiphytes of the seedling plants and, even, go out to the culture medium (Kaga et al. 2009). Many of them behave like PGP bacteria (Truyens et al. 2015; Sánchez-López et al. 2018a; White et al. 2018b). Verma and White (2017) suggested that conservation and management of seed-vectored endophytes could make more sustainable and productive crops. It has been recently proposed (Glassner et al. 2018) that bacteria distribution within the seed is not random, but follows a pattern based on the anatomy of the seed. The microbiome of plant seeds could provide the source of microbes for successful plant growth, before being augmented by microbes from the soil (Mitter et al. 2017). It is very interesting what previously has been suggested by Klaedtke et al. (2015) that ecological niche-based processes determine seed-associated microbial assemblages, that environmental factors are a key driver of these selective forces, and that microbial structure is explained by host genotype. However, dynamics of endophytic bacteria communities were influenced by plant growth stage, and microbial diversity was reduced during seedling growth (Shi et al. 2014).

The most common genera of cultivable species from seeds belong to *Pseudomonadaceae*, *Methylobacteriaceae*, *Burkholderiaceae*, *Bacillaceae*, and *Enterobacteriaceae* (Ferreira et al. 2008; Liu et al. 2012; Kaga et al. 2009).

However, next-generation DNA sequencing has allowed us to confirm the high richness and diversity of the seed endosphere (Shi et al. 2014; Sánchez-López et al. 2018a, b). These results seem to indicate that only a small percentage can be grown under laboratory conditions. Thus, in *J. montana*, only 6% of a total of 87 OTUs detected by Illumina could be cultivated (data not shown).

17.4.1 Is There Any Evidence that Endophytes in Seeds Improve Plant Response Under As Stress Conditions?

Little is known about the influence of seed endophytic bacteria on germination and early development of host plants under conditions of metal toxicity (Mastretta et al. 2009). Diversity and richness of the seed endophytic community decreased with increasing number of generations under Cd stress conditions being able to identify Cd-indicator endophytic bacteria (Truyens et al. 2014, 2015). Recently, it has been found that under multi-metal stresses, plants possessed similar bacterial endophytic communities across three consecutive seed generations, with few specific distinctive species of each generation and a high percentage of shared core strains (Sánchez-López et al. 2018b). In general, endophytes in seeds have positive effects on plant growth, with an increase in biomass production under conditions with and without metal stress and, also, metal accumulation such as Cd (Mastretta et al. 2009).

There is evidence that endophytic microbes from seeds improve plant response to metal stress, but it still remains hypothetical whether the seed microbiome of As-tolerant plants could metabolize and detoxify As. Thus, both partner seed microbiome and seedling plant could combine their genes involved in this process (Fig. 17.2). As a result a metaorganism would arise whose physiological characteristics would allow the success of germination and early development. The selective incorporation of microorganisms from soil, air, predators, pollinators, etc. would implement the endosphere genetic pool and, with it, increase the chances of survival.

To test whether the microbiota of the seed has the capacity to tolerate high concentrations of arsenic and if they have PGP bacteria characteristics, we selected *Jasione montana* L. This Mediterranean As-tolerant plant can grow in high concentrations of arsenic (García-Salgado et al. 2012; Gutiérrez-Ginés et al. 2015) although the strategy of detoxification is controversial. While some authors classify it as arsenic accumulator plants (Porter and Peterson 1975; Benson et al. 1981), others consider that it behaves as an excluder plant (García-Salgado et al. 2012). Recent preliminary results from our research team point out that the response of plants to As stress could be genotype-specific instead of species-specific (data not shown).

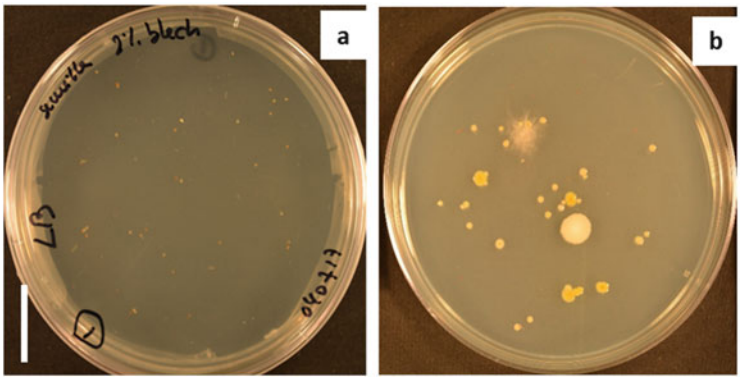

Fig. 17.4 *J. montana* seeds wash in 2% sodium hypochlorite (**a**) or in water (**b**) for 5 min. Scale = 1 cm

17.4.2 Protocol to Isolate Endophytic Bacteria from *J. montana* Seed

The seed microbiome is embedded within host plant, and physical separation and isolation are often difficult tasks (Utturkar et al. 2006). Moreover, the obligatory bacterium cannot be grown out of the seeds, and many others, even if they are cultivated, have a shorter half-life than non-endophytic culturable bacteria (data not shown). According to Nejad and Johnson (2000) and Hallmann et al. (2006), we adjusted the isolation method to endophytic bacteria in seed from *J. montana*. The most effective and simple process for external sterilization to remove the surface microbes was washing with 2% sodium hypochlorite and incubating during 5 min in an orbital shaker. After this, three rinses were carried out in sterile water at the same conditions. To control the degree of superficial sterilization, seeds were plated in Luria-Bertani (LB) agar. After 20 days, no colonies were visualized, whereas, when the seeds were not sterilized, 11 bacteria and 9 fungi strains were isolated (Fig. 17.4).

The ability of seeds of *J. montana* to germinate and grow varies depending on the culture medium used. When basal germination media such as 0.7% agarose were used, the germination was 90 ± 5.5% with an average seedling length after germination of 9.0 ± 1.33 mm/month. The germination percentage was similar when some specific bacteria media were used as germination medium, such as Pikovskaya (PA; Pikovskaya 1948) or Tryptic Soy Agar (TSA10; Nejad and Johnson 2000) (Fig. 17.5a, b, d, f). When PA was used, seedlings were better developed (18 ± 0.85 mm/month, Fig. 17.5a, f). This allows us to hypothesize that perhaps, the seeds contained bacteria with the capacity to solubilize inorganic phosphate, which could be favoring the incorporation of P and, therefore, their growth (Han et al. 2016). When yeast extract sucrose (YES) media were selected, the rate of germination (70.43 ± 10.42) and the growth decreased considerably (Fig. 17.5c, f). When LB media was used, there was no germination at all (Fig. 17.5e, f). Osmotic compounds,

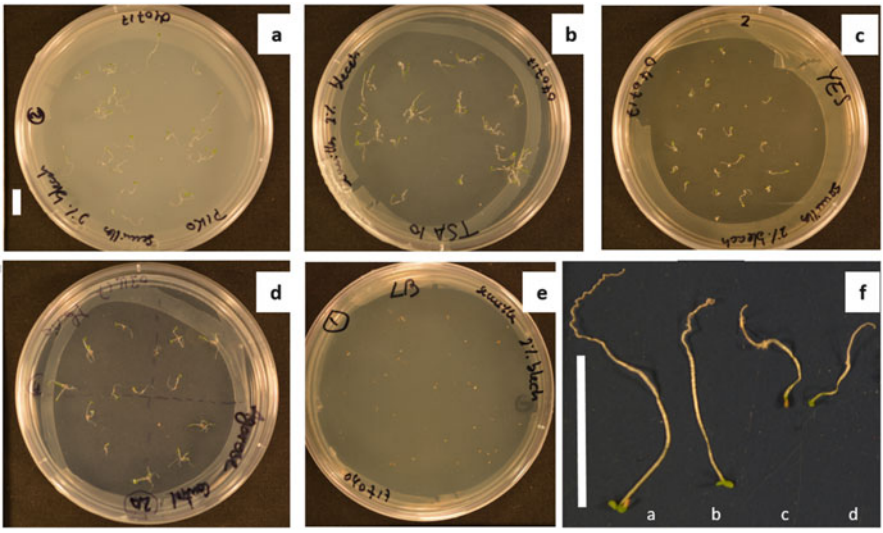

Fig. 17.5 *J. montana* seedling plants germinated on PAM (**a**), TSA10 (**b**), YES (**c**), 0.7% agarose (**d**), and LB (**e**). Seeding plant length on PAM (**f**)

such as sugars and mineral salts, in these media (YES, LB) can contribute to the negative water potential of the media, hindering germination of seeds and development of seedlings.

Only when seeds were germinated and bacteria were released outside, some colonies were visualized, and endophytes could be isolated. LB media was not a good medium for endophyte isolation, since the seeds did not germinate, although it was a good medium to maintain the strains after isolation. One exception was MC-13 strain, which was isolated using seedling maceration methods and maintained on Potato Dextrose Agar (PDA).

To increase the number of colonies isolated, a maceration method was used since this method increases the culturable bacteria spectrum (Hallmann et al. 2006). We recommend to modify this method using superficially sterilized and germinated seeds in 0.7% medium agarose instead of "non-germinated seed." This modification improves the number of bacterial strains isolated. After germination, seeds are triturated with mortar and pestle under sterile conditions. It is recommended to carry out this process at low temperatures (on ice) to reduce the effects of hydrolytic enzymes as much as possible. After maceration, the triturate should be quickly diluted to reduce the concentration of toxic compounds. Hence, it was added to a glass flask with 100 ml of LB and 10 mM arsenate (w/v, final concentration). With the As treatment, we are favoring isolation of a consortium that is resistant to arsenic. Twenty-four hours later, the bacteria were isolated using the serial dilutions method and loop depletion, using a large battery of culture media.

This method is effective because many bacterial endophytes are in an intimate symbiotic mode of growth within plant cells and grinding the tissue breaks the

Table 17.1 Characterization and molecular identification of endophytes bacterial strains isolated from *Jasione montana* seeds

Strains	Ident. molecular (% similarity)	As (V) MIC (mM)	Phosphate solubilizer	Auxin production Average (ug/ml/D.O.)	% inhibition against *Alternaria* sp. LB/PDA
MC-12	*Pantoea eucalypti*^{T}	200	++	18.96 ± 8.79	40/35
	(99.5%, NR116112)				
MC-10	*Bacillus siamensis*^{T}	1	+	1.10 ± 0.12	100/82.6
	(100% KY643639)				
MC-13	*Pantoea* sp.	200	+	2.30 ± 1.56	0/0
	(96% HG001277.1)				
MC-14	*Acinetobacter radioresistens*^{T} (100%, NR114074)	200	−	0.83 ± 0.03	26.7/0
AD-37.2	*Bacillus aryabhattai*^{T}	1	−	10.09 ± 0.16	40/13
	(100%, EF114312)				

++, high phosphatase activity; +, intermediate activity; −, nonactivity

symbiosis with the plant. Besides, putting triturate in liquid culture medium dilutes inhibitors from plant and puts bacteria in a high nutrient, reduced oxygen environment to favor nonsymbiotic growth.

17.4.3 Molecular Identification and Characterization of Isolated Strains

Five As-resistant strains (*Bacillus siamensis*, *Bacillus aryabhattai*, *Pantoea eucalypti*, *Pantoea* sp., *Acinetobacter radioresistens*, see Table 17.1) were identified using 16S rDNA as molecular marker (Molina et al. 2009). All of them except one (MC-13) were identified with a similarity percentage ≥99.5% with type strains (Table 17.1). All have been found to produce IAA, especially *Pantoea eucalypti* (MC-12) with fungal mycelial growth suppression against *Alternaria* sp., a potential pathogen isolated from the surface of the *Jasione* seeds. *Bacillus siamensis* (MC-10) showed high antifungal capacity with 100% on inhibition on LB and 83% on PDA media. MC-13 could not be tested to antifungal capacity due to its low viability in culture. Moreover, *Bacillus siamensis* MC-10, *Pantoea* sp. MC-13, and, especially, *P. eucalypti* MC-12 were phosphate-solubilizing bacteria. These results may explain why seedlings had better development in this medium (Fig. 17.5a), confirming that seed endophytes favor seedling growth (Verma and White 2017).

When the bacteria were grown in increasing concentrations of arsenic, *P. eucalipti* MC-12, *B. siamensis* MC-10, and *Acinetobacter radioresistens* MC-14 survived at very high As concentrations, with an AsV-MIC (As minimum inhibitory concentration) of 200 mM, while *Pantoea* sp. MC-13 and *Bacillus*

aryabhattai AD-37.2 were able to grow at relatively low concentrations of the contaminant. Although, for the time being, we do not know which of the detoxification mechanisms these bacteria use, we consider them as potential helpers of the germination and development of seeds against high concentrations of arsenic in soil (AEB). New bioaugmented tests, as well as the determination of the metabolic pathways involved in the detoxification of arsenic in both symbionts (biome and plant), could help us to confirm this hypothesis.

Based on these results, we can point out that some endophytic bacteria of the *J. montana* seed can offer both indirect (solubilization of inorganic phosphate, IAA production, inhibition of the growth of phytopathogenic fungi) and direct (arsenic tolerance) advantages to the host plant under arsenic stress conditions. Thus, vertical transmission of these bacteria through successive generations can ensure germination and early development, when seeds fall on soils contaminated with arsenic. The presence of this type of bacteria in the seed could favor colonization in contaminated soils assuming an adaptive advantage against other plants with a different microbial community not adapted. It is expected that *J. montana* will be an adequate ecophysiological niche for these microorganisms. In this way, the interrelationships between the symbionts configure the appropriate phenotype for this metaorganism to be able to grow in soils highly contaminated with arsenic. Bioaugmentation and biochemical tests are needed to confirm this hypothesis.

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