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**SEED ENDOPHYTES OF *JASIONE MONTANA*: ARSENIC DETOXIFICATION
WORKERS IN AN ECO-FRIENDLY FACTORY**

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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 bacteria, are able to use arsenic as a donor or acceptor of electrons through respiratory processes (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 to organic forms less toxic and volatile it. These mechanisms allow them 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. In addition, we isolate and characterize endophytic bacteria from seeds, obtained from plants that grow in As contaminated soils, shows 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

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X.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, industrial metal smelting to production of pesticides or fertilizers, wood preservatives and cotton desiccants (Mukhopadhyay et al 2002; Ratnaik 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 are found in inorganic soluble forms such as AsIII and AsV. They can be easily incorporated into living organisms, and can be accumulated and/ or bio-transformed (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.

X.2 Plants as metaorganisms

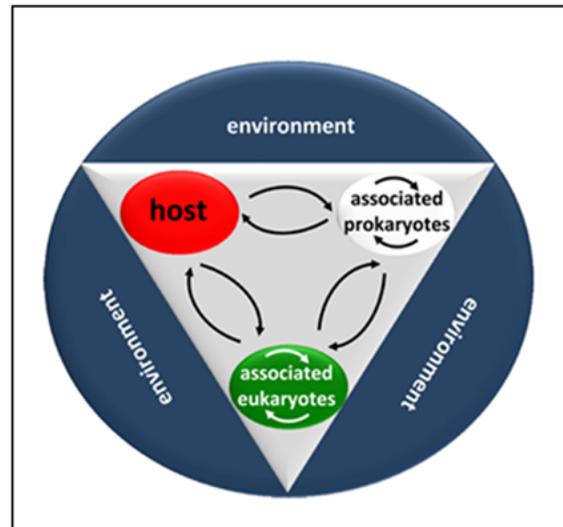
Plants can be considered complex multi-genomic organisms (metaorganisms) formed by the plant itself, its microbiome and the set of interspecific interactions that are established (Fig. 1; Bosh and McFall-Ngai 2011; Thijs et al 2016). The microbiome in turn is complex, made up of the rhizosphere (microorganisms 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 have fundamental roles in the physiology of the plant (Compant et al 2005; Sessitsch et al 2012; Kembel et al 2014; Hardoim et al 2015).

Plants offer carbon sources, nutrients and an eco-niche for the development of microorganisms (bacteria, fungi, yeast). Some microorganisms are pathogens, but others collaborate and promote the growth of the host plant. Plant growth promoting bacteria (PGP) 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 2013). PGP bacteria also are able to produce antibiotics and antimicrobial volatile organic compounds against fungi, bacterial phytopathogens (Sheoran et al 2015; Verma et al 2017; White et al 2018) 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 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. Other important functions that endophytic PGP bacteria 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 in press) 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 deaminase (ACC) 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-bacterium 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 2017).. 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-metazoan response under arsenic stress conditions. Although the role of fungi (Thijs et al 2017) in the metaorganism is important, in this chapter we will focus primarily on plant-prokaryotes interactions.



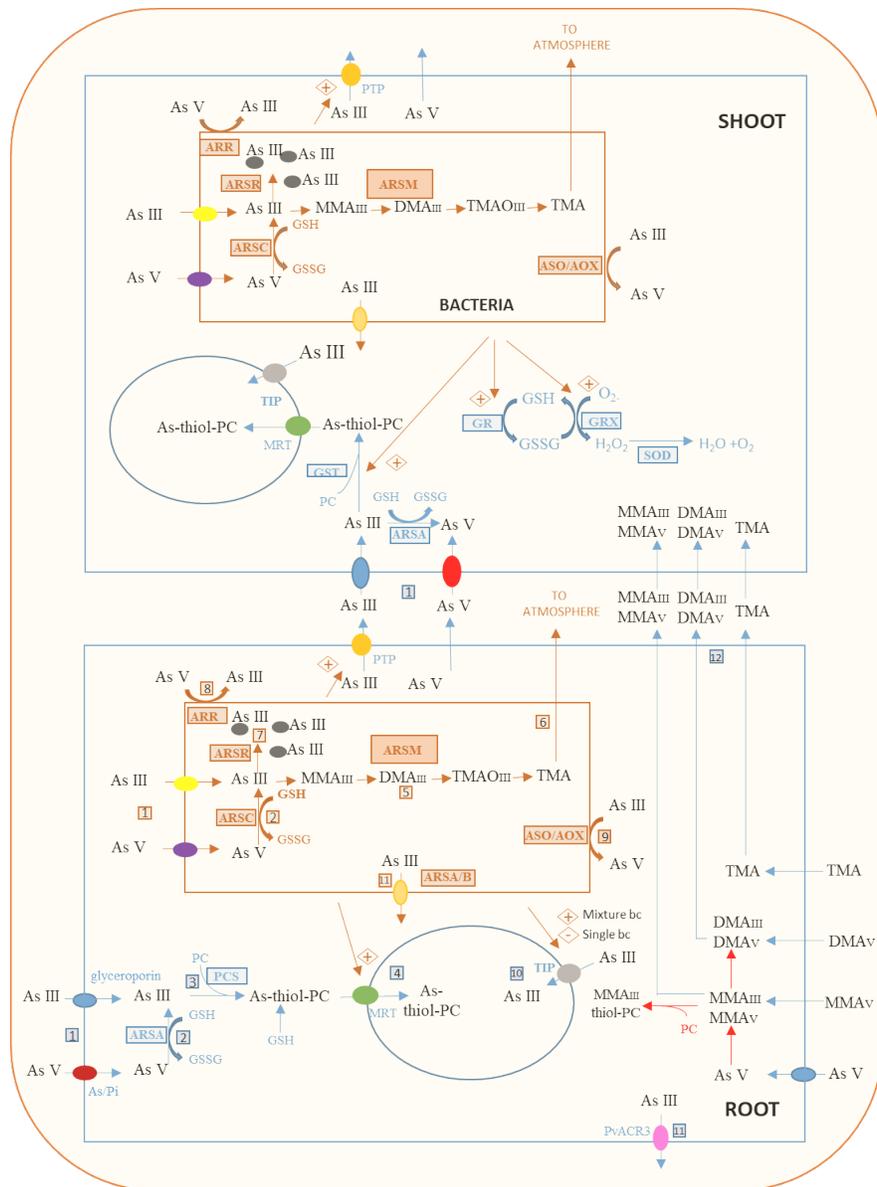
X.3 As-Tolerant Organisms

X.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; Montoya et al 2015). Chemical oxidation, is slow, 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, 2012).

Several mechanisms are involved to reduce arsenic toxicity in bacteria (Fig. 2):

- 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

Fig. X.2 Possible detoxification and elimination processes in endophytic bacteria and plants. The brown arrows show some of the processes described in bacteria 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 (bacteria). 3. Phytochelatin produced by phytochelatin synthases (PCS). 4. As-Thiol_PC transport into vacuoles. 5. methyltransferase (ARSM) involve to sequential methylation. 6. Volatile compounds release

(TMA). 7. Transcriptional regulator ARSR involve to 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. Transport of free AsIII to the vacuole for its storage. 11. AsIII expulsion by a membrane transporter in bacteria (ARSA/B) and plants (AvACR3). 12. Transport of As methylated molecules from the root to the shoot. Bacterial consortium could switch on / of (+/-) modulating different detoxification mechanisms differentially in roots and shoots. This approach trade off accumulation in roots or mobilization towards shoots

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 to methyltransferase which carries out the sequential methylation yield mono (MMA), di (DMA) and trimethylarsenic (TMA) acid (Bentley and Chasteen 2002) and then trimethylarsenine 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 towards the formation of volatile compounds, which are easily eliminated by facilitated diffusion.

- Moreover, AsIII it seems can be detoxified by complexation or chelation with Cys-rich peptides (Paéz-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 organoarsenical oxidase that detoxifies trivalent methylated MAsIII and aromatic arsenicals by oxidation to the less toxic pentavalent species MAsV.

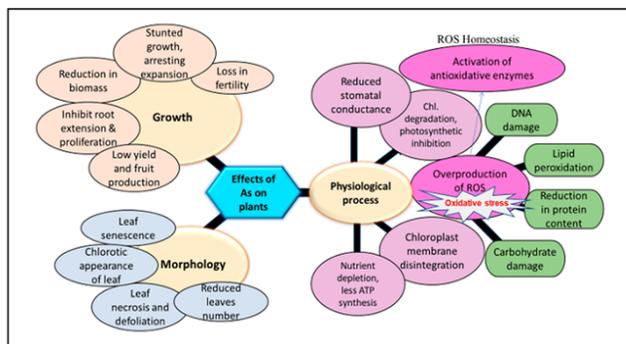
- Recently, Nadar et al (2016) and Pawitwar et al (2017) identified a bacterial gene arsI responsible for aerobic demethylation of methylarsenite MAsIII to inorganic form AsIII.

X.3.2 Arsenic metabolism in plants

Arsenic is responsible for some important changes in plants (Fig. 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

Fig. X.3 Arsenic toxicity in plants: morphological (reduction in leaf number, chlorosis, necrosis 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



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. 2):

- Plants (and bacteria) take up arsenate AsV and arsenite AsIII through phosphate transporters and aquaglyceroporins, respectively (Asher and Reay 1979; Meharg et al 2003; Han et al 2017).
- Synthesis of GSH to produce phytochelatins (polymers of GSH, PCn) to bind AsIII (Srivastava 2006), which is obtained from AsV reduction by arsenate reductase (ARSA) using GSH (Ellis et al 2006) or H₂O₂ 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 2017).
- 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). Mirsha 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 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, maintain 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. 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 bacteria may be directly involved in this biotransformation (Ritchie et al 2004).

X.3.3 Is there evidence that plant microbiota may confer tolerance of plants to arsenic?

As-resistant bacteria (AEB) have effective mechanisms for detoxification of As, not only for themselves, but potentially also for organisms associated with them (Paéz-Espino et al 2009). For that reason, when plants grow in soil contaminated 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 metalloids 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 PGP bacteria 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. They have been re-inoculated increasing As and P uptake, growth and increasing the capacity to accumulate As in fronds (Hans 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, 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, As(III) 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. 2). Therefore, bacterial consortium or single bacterium from As-resistant plants could be potentially useful for inoculation in non-resistant 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".

X.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 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 et al 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; Sanchez-Lopez et al 2018a; White et al 2018). 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 2017) 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, and 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 Pseudomonaceae, Methylobacteriaceae, Burkholderiaceae, Bacillaceae and Enterobacteriaceae (Mastretta et al 2006; Ferreira et al 2008; Cottyn et al 2009; 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; Sanchez-Lopez 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).

X.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 (Sanchez-Lopez 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. 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-Gines and Hernández 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 plants (Garcia-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 do not shown).

X.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

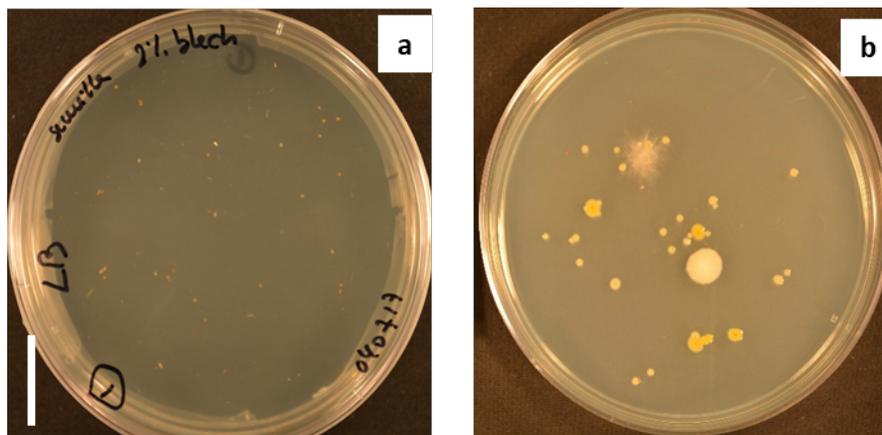


Fig. X.4 *J. montana* seeds wash in 2% sodium hypochlorite (a) or in water (b) for 5 mins. Scale= 1cm

often difficult tasks (Utturkar et al 2016). 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 surface sterilization to remove the surface microbes was washing with 2% sodium hypochlorite and incubated during 5 mins 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 agar (LB). After 20 days, no colonies were visualized whereas, when the seeds were not sterilized 11 bacteria and 9 fungi strains were isolated. (Fig. 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 was used, the germination was $90\pm 5.5\%$ 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. 5a, b, d, f). When PA was used, seedlings were better developed (18 ± 0.85 mm/month, Fig. 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 was selected, the rate of germination (70.43 ± 10.42) and the growth decreased considerably (Fig. 5c, f). When LB media was used, there was no germination at all (Fig. 5e, f). Osmotic compounds, 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.

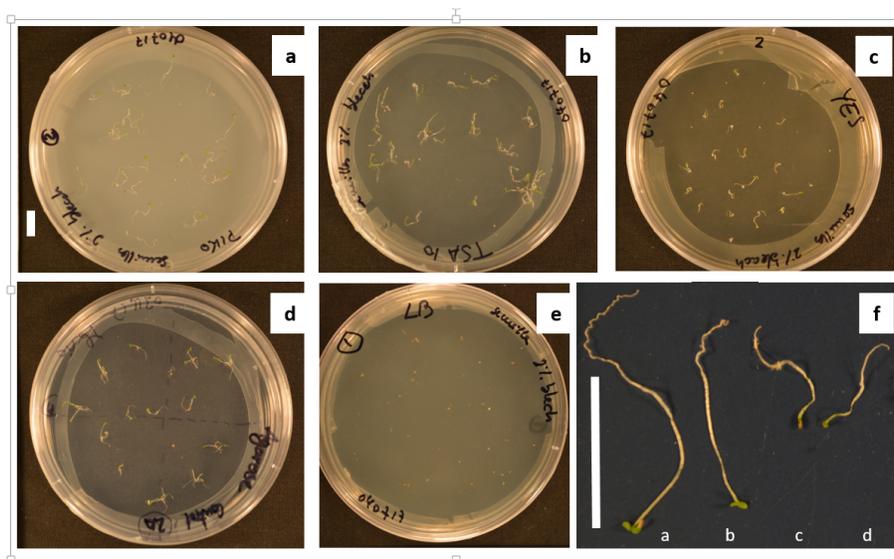


Fig. X.5 *J. montana* seedling plants germinated on PAM (a), TSA10 (b), YES (c), 0.7% agarose (d) and LB (e). f, seedling plants length on PAM (a), TSA10 (b), YES (c) and 0.7% agarose (d).

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 except 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 '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. 24 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 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 non-symbiotic growth.

X.4.3 Molecular identification and characterization isolated strains

Five As-resistant strains (*Bacillus siamensis*, *Bacillus aryabhatai*, *Pantoea eucalypti*, *Pantoea sp.*, *Acinetobacter radioresistens*, see Table 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 $\geq 99.5\%$ with type strains (Table 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. 5a), confirming that seed endophytes favor seedling growth (Verma and White, 2017).

When the bacteria were grown in increasing concentrations of arsenic, *P. eucalypti* 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 aryabhatai* 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.

Table X.1 Characterization and molecular identification of endophytes bacterial strains isolated from *Jasione montana* seeds

	Ident. Molecular (% similarity)	As(V) MIC	Phosphate solubilizer	Auxin production	% inhibition against <i>Alternaria</i> sp.
Strains				Average (ug/ml/D.O.)	LB/PDA
MC-12	<i>Pantoea eucalypti</i> ^T (99.5%, NR116112)	200 mM	++	18.96±8.79	40/35
MC-10	<i>Bacillus siamensis</i> ^T (100% KY643639)	1 mM	+	1.10±0.12	100/82.6
MC-13	<i>Pantoea</i> sp. (96% HG001277.1)	200 mM	+	2.30±1.56	0/0
MC-14	<i>Acinetobacter radioresistens</i> ^T (100%, NR114074)	200 mM	-	0.83±0.03	26.7/0
AD-37.2	<i>Bacillus aryabhatai</i> ^T (100%, EF114312) Shibaji et al 2009	1mM	-	10.09±0.16	40/13

++: high phosphase activity, +: intermediate activity, -: non activity.

Based on these results, we can point out that some endophytic bacteria of the *J. montana* seed can offer both indirect (solubilization phosphate inorganic, 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 eco-physiological 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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