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Metals in soil *versus* plant-microbe interactions: Biotic and chemical interferences

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Abstract

The interactions of soil microorganisms with plants have been increasingly attracting attention during the past decade both in basic research and in applied fields, particularly those related to agricultural and environmental biotechnology. Plant-microbe interactions can be reasonably divided into a few major categories: (i) the physiological and biochemical properties and responses of the macropartner (the host plant); (ii) the corresponding properties and behaviour

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of the micropartner (consortia of plant-associated microorganisms, in particular, in the rhizosphere), as well as (iii) any processes or phenomena directly related to their interactions per se, e.g. remote exchange of molecular signals and their perception, quorum sensing and its inhibition (including chemical and enzymatic "quorum quenching" or "anti-quorum sensing"), contact and intercellular interactions, the effects and role of the chemical composition and conditions of the medium, etc.

Soil microbiota plays an essential role in many processes central to environmental biotechnology that are directly related to soil bioremediation and, especially, phytoremediation, in which plant-microbe interactions have been documented to be of vital importance. Such highly persistent and hardly avoidable pollutants as heavy metals, in contrast to organic contaminations, cannot be biologically or chemically degraded, but can only be deactivated (i.e., detoxified and/or immobilised) within the soil or removed from the contaminated site. On the other hand, they can directly or indirectly affect plant-microbe interactions in the rhizosphere. Their direct influence involves possible toxic or other detrimental effects of particular chemical metal species on the biochemistry and physiology of plant-associated microorganisms and on the growth and development of plants. These effects comprise the biotic interferences mentioned in the title. The chemical reactivity of particular metal species or their catalytic effects, which result in chemical binding or redox degradation of biogenic organics involved in plant-microbe interactions (i.e., in the aforementioned remote exchange of molecular signals, quorum sensing within bacterial communities, release and exchange of nutrients, etc.), may thus be classified as indirect effects comprising chemical interferences. This chapter presents discussion and some examples of recent experimental evidence for environmentally important effects, either incited or directly caused by the presence of heavy metals in soil, and their possible consequences for the efficiency of functioning of plant-microbe associative symbioses in the rhizosphere – the overall tuning of this highly complicated "underground orchestra".

1. Introduction

The highly sophisticated and diversified field of bioscience comprising the interactions of microorganisms with their hosts (higher organisms) has been increasingly attracting attention during the past decade both in basic research and in applied fields, particularly those related to agricultural and environmental biotechnology (see, e.g. Stacey & Keen [127] and later volumes of the series, as well as reviews [42, 51, 54, 67, 113] and references therein). As for plant-microbe interactions, the subject can be reasonably classified and accordingly divided into a few major categories, *viz*:

(*i*) the physiological and biochemical properties and responses of the macropartner (the host plant) [11, 22, 90, 91, 132],
(*ii*) the corresponding properties and behaviour of the micropartner (consortia of plant-associated microorganisms, in particular, in the rhizosphere ([26,125]; see also the recent excellent comprehensive reviews by Barea *et al.* [5] and Brencic & Winans [13]), as well as
(*iii*) any processes or phenomena directly related to their interactions *per se*, e.g. remote exchange of molecular signals and their perception, quorum sensing and its inhibition (including chemical and enzymatic "quorum quenching" [30, 38, 140] or "anti-quorum sensing" [1, 132]), contact and intercellular interactions [71, 126], the effects and role of the chemical composition and conditions of the medium, etc. [12, 36, 94, 103, 126, 137].

This chapter presents discussion and some examples of recent experimental evidence for environmentally important effects either incited or directly caused by the presence of heavy metals in rhizosphere soil, and their possible consequences for the efficiency of functioning of plant-microbe associative symbioses. These effects should undoubtedly be further thoroughly studied together using a broad range of modern methods and instrumental techniques in a multidisciplinary approach [53], as their concerted action can dramatically deteriorate the overall tuning of the highly complicated "underground orchestra" – plant-microbe associations.

2. Biotic and chemical interferences of heavy metals in plant-microbe interactions: Two sides of the same medal

The phenomenon of plant-microbe interactions is nowadays commonly accepted as being of undoubted significant importance both for the macropartner (higher plants) and for the plant-associated micropartners [73, 88, 126]. The latter can induce antagonistic (in case of phytopathogens) or symbiotic interactions. Among the microorganisms beneficial to plants, besides the well-known rhizobium-legume symbioses, there is a vast and diverse group of free-living plant-growth-promoting rhizobacteria (PGPR) which have been documented to live in association with higher plants [6, 87, 88]. On the other hand, their survival, propagation and functioning in soil and, in particular, in the rhizosphere, which noticeably differs from the bulk soil in a range of biochemical, chemical and physical processes [40, 142], is dependent not only on the host plant [40, 49, 101] but also on the conditions and properties of the medium (soil composition and temperature, water content, pH; the presence and concentrations of nutrients and pollutants, as well as of other competing microorganisms) [37, 40, 50, 108, 119, 142].

In the last decades, the essential role of soil microbiota has been emphasized for many processes central to environmental biotechnology that are directly related to soil bioremediation and, especially, phytoremediation, in which plant-microbe interactions have been documented to be of vital importance [2, 34, 51, 54, 69]. Note that in case of phytoextraction, inoculation of plants with PGPR may be beneficial mainly due to plant growth stimulation and a corresponding increase in the above-ground biomass, rather than to an increase in metal concentrations in plant tissues, which may actually be weakly influenced [146].

Such highly persistent and hardly avoidable pollutants as heavy metals, in contrast to organic contaminations [67], cannot be biologically or chemically degraded, but can only be deactivated (i.e., detoxified and/or immobilised) within the soil or removed from the contaminated site (see, e.g. the excellent reviews by Ruml & Kotrba [117] and Vassilev *et al.* [136]). On the other hand, they can directly or indirectly affect plant-microbe interactions in the rhizosphere.

Their direct influence involves possible toxic or other detrimental effects of particular chemical metal species on the biochemistry and physiology of plant-associated microorganisms [69, 112] and on the growth and development of plants [14, 45, 120, 122]. These effects comprise the *biotic interferences* mentioned in the title. The chemical reactivity of particular metal species or their catalytic effects, which result in chemical binding or degradation of biogenic organics involved in plant-microbe interactions (i.e., in the aforementioned remote exchange of molecular signals, quorum sensing within bacterial communities, release and exchange of nutrients, etc. [37]), may thus be classified as indirect effects comprising *chemical interferences*. Some possible major biological and chemical processes involving heavy metals, plants and plant-associated soil-borne microorganisms [67, 148] are shown in Figure 1.

It is clear that any purely chemical (i.e., abiotic) processes, induced in the rhizosphere by the presence or formation of chemically active metal species, which result in chemical depletion, inactivation or degradation of any biomolecules directly involved in plant-microbe interactions *via* their binding and/or redox transformation, would inevitably affect these biologically specific interactions. However, in the rapidly increasing pool of basic and applied research data related to plant-microbe interactions (see, e.g. the recent highly informative reviews by Somers *et al.* [126], Khan [69], Kadouri *et al.* [48], Morgan *et al.* [91], Biró *et al.* [11], Gunatilaka [37], Mukerji *et al.* (Eds.) [92], Mur *et al.* [94], Rodriguez *et al.* [113], Watt, Silk & Passioura [142] and references therein), such chemical interferences seem to have been paid significantly lesser attention hitherto than they really deserve [51, 54, 60]

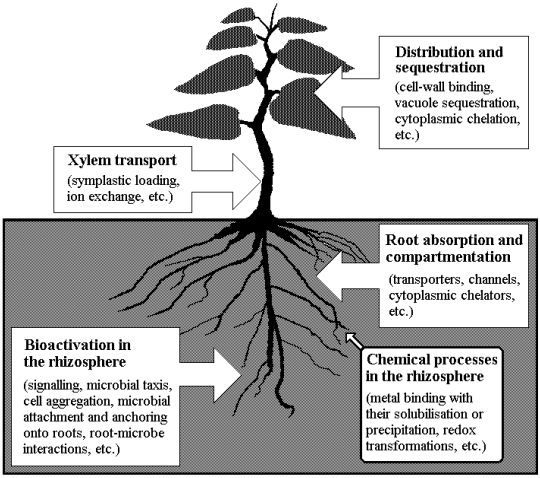

Figure 1. Scheme of major biological and chemical processes involving heavy metals, plants and plant-associated rhizospheric microorganisms.

considering their possible contribution to the overall effects. This disbalance in approaching the whole problem, leading to a virtual disbalance in understanding the diversity of molecular mechanisms underlying the processes and phenomena in highly sophisticated soil-plant-microbe systems, still remains to be corrected by increasingly involving expertise from chemical and physical sciences and applying a complex of relevant modern instrumental techniques.

3. Bioleaching and chemical transformations of heavy metals and radionuclides mediated by soil rhizosphere microorganisms

Soil microflora is well documented to be an important and active participant of biogeochemical processes, leading to diverse chemical transformations of soil minerals [19, 108, 113]. In this respect, the rhizospheric microbial communities supported by host plants (along with a contribution from plant-root exudates) can demonstrate even higher activities [92]. The rhizosphere, as compared to the bulk soil, is highly populated by various microorganisms mainly comprising bacteria (predominatingly Gram-negative bacteria) and mycorrhizal fungi (Figure 2) [67, 87] showing higher metabolic activity [88], even in polluted soils [11, 78] (note, however, that on the rhizoplane, in most close proximity to the root surface itself, as well as in the

endorhizosphere, under direct influence of the plant, the complexity and diversity of microorganisms may be significantly lower than in the "bulk rhizosphere" [39, 141]). Those plant-associated rhizobacteria and mycorrhizae may significantly increase the bioavailability of various heavy metal ions for their uptake by plants. Also, they are known to catalyse redox transformations leading to changes in heavy-metal bioavailability [148].

Some of diverse routes leading to microbially mediated bioleaching and biotransformation of heavy metals and radionuclides entrapped within soil minerals [84, 150] are represented by the following processes [60]:

(*i*) microbial dissimilatory reduction and concomitant solubilisation of metal ions (e.g., Fe, Mn and many other less abundant redox-active metals) via different mechanisms as discussed below (for reviews see, e.g. [55, 83, 85]);
(*ii*) production and excretion of siderophores, carboxylic acids, acidic exopolysaccharides and other organics capable of binding and/or reducing metal cations [28, 37, 108, 142], as well as
(*iii*) contact interactions of microbial cells with metal complexes and solid compounds via microbial cell-wall biomacromolecules, sometimes with further involvement of metal ions in cellular metabolism or their inactivation via special intracellular mechanisms, etc. [44, 63, 80, 110].

The bioavailability of iron, one of the few microelements vitally important for virtually all organisms [28, 123], which is commonly abundant in most soils, is yet well known to be extremely low within a wide range of environmental conditions. This is due to the very low solubility of ferric oxide

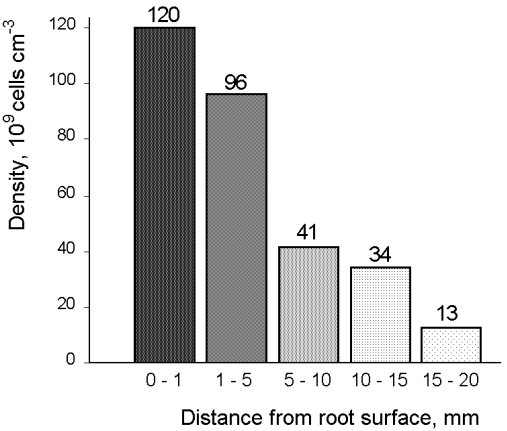

Figure 2. Estimated microbial population density in the rhizosphere as a function of distance from the root surface (adapted from [67, 104]).

or hydrated oxide species that are most stable under aerobic and microaerobic conditions. Thus, iron(III) largely represented by oxides or oxyhydroxides of variable crystallinity is an essential component of the majority of soil minerals.

A variety of soil bacteria were documented to couple oxidation of soil organic matter, including such strong pollutants as aromatic and polyaromatic hydrocarbons, to dissimilatory reduction of many redox-active metals, primarily iron(III) and manganese(IV) as commonly the most abundant (for reviews see, e.g. [83, 85, 86]). These biogenic redox processes occurring in soils, sediments or aquifers can be utilised for bioremediation of organic pollution [83, 86].

As for iron(III) chemical species, such dissimilatory reduction processes can be realised via three most general mechanisms [55, 83] schematically depicted in Figure 3:

(1) *contact reduction*, when the bacterial cell attaches directly to the surface of iron(III)-bearing soil particle, with further enzymatically driven reduction of iron(III) to iron(II) at the surface of the "parent" soil particle;

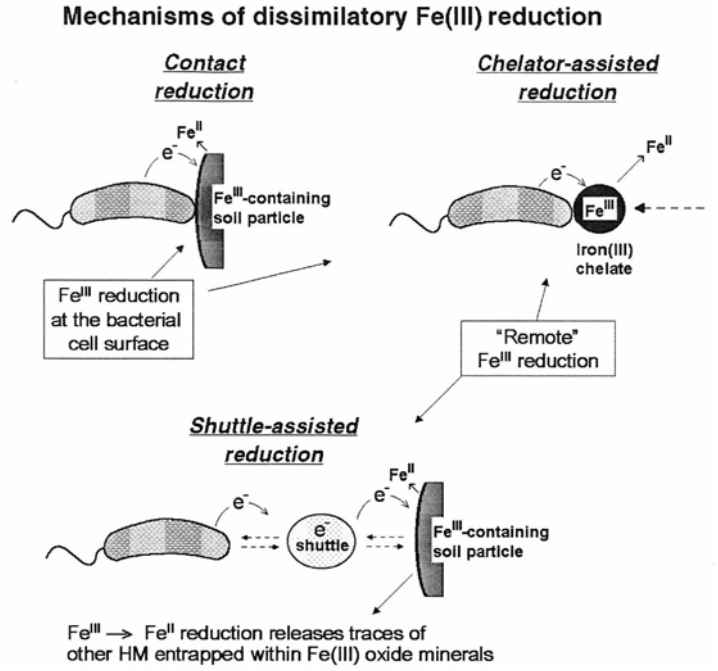

Figure 3. Mechanisms of dissimilatory microbial reduction of soil iron(III).

(2) *chelator-assisted contact reduction*, when iron(III) bound in a complex by a chelating agent in a soluble form is transported to the bacterial cell, with subsequent dissimilatory iron(III) reduction at the cell surface similar to the reduction step in the above-mentioned case;
(3) *shuttle-assisted reduction* involving specific compounds capable of redox cycling that serve as an electron shuttle between the bacterial cell and iron(III)-bearing mineral.

As can be seen from the scheme, all the three mechanisms are clearly fundamentally different. Nevertheless, as for common features and distinctions, the first two mechanisms involve iron(III) reduction at the bacterial cell surface while the third one does not, which represents its main difference in the mode of electron transfer to iron(III). On the other hand, for mechanisms (2) and (3), the bacterial cell and the mineral particle (as a source of Fe^{III}) need not be in contact as in the case of mechanism (1). For mechanisms (1) and (3), the iron(II) resulting from the reduction of iron(III) can either remain within the soil particle (for instance, in the form of insoluble mixed-valence partly reduced iron oxides, e.g. magnetite Fe_3O_4) or be solubilised, as ferrous iron species are commonly much more soluble than ferric species in circumneutral or weakly acidic media [23].

For mechanism (2), the resulting iron(II) commonly remains in solution as a complex of the same chelating agent. Note that in this case the reduced ferrous iron, and also some other redox-active d-transition metals in a lower oxidation state, become significantly more bioavailable, as they are commonly much more weakly bound by chelators than in the oxidised form (see [23, 83] and references therein).

Shuttle-assisted reduction (mechanism (3)) is known to commonly involve redox-active organic molecules either specially produced or excreted by soil microorganisms [83, 128] or naturally present in the soil environment, such as humic substances [83, 86]. The majority of "electron shuttle" (macro)molecules have quinone/hydroquinone moieties responsible for redox transformations and reversible electron transfer cycling. Nevertheless, Gorby & Bolton [35] have demonstrated that microbially reduced Co^{II}–EDTA complex (much less stable than its oxidised Co^{III} form, which is typical of metal(II)/metal(III)–EDTA complexes) can also transfer electrons to solid Mn(IV) oxides and abiotically reduce them, thus effectively acting as an electron shuttle between the bacterial cell and the mineral. Therefore, soil-borne Mn(IV) oxide minerals could play a role in maintaining concentrations of highly soluble, stable and mobile $[Co^{III}EDTA]^-$ species in the subsurface.

In addition to biotic (enzymatic) dissimilatory reduction by bacteria-reducers, humic substances, as well as their lower-molecular-weight precursors, are able to abiotically (chemically) reduce e.g., highly soluble Cr^{VI}

to less mobile Cr^{III} [96, 151] which is prone to hydrolytic precipitation at pH values close to physiological. Iron(III) exhibits a catalytic effect in these processes which depends on the type of humic substances [151].

It is interesting to note that some dissimilatory metal-reducing bacteria were found to be capable of sensing the depletion of soluble electron acceptors, with the subsequent synthesis of pili and flagella when growing on insoluble iron(III) or Mn(IV) minerals (but not on their soluble forms) [83]. Thereby the bacteria can adapt to the conditions by "switching on" their active chemotaxis mechanism to move towards the insoluble sources of electron acceptors (solid Fe^{III}- or Mn^{IV}-containing mineral particles), with subsequent attachment, thus passing from shuttle-assisted reduction (mechanism (3); see above), in view of shuttle deficiency, to contact reduction by mechanism (1). Note that similar chemotactic activity of PGPR is essential for their colonisation of plant roots for establishing an efficient associative symbiosis [126].

Note also that Mn^{III} from relatively stable and poorly soluble MnOOH-containing minerals (e.g., manganite, γ-MnOOH) has been reported to be non-reductively dissolved by bacterial hydroxamate siderophores at pH > 6.5 [25]. In weakly acidic media (already at pH < 6.5) the Mn^{III}–siderophore complex decomposes by internal electron transfer, yielding Mn^{II} and oxidation products of the siderophore. Nevertheless, Mn^{II}–siderophore complex can be readily oxidised in air back to its Mn^{III} complex [24]. These processes are likely to play a vital role in biogeochemical cycling that involves, in particular, redox-active elements and bacterial siderophores, affecting also the chemical stability of the latter via oxidation (thus providing an example of possible chemical interferences involving PGPR siderophores).

The value of the specific rate (on the molar basis) of dissimilatory Fe^{III} reduction was reported [18] to be ca. 3.6-fold and ca. 7.2-fold higher than that of nitrate and sulphate, respectively. Considering the number of electrons required for the reduction, these data demonstrate that the reaction $Fe^{III} \rightarrow Fe^{II}$ ($1e^-$) can be somewhat twice as efficient as the reaction of nitrate-to-nitrite reduction $NO_3^- \rightarrow NO_2^-$ ($2e^-$) or approximately equal to any of the overall processes of sulphate reduction either to elementary sulphur or to sulphide: $SO_4^{2-} \rightarrow S^0$ (total $6e^-$) or $SO_4^{2-} \rightarrow S^{2-}$ (total $8e^-$).

It should be emphasized that the aforementioned microbial dissimilatory reduction of ferric species facilitates the release of traces of other metals often present as admixtures within ferric oxide minerals [150]. Traces of heavy metals (in particular, ^{60}Co or other heavy-metal radionuclides) are known to be entrapped within or adsorbed at the surface of ubiquitous natural iron(III) oxide minerals [20]. Similar data were reported for cobalt species associated with manganese oxide particles [131]; it has been shown that dissolved Co is released during the bacterially driven reductive dissolution of Mn oxides and,

together with the reduced manganese, can further accumulate via oxidative precipitation at the oxic–anoxic interface.

Besides the direct toxic effects of heavy-metal and, in particular, radioactive elements on soil microbiota [116] and plants [120, 122], their mobilisation (solubilisation) and leaching from disposal sites presents a significant environmental threat [83]. It is related to very complicated biogeochemical processes [19] involving, on the one hand, the abiotic "macrocomponents" of the ecosystem (inorganic minerals with entrapped and/or adsorbed heavy metals and radionuclides) and, on the other hand, the soil-borne and/or aqueous-phase microbiocenoses and plant root systems.

The recent comprehensive study by Zachara *et al.* [150] involving radioactive labels (^{57}Co and ^{60}Co used for separately labelling solid and aqueous phases) showed that cobalt traces, initially present within the mineral phase as cobalt(III) oxyhydroxides, were readily reduced in the course of microbially driven processes. The resulting cobalt(II) was in most cases solubilised in preference to iron(II) formed under the same conditions; similar results were obtained also for trace nickel impurities. It was also noted that natural crystalline iron(III) oxyhydroxides are more reducible and adsorb metal cations more weakly than their synthetic analogues owing to structural imperfections characteristic of natural minerals (see [150] and references therein).

Note that the radioactive ^{57}Co nuclide, besides the possibility of its use in research as a separate radioactive label (similar to and together with another cobalt radionuclide, ^{60}Co, as used by Zachara *et al.* [150]), also provides a unique opportunity for using it as a Mössbauer-active nuclide in the highly sensitive, selective and informative non-destructive emission variant of Mössbauer (nuclear gamma-resonance) spectroscopy (EMS) (see [57, 58] and references therein). Until recently, this powerful nuclear chemistry technique was virtually out of use in life sciences [52], which relates primarily to the necessity of using a radioactive element (^{57}Co) in samples under study. Nevertheless, the EMS technique is highly sensitive to quantitative and qualitative transformations of various chemical and structural forms of cobalt species, thus presenting the possibility for trace-level bioanalytical and chemical speciation of this metal, which has a broad range of physiological and biochemical functions [144, 145], both *in vitro* and *in vivo* [52, 56]. EMS measurements, using the ^{57}Co nuclide and the ubiquitous plant-growth-promoting rhizobacterium *Azospirillum brasilense*, were reported to provide valuable information on primary adsorption of cobalt traces by live bacterial cells and ongoing metabolic transformations of the cation, as compared to its purely chemical binding to dead-cell biomass and complexation in the cell-free culture medium [63]. These model EMS studies, together with the literature data [131, 150], reveal possible biotransformation routes of Co radionuclide

traces that can result in their microbially mediated migration in rhizosphere soils and aquifers.

As-yet poorly understood processes of bacterial mobilisation of heavy metals and radionuclides [83, 116] may in principle be advantageous and biotechnologically beneficial when occurring in the rhizosphere of metal-tolerant plants (and special hyperaccumulator plants), thereby facilitating phytoremediation processes [51, 69, 136, 148]. However, in unplanted, poorly planted soils or in the presence of non-metal-tolerant plants the bioleaching and solubilisation of heavy metals and radionuclides, besides their generally enhanced bioavailability and biotoxicity, can lead to a virtually unpredictable outspread of the contamination plume or to penetration of the pollutants into ground waters.

Interaction of metal ions with biological matter is essential both as *in-vivo* processes (either vitally important or deleterious) for all organisms [124], and in related fields (biogeochemistry, bioremediation and phytoremediation, biomining, biotechnology of metal extraction, sorption and recovery, etc.). It has to be emphasized that lack of understanding of molecular mechanisms underlying the effects of soil microorganisms and plant-root exudates on the state of metal compounds in the rhizosphere can be overcome by widely using a variety of modern powerful physicochemical techniques in environmental and life sciences, as mentioned above. On the other hand, knowing the mechanisms and routes of metal transformations may open ways for a variety of practical applications (for recent reviews see [34, 37, 51, 69, 86, 87, 113] and references therein).

4. Biotic interferences of heavy metals: Biasing the underground orchestra to play out of tune

In the course of evolution, both microorganisms and plants have developed diverse strategies helping them overcome or adapt to unfavourable environmental conditions. A number of PGPR and other microorganisms have been reported to be resistant to relatively high concentrations of heavy metals [7, 8, 10, 11, 78, 97, 116] and remain active in moderately acidic soils [9, 43, 149] which are wide-spread, comprising over 30% of only arable territories [139]. Such phytostimulating rhizobacteria could contribute to plant growth promotion in metal-contaminated sites both indirectly, by increasing the overall fertility of the contaminated soil, and, in certain cases of complex pollution, also directly, not only inducing the unique active "ready-for-battle" state of the plant (called "priming") [107] as well as providing nutrients, phytohormones and exhibiting other PGPR-related traits [115, 126] but also catabolising certain organics and/or partly oxidised intermediate biodegradation

products (see [55, 95] and references therein). Considering the beneficial effects of phosphate solubilisation by PGPR [108, 113], note also that, as shown by Ratti *et al.* [111], joint inoculation of plants with arbuscular mycorrhizal fungi and phosphate-solubilising and nitrogen-fixing bacteria may be advantageous in phosphate-deficient alkaline soils amended with insoluble $Ca_3(PO_4)_2$ which otherwise is not used by the plant.

Considering biotic interferences, the responses of soil bacteria will mostly be discussed here, as this "micropartner" of plant-microbe associative symbioses is generally much more adaptable and more quickly responding to the unfavourable conditions in the environment. In particular, significant attention is paid to PGPR of the genus *Azospirillum*, ubiquitous soil diazotrophic phytostimulators known since 1978 and extensively studied through the last decades, that comprise nine species characterised hitherto (see [6, 126] considering seven species known earlier and [106, 147] for the two species isolated recently).

It has been well documented that the biosynthesis of auxin phytohormones with their excretion into soil makes a major contribution to bacterial plant-growth-promoting effects [10, 11, 29, 126]. In addition, some plant-associated bacteria were shown to improve host plant growth and development in heavy-metal-contaminated soil by mitigating toxic effects of heavy metals on the plants [7, 15].

As for PGPR of the genus *Azospirillum*, previous studies have shown that these phytostimulating microaerophilic rhizobacteria are relatively tolerant to submillimolar concentrations of heavy metals at which, in many cases, growth of the bacterial culture is not significantly suppressed (see, e.g. [63] and references therein). Moderate concentrations of heavy metals in soil can often be found as a result of contamination and/or bioleaching of minerals, and assessing their impact on the bacterial metabolism is of importance for a deeper insight into their biology and presents an obvious biotechnological and agricultural interest, considering the plant-growth-promoting abilities of azospirilla [6, 29, 87, 126]. In particular, *A. brasilense* represents a relatively rare chance to compare two strains of the same species which essentially differ in their mode of plant-root colonisation. For instance, *A. brasilense* strain Sp245 is known to be a facultative endophyte (capable of penetrating to and colonising the plant root interior), whereas non-endophytic strain Sp7 colonises the root surface only [72].

The levels of the auxin phytohormone (indole-3-acetic acid, IAA) production for each of the two strains were analysed in a standard medium (control) and in the presence of 0.2 mM $CuSO_4$ or $CdCl_2$ under microaerobic conditions that are favourable for these microaerophilic bacteria [64]. In parallel, the bacterial growth rate (in terms of colony-forming units, CFUs) was also assessed (Figure 4).

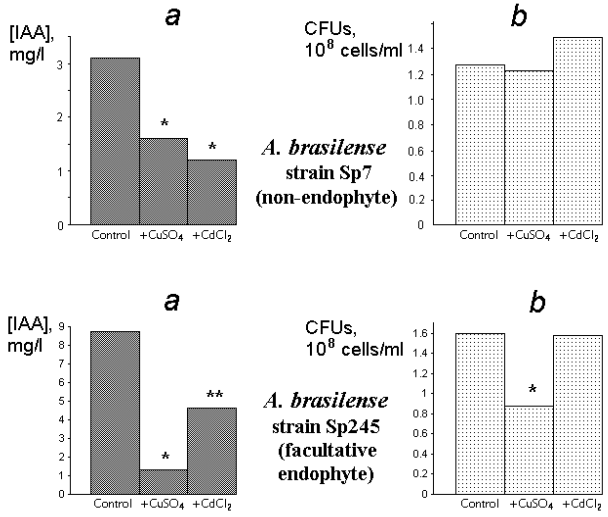

Figure 4. Indole-3-acetic acid (IAA) production levels (*a*) and bacterial growth rate (*b*) for *Azospirillum brasilense* strain Sp7 (non-endophyte; upper diagrams) and strain Sp245 (facultative endophyte; lower diagrams) grown in the malate salt medium (control) and in the medium with 0.2 mM Cu^{2+} or Cd^{2+} under microaerobic conditions [64]. Different asterisks show statistically significant differences from the control and/or between the samples (confidence probability 0.95).

For strain Sp7, both copper(II) and cadmium ions were found to result in a significant (2- to 3-fold) decrease in the level of IAA production, whereas the bacterial growth rate was virtually not affected. For strain Sp245, the overall level of IAA production in the control was approximately 3 times higher than for Sp7 (see Figure 4,a). Nevertheless, in this respect strain Sp245 appeared to be more sensitive to heavy metals: a noticeable decrease in IAA production was observed under the effect of both the metals, especially with Cu^{2+}; note also that for strain Sp245 copper(II) was somewhat more toxic decreasing also the bacterial counts (see Figure 4, lower diagrams).

Thus, the observed decrease in IAA production by the bacteria under the influence of copper(II) and cadmium(II) may directly affect the efficiency of associative plant-bacterial symbioses in heavy metal-polluted soils. Whereas for the non-endophytic strain Sp7 this detrimental influence of the soil pollutant is inevitable, it has still to be confirmed experimentally whether the possibility for the endophytic localisation of strain Sp245, "protected" under the plant tissue, would be advantageous to more successfully resist heavy-metal toxicity.

Note that the *A. brasilense* strains under study have shown some heavy-metal tolerance, as in three cases out of four (see Figure 4) the bacterial growth rate was not inhibited by heavy metals under microaerobic conditions (see below). Nevertheless, in this respect azospirilla are relatively less studied, so that the mechanism of their heavy-metal resistance, reported for better studied microorganisms [97, 114], has still to be clarified.

The effects of cadmium(II) and copper(II) at 0.2 mM on IAA production by *A. brasilense* strains Sp245 studied under *aerobic conditions* were also reported recently [133], along with the corresponding CFU numbers. It was found that for the culture grown in the presence of 0.2 mM cadmium(II) or copper(II), the IAA concentration was lowered by 38% or 46%, respectively. On the other hand, it was also found that the CFU number was simultaneously lowered ca. 2-fold and 3.7-fold for Cd^{2+} and Cu^{2+}, respectively. Thus it may be concluded that the decrease in auxin concentrations in the culture medium found in the presence of 0.2 mM Cd^{2+} or Cu^{2+} under *aerobic* conditions resulted rather from HM-induced culture growth suppression but not from a decrease in the average specific per-cell IAA production rate, which actually was not lowered.

In an earlier work [135], in order to study the expression of the indole-3-pyruvate decarboxylase gene (*ipdC*), an *A. brasilense* strain bearing a translational *ipdC-gusA* fusion (pFAJ64) was constructed. Induction of the *ipdC* gene encoding the key enzyme in IAA biosynthesis, indole-3-pyruvate decarboxylase, was found to be inhibited (along with the inhibition of IAA biosynthesis) under aerobic conditions in the logarithmic growth phase [102]. In another recent report [105], IAA biosynthesis by *Azospirillum* strains (including *A. brasilense* Sp7 and Sp245) was confirmed under aerobic conditions in the presence of tryptophan (0.1 mg ml^{-1}). In the late stationary phase (72 h), *A. brasilense* strains were found to produce 16.5 to 38 μg IAA per mg protein, whereas several *Gluconacetobacter* spp. and *Pseudomonas stutzeri* produced less IAA (1.0 to 2.9 μg IAA per mg protein) [105]. Thus, the experimental data discussed above provide evidence that, in the late stationary phase, aerobically cultivated *A. brasilense* can still produce significant amounts of auxin, despite the fact that aerobic conditions may be regarded as a stress for microaerophilic azospirilla.

To compare heavy-metal resistance of some plant-associated soil bacteria, the values of minimum inhibitory concentrations (MIC) and minimal lethal concentrations (MLC) for several PGPR grown on the agar medium (aerobic conditions) [7, 133] are presented in Table 1.

It should be noted that the MLC of cadmium(II) found for both the two *A. brasilense* strains (5 mM) was much higher than those reported for the other associative rhizobacteria (0.35 to 0.6 mM), i.e. by an order of magnitude, while

Table 1. Heavy-metal resistance of some PGPR grown on the agar medium [7, 133].

Strain	MIC / MLC values (mM) for different heavy metals					
	Co^{II}	Ni^{II}	Cu^{II}	Zn^{II}	Cd^{II}	Pb^{II}
Azospirillum brasilense Sp7	0.1 / 0.5	– / –	0.1 / 0.5	– / 5.0	– / 5.0	– / –
Azospirillum brasilense Sp245	0.1 / 0.5	– / –	0.1 / 0.5	– / 5.0	– / 5.0	– / –
Azospirillum lipoferum 137	– / –	0.15 / 0.40	0.05 / 0.15	0.40 / 1.00	0.03 / 0.35	0.20 / 0.70
Arthrobacter mysorens 7	– / –	0.05 / 0.25	0.20 / 0.70	0.50 / 1.50	0.05 / 0.60	0.40 / 1.00
Agrobacterium radiobacter 10	– / –	0.15 / 0.50	0.10 / 0.45	0.15 / 0.80	0.06 / 0.50	0.20 / 0.80
Flavobacterium sp. L30	– / –	0.025 / 0.20	0.07 / 0.30	0.40 / 1.00	0.002/ 0.01	0.10 / 0.50

Note: The minimum growth-inhibitory (MIC) and minimum lethal (MLC) concentrations of heavy metals are shown on the left and on the right of the "/" symbols, respectively (the dash sign "–" means that the values were not determined).

for the Cd-sensitive rhizobacterium *Flavobacterium* sp. L30 the MLC was 0.01 mM [7]. By their cadmium resistance, the following soil bacteria isolated from the rhizoplane of Indian mustard (*Brassica juncea* L. Czern.) plants [8] were closer to *A. brasilense*: *Variovorax paradoxus* (MLC = 0.6 to 3.5 mM for different strains), a *Rhodococcus* sp., a *Ralstonia* sp., a *Flavobacterium* sp., a *Pseudomonas* sp. and some other strains (MLC = 2.0 to 4.0 mM).

The two *A. brasilense* strains were also significantly more resistant to zinc(II): the MLC value for both strains Sp7 and Sp245 was found to be 5 mM (see Table 1), which is several times higher than that for *A. lipoferum*, *A. mysorens*, *A. radiobacter* and *Flavobacterium* sp. (0.8 to 1.5 mM). Nevertheless, for the latter four bacteria the MIC and MLC of Cu^{2+} were in the range of 0.05 to 0.2 mM and 0.15 to 0.7 mM, respectively. The data on copper(II) MIC and MLC obtained for *A. brasilense* (0.1 and 0.5 mM, respectively) is within the above-mentioned range (see Table 1).

Therefore, considering the data reported in the literature for various associative rhizobacteria, *A. brasilense* strains Sp7 and Sp245 show a relatively high resistance to copper(II) and a very high resistance to cadmium(II) and

zinc(II). As compared to *E. coli*, a well-studied microbiological object [97], *A. brasilense* strains Sp7 and Sp245 were more resistant to cadmium(II) but much more sensitive to cobalt(II) and copper(II), while the resistance of the two *A. brasilense* strains and of *E. coli* to zinc(II) was similar. It is also interesting to note that, as compared to *Pseudomonas putida* (strain ATCC 33015) [116], which is regarded as a highly metal-tolerant bacterium having potential for use in bioremediation, the two *A. brasilense* strains discussed above (see Table 1) still show noticeably higher resistance to cobalt(II), copper(II) and cadmium(II) and similar resistance to zinc(II). Note also for comparison that an increased growth of *P. putida* was observed in the presence of low concentrations of Cu^{II}, CrO_4^{2-} (by ca. 20% at 0.06 and 0.08 mM, respectively) and even uranium(VI)–citrate complex (by ca. 20% at 0.4 mM and by over 50% at 0.8 to 1.3 mM, although with an abrupt inhibition already at 1.7 mM, with virtually no effect of Na citrate up to 0.23 M) [116]. The mechanisms of these stimulating effects are yet unclear, at least for the non-nutrient metals (Cr^{VI} and U^{VI}).

To conclude, the results reported in the literature provide evidence for a relatively high resistance of the bacteria of the genus *Azospirillum* to heavy metals, even under aerobic conditions less favourable for these microaerophilic bacteria (and probably still higher resistance under microaerobic conditions, that are to be mostly expected in the rhizosphere). This feature, together with their capability of degrading oil hydrocarbons and still producing comparable amounts of IAA under crude-oil contamination [95], suggests that their application as PGPR might be advantageous for their host plant to more efficiently counteract the detrimental effects of complex soil pollution, including their possible role as "micropartners" in soil phytoremediation.

Comparing the non-endophyte *A. brasilense* Sp7 and the facultative endophyte *A. brasilense* Sp245, it should be mentioned that their overall metabolic responses in the case of a moderate heavy-metal stress were found to be significantly different [64]. Comparing the infrared spectroscopic images of whole cells, grown in a rich malate salt medium supplemented with 3 g l^{-1} NH_4Cl in the absence (control) and presence of cobalt(II), copper(II) or zinc (at 0.2 mM), each of the heavy metals was found to induce in the non-endophyte *A. brasilense* Sp7 (but not in the facultative endophyte *A. brasilense* Sp245 under the same conditions) a significantly enhanced accumulation of polyhydroxyalkanoates (PHAs), including dominating poly-3-hydroxybutyrate (PHB) [63, 64]. These biopolyesters are well-known important intracellular bacterial carbon and energy storage materials that are involved in stress endurance and alleviation via yet unknown mechanisms, and their production by PGPR may be a mechanism favouring their establishment, proliferation, survival and competitive abilities in the rhizosphere [46, 48]. Thus, bacterial

PHA production has been proposed to be of critical importance for improving the storage, efficiency and reliability of commercial plant-growth-promoting bacterial inoculants for agricultural uses (for recent reviews see [48, 70, 81]).

Note, however, that polyhydroxyalkanoates are commonly known to accumulate in bacterial cells mostly under starvation or nutritional stress (e.g., at a high C/N ratio which is typical of the rhizosphere, where it was estimated to be around 20) [47, 48]. The induction of PHB and/or PHA biosynthesis and accumulation in bacterial cells in a rich medium under a moderate heavy-metal stress, as found for the non-endophyte *A. brasilense* strain Sp7, is a novel trait [59, 62]. In the non-endophytic strain, it may be a specific flexible adaptation strategy related to the localisation of the bacteria either in the rhizosphere soil or, after their attachment to plant roots, on the rhizoplane, i.e. always in direct contact with rhizospheric soil components (whereas this trait is not observed in the facultatively endophytic strain *A. brasilense* Sp245 [64]). This corresponds to the documented capability of strain Sp7 to outcompete other co-inoculated strains [72]. In line with the above environmentally significant behavioural distinctions, *A. brasilense* strain Sp245 was found to be serologically dissimilar to *A. brasilense* Sp7, whereas other *A. brasilense* strains Sp7 and CD (a closely related strain) as well as even *A. lipoferum* Sp59b all showed antigenic similarity, as demonstrated using double radial immunodiffusion and enzyme-linked immunosorbent assay [74].

The dissimilarity in the response of the two *A. brasilense* strains (non-endophyte Sp7 and facultative endophyte Sp245) to a moderate heavy-metal stress is remarkable, especially considering the comparable uptake level of each of the heavy-metal cations in cells of strain Sp7 and Sp245 at metal concentrations 0.2 mM in the cultural liquid (ca. 0.12 and 0.13 mg Co, 0.48 and 0.44 mg Cu, 4.2 and 2.1 mg Zn per gram of dry biomass, respectively, that is one to three orders of magnitude higher than the corresponding metal contents in cells grown in the standard control medium with only background impurities of the metals) [63]. These dissimilarities in their behaviour may thus be related to different adaptation abilities of the strains under stress conditions owing to their different ecological status and, correspondingly, different ecological niches they can occupy in the rhizosphere. Note that for *A. brasilense* Sp245, the differences in heavy metal uptake between the wild-type strain and its two mutant strains, each deficient in the synthesis of either of the two O-specific polysaccharides of its cell-surface lipopolysaccharide (LPS) [134], point to the importance of the structure of the cell-surface biopolymers in the processes of heavy-metal binding and accumulation. Similar conclusions were made by Langley & Beveridge [80] when studying metal binding in *Pseudomonas aeruginosa* PAO1 and its three isogenic LPS mutants.

5. Chemical interferences: An inexpugnable hindrance?

The above considered biotic interferences, induced by the presence of heavy-metal or radionuclide pollution, can in principle be overcome or attenuated by adapting the properties and behaviour of the partners of plant-microbe associative symbioses. However, the concomitant chemical interferences seem to be virtually inexpugnable. Indeed, the chemical properties and reactivity of the biotically produced substances, that are directly involved in plant-microbe interactions, towards metal ions in soil can hardly be changed or otherwise influenced under given environmental conditions. Nevertheless, in order to be able to better understand the functioning mechanisms and tuning of the "subsurface orchestra" (plant-microbe associative symbioses) displaying its "love parade beneath our feet" [126], the possible chemical interferences have to be thoroughly investigated as well.

Besides the above-described directly biotic reduction of iron(III) mediated by specific soil bacteria – dissimilatory reducers, a range of secondary metabolites produced both by soil microorganisms and by plant roots can abiotically reduce Fe^{III}. For instance, direct experiments using the powerful and sensitive ^{57}Fe Mössbauer spectroscopic technique showed that such low-molecular-weight organic substances (which are known to occur in soil, e.g. among plant-root or bacterial exudates) as anthranilic (*o*-aminobenzoic) acid, indolic compounds including tryptophan, indole-3-acetic acid (a phytohormone of the auxin series produced by many soil microorganisms) and other auxins (see [60, 76, 77] and references therein) can abiotically reduce Fe^{III} in acidic media (under pH 5) even under aerobic conditions.

In Figure 5, gradual iron(III) reduction to iron(II) by a series of indole-3-alkanoic acids in acidic aqueous solutions is shown, as follows from ^{57}Fe Mössbauer spectroscopic measurements [76]. Already 15 min after mixing dissolved iron(III) salt and an indole-3-alkanoic acid, iron(II) was readily detectable in the spectra of filtered and rapidly frozen solutions (11% to 24% of total dissolved iron, except ~2% for indole-3-carboxylic acid) by its characteristic doublet with a large quadrupole splitting (Figure 5,a–d). After two days under these conditions, iron(II) dominated in the solutions comprising ca. 51% to 100% of total dissolved iron (Figure 5,f–h), except ca. 19% for indole-3-carboxylic acid (see Figure 5,e), for which the reduction rate was always definitely lower than for the other indolic acids studied. It is noteworthy that for indole-3-acetic acid, practically full reduction is observed within 1–2 days, which corresponds to its more easily proceeding oxidative decarboxylation [118]. In all cases, the parameters of the iron(II) doublet closely corresponded to ferrous hexaaquo complex [76, 77]. Thus, the reduced iron ions in solution were most probably in the hydrated form not coordinated to the indolic auxins or their oxidation products.

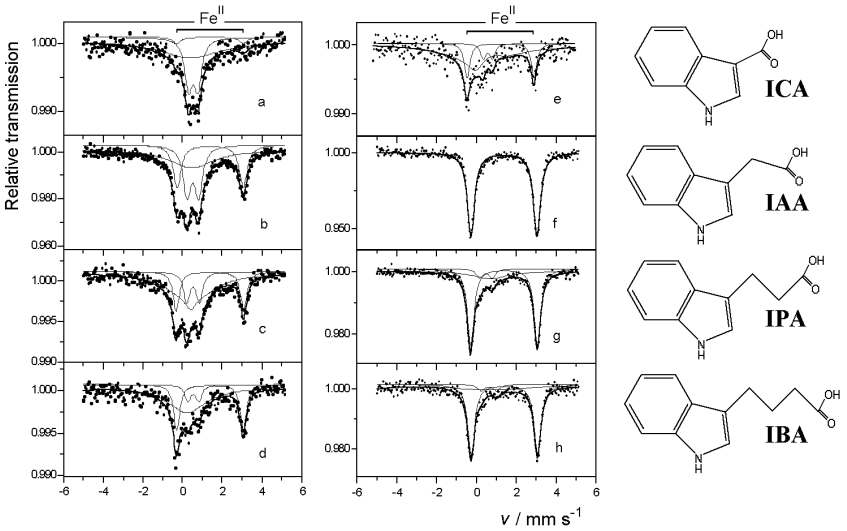

Figure 5. Mössbauer spectra of $^{57}Fe^{III}$ nitrate and indole-3-alkanoic acid aqueous solutions filtered and rapidly frozen (at T = 80 K) 15 min (a–d) and 2 days (e–h) after mixing the reagents (1:3 molar ratio; final pH 2–3). Spectra (a), (e) – indole-3-carboxylic acid (ICA); (b), (f) – indole-3-acetic acid (IAA); (c), (g) – indole-3-propionic acid (IPA); (d), (h) – indole-3-butyric acid (IBA). The position of the Fe^{II}-related doublets is indicated in the upper plots by square brackets (adapted from [76]).

Raising the pH was found to result in a slower reduction, which, nevertheless, was still noticeable under pH 5 [60]. Since acidic soils are rather widely distributed, as was mentioned above [139], besides highly metal-polluted soils which often have lower pH, such abiotic Fe^{III} reduction involving organic molecules of biotic origin may significantly contribute to iron transformation in soil. Note that the resulting pool of more soluble iron(II) can further abiotically reduce toxic and mobile high-valent metals and metalloids [51] and contribute to reductive degradation of chlorinated and nitroaromatic organics [55]. On the other hand, these processes involving ubiquitous soil iron(III) may facilitate chemical (i.e., abiotic) oxidative degradation of auxins lowering the pool of these phytohormones in soil.

While enzymatic oxidation of auxin phytohormone catalysed by plant peroxidases, regarding its mechanism and products, has been under intensive investigation owing to basic interest as well as possible biomedical applications (see, e.g. [33, 118, 130] and references therein), chemical oxidation products of auxins are much less studied. Some products of chemical oxidation of indole-3-acetic acid occurring in the presence of iron(III) were

recently isolated and studied using analytical and spectroscopic techniques [60]. The formation of oxindole-3-acetic acid was shown, which formed a poorly soluble complex with Fe^{III} similar to that with indole-3-acetic acid [61, 121]. From the reaction medium, using extraction with isobutanol and further chromatographic separation, two other oxidation products were isolated. One of the products, on the basis of infrared and ^1H NMR spectroscopic data [60], was identified as 3-methyl-2-oxindole (Figure 6); traces of the other extracted product gave a mass spectrum which suggests that the oxidation product was formed by further oxidative splitting of the pyrrolin-2-one cycle.

It should be noted that both oxindole-3-acetate and 3-methyl-2-oxindole, which were found to be formed in the course of chemical oxidation of indole-3-acetic acid in the presence of Fe^{III} (see Figure 6), had been reported among products of both enzymatic [33, 100] and electrochemical oxidation of indole-3-acetic acid [41] at physiological pH (along with 3-methylene-2-oxindole), i.e. under different conditions and electron transfer modes. Nevertheless, the exact mechanism of chemical oxidation of IAA, in particular, under environmentally relevant conditions has to be elucidated in more detail.

The aforementioned oxidative degradation of indole-3-acetic acid and other auxins can lead to a reduction in their concentrations in soil and thus be of ecological importance. Note that indole-3-acetic acid was proposed to act as a "reciprocal signalling molecule" between PGPR, which produce and excrete it, and plants [79], hence its chemical degradation would also affect plant-microbe signalling efficiency. On the other hand, some experimental evidence was reported for an increased auxin activity of indole-3-acetate complexes with lanthanoids at micromolar concentrations [143]. It has yet to be elucidated whether any other auxin–metal complexes can exhibit an increased auxin activity and what could be the reason for this effect.

Another rapidly developing field within the "underground hemisphere" of plant-microbe interactions, that can be affected by chemical interferences in soil, includes biosynthesis and exudation of intercellular bacterial signaling (quorum-

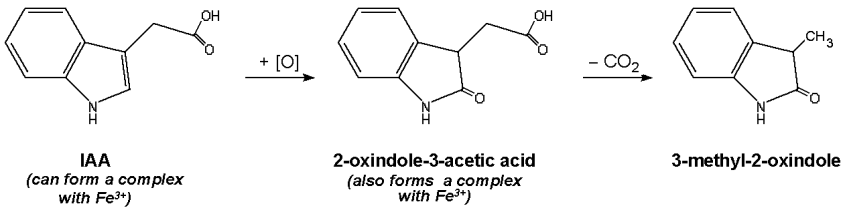

Figure 6. Scheme and some products of chemical oxidation of indole-3-acetic acid (auxin) in aqueous solution in the presence of iron(III) [60].

sensing) molecules, as well as a variety of already known, hitherto presumed and as yet undisclosed biomolecules by which plant root-microbe signalling and interactions are implemented [3, 4, 13, 126, 129] or by which plants mimic, quench or otherwise interfere with bacterial signals [1, 13, 65, 90, 132]. The majority of signalling molecules, extracellular bacterial autoregulators and autoinducers known hitherto comprise *N*-acyl-L-homoserine lactones (AHLs) [126, 137, 138] and their common structural element α-amino-γ-butyrolactone [93], alkylhydroxybenzenes (AHBs) [27, 93], cyclic dipeptides and quinolones, α,β-unsaturated fatty acids or fatty-acid esters, furanosyl and other borate diesters [71, 126], cyclic lipopeptides [109], amino acid derivatives (opines) and *myo*-inositol derivatives (rhizopines) [126], etc., mostly used by Gram-negative bacteria, as well as linear and cyclic peptides, etc., used by Gram-positive bacteria [71]. In particular, noteworthy is the active role of PGPR cell-surface polysaccharide-containing macromolecular complexes [6, 75, 126] and cell-surface lectins (specific polysaccharide-binding glycoproteins) [66, 98, 126] in bacterial interactions with host-plant roots involving also plant lectins. As an example, wheat lectin (wheat germ agglutinin, WGA), which is exposed on the root surface and can also dissolve therefrom in the aqueous medium, has been documented to induce a number of metabolic responses in azospirilla at low concentrations and therefore is proposed to act as a molecular signal for these wheat-associated bacteria (see [3, 4] and references therein).

Many of the aforementioned substances are evidently good complexing agents for metal ions, the binding of which can block the corresponding available functional groups in both receptors and target biomolecules (or in target cells), thus switching them off and retarding the relevant biospecific interactions. In addition, some of the biomolecules can relatively easily be oxidised either by direct action of metal species or via their catalytic activity. Nevertheless, most of the current research on the chemistry of signalling molecules has so far been focused on developing instrumental approaches and/or analytical methods for their characterisation [12] and quantification [16, 17, 31, 32, 82].

It should be noted that some biomolecules involved in rhizosphere communication have been demonstrated to have additional functions, e.g. biosurfactant activity of long-chain AHLs affecting bacterial swarming and inducing liquid flows as a result of surface tension gradients at biologically relevant concentrations [21]; potential antibacterial activity of 3-oxo-AHLs and their degradation products [68, 138]; adaptogenic functions of low-molecular-weight AHB autoregulators of microbial stress response with antioxidant properties [99] as well as capability of stabilising enzymes in aqueous media and increasing their catalytic activity, as was shown for C_7-AHB and microbial protease, cellulase and α-amylase [89], where both the

enzymes and their polymeric substrates formed complexes with C_7-AHB, thereby influencing the efficiency of hydrolytic reactions. These complementary traits accordingly enhance the role of possible chemical interactions (e.g., metal binding) or degradation of the above signalling molecules in affecting plant-microbe interactions. Nevertheless, this field as yet remains virtually unexplored, and therefore much in the chemistry of biogenic substances involved in biospecific interactions, communication and signalling still has to be further understood in order to unravel the related molecular mechanisms of possible chemical interferences in the rhizosphere.

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