





## Opinion

# Significance of nitrogen-fixing actinorhizal symbioses for restoration of depleted, degraded, and contaminated soil

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Atmospheric nitrogen (N<sub>2</sub>)-fixing legume trees are frequently used for the restoration of depleted, degraded, and contaminated soils. However, biological N<sub>2</sub> fixation (BNF) can also be performed by so-called actinorhizal plants. Actinorhizal plants include a high diversity of woody species and therefore can be applied in a broad spectrum of environments. In contrast to N<sub>2</sub>-fixing legumes, the potential of actinorhizal plants for soil restoration remains largely unexplored. In this Opinion, we propose related basic research requirements for the characterization of environmental stress responses that determine the restoration potential of actinorhizal plants for depleted, degraded, and contaminated soils. We identify advantages and unexplored processes of actinorhizal plants and describe a mainly uncharted avenue of future research for this important group of plant species.

## Potential of woody plants for phytoremediation

Anthropogenic activities such as poor agricultural practices, mining, deforestation, and land-use change have resulted in soil erosion, nutrient leaching, and soil contamination with heavy metals and/or organic pollutants that together with climate change strongly reduce soil fertility and restrict plant growth and development [1–3]. For restoration of contaminated soils, **phytoremediation** (see Glossary) has been proven to constitute a useful, low-cost, but time-consuming alternative to physical or chemical decontamination, particularly since the latter approaches are labor intensive, prone to cause secondary pollution, and inappropriate to remediate large contaminated areas (see [4] and literature cited therein). In this context, afforestation with fast-growing pioneer tree species such as poplar has been successfully applied for the mitigation of these environmental constraints. Forest trees can support essential soil functions and ecosystem services by topsoil conservation, improved nutrient availability, heavy-metal decontamination, water retention, enhanced **carbon sequestration**, and biodiversity conservation, thereby restoring soil fertility [5,6], particularly by **mycorrhizal symbioses** [7]. In addition, they can prevent the release of contaminants into the **hydrosphere** due to their deep rooting system. However, current use of tree species for phytoremediation of heavily nutrient-depleted soils is largely restricted to black locust (*Robinia pseudoacacia* L.), a fast-growing, N<sub>2</sub>-fixing **legume** tree species. In this context, the ability of black locust to form a tripartite symbiotic association {legume, mycorrhizal fungi [particularly **arbuscular mycorrhizal fungus (AMF)**], plus **rhizobia**} made such species a feasible model for ecophysiological studies of woody legumes [8]. Such a tripartite symbiotic association confers on host black locust not only additional resources from its symbiotic partners but also other benefits (e.g., enhanced plant defense, counteracting environmental constraints) [8]. Its cultivation can not only improve the nitrogen (N) and phosphorus (P) content of the soil directly, but also can support

## Highlights

The potential of atmospheric nitrogen (N<sub>2</sub>)-fixing actinorhizal plants associated with *Frankia* spp. for phytoremediation in depleted, degraded, and contaminated soil is largely unexplored and constitutes a vital avenue for future studies.

Features of biological N<sub>2</sub> fixation by legumes associated with rhizobia and actinorhizal plants are compared.

Current knowledge on the significance of N<sub>2</sub> fixation by actinorhizal–*Frankia* symbioses for the compensation of environmental constraints is summarized.

Unexplored processes that determine the restoration potential of actinorhizal plants for depleted, degraded, and contaminated soils are identified.

Potential advantages and essential basic research requirements for the application of actinorhizal plant species for pedosphere phytoremediation are provided.

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the nutrition of co-cultivated plant species with these essential macronutrients [9,10]. In addition, black locust can adsorb high concentrations of heavy metals in soils. Therefore, this tree species is suitable for habitat restoration of heavy-metal-polluted as well as heavily nutrient-depleted sites [6,10]. However, high biomass accumulation by black locust and, hence, appropriate phytoremediation capacity is largely restricted to temperate climate conditions. Moreover, black locust is an invasive alien species native to the southeastern part of North America, but extremely widespread from Europe to Eastern Asia [11]. Therefore, additional approaches are required for the restoration of heavily nutrient-depleted and contaminated soils by the use of native  $N_2$ -fixing trees/bushes for phytoremediation that are also advantageous for the conservation of biodiversity. The aim of this Opinion is to summarize current knowledge on the significance of actinorhizal symbioses of plants for the restoration of depleted, degraded, and contaminated soils and to identify essential basic research requirements that can support practical applications to increase soil fertility.

### Significance of $N_2$ -fixing symbioses

$N_2$ -fixing symbioses between plants and prokaryotes are an outstanding evolutionary adaptation that allows plants to acquire  $N_2$  directly from the atmosphere and to introduce reduced N into the biosphere with an enormous contribution to soil fertility [12–14]. **BNF** is thought to account for more than 97% of N inputs into unmanaged terrestrial ecosystems [15,16]. Three types of prokaryotes participate in  $N_2$ -fixing symbioses with plants, namely: (i) Gram-negative filamentous cyanobacteria of the genus *Nostoc* [17]; (ii) a **polyphyletic group** of soil proteobacteria collectively referred to as *Rhizobium* spp.; and (iii) Gram-positive soil bacteria of the genus *Frankia* [18]. The highly specific and most efficient processes for  $N_2$  fixation involve the formation of root **nodules**, specialized organs where bacterial symbionts colonize intracellularly and generate appropriate conditions for the conversion of  $N_2$  into ammonia ( $NH_3$ ) by the enzyme **nitrogenase** [19]. Fixed N will be delivered to host plants in exchange for reduced carbon (Figure 1). Plant species with  $N_2$ -fixing nodules are distributed across only ten lineages in the related taxonomic orders Fabales, Fagales, Cucurbitales, and Rosales [20]. Both legumes (Fabales) and the non-legume *Parasponia* (Rosales) possess the ability to form nodules with rhizobia, whereas species of eight lineages in the orders Fagales, Cucurbitales, and Rosales form  $N_2$ -fixing nodules with the actinobacterial genus *Frankia* and are accordingly named actinorhizal plants [21]. By far, the  $N_2$ -fixing symbioses are best known from associations involving rhizobia and legumes, particularly the two model species *Medicago truncatula* Gaertn. and *Lotus japonicus* Regel [22].

Actinorhizal symbioses are much less studied in terms of the number of plant species involved and their economic importance than *Rhizobium*–legume associations. Actinorhizal plants share the feature of inhabiting depleted and contaminated soils and play an important ecological role in early successional habitats [23]. The establishment of  $N_2$ -fixing symbioses requires a harmonized molecular signal exchange between bacteria and plants enabling the infection process and the **organogenesis** of nodules. In this context, the significance of P in nodule initiation and growth is controversial (Figure 1). In *Rhizobium*–legume associations, the inorganic P content of the soil and the P nutrition of the plant partner can be improved, probably by enhanced mobilization of soil resources [10]. For actinorhizal symbioses, *Discaria trinervis* (Poepp.) Reiche is reported to possess an enhanced P requirement for vegetative growth and nodule formation [24]. Other actinorhizal plants such as *Alnus* and *Casuarina* were shown to be highly dependent on adequate P supply to establish symbioses with *Frankia* [25]. However, information on the consequences of **nodulation** with *Frankia* for soil inorganic P content and P nutrition of the plant partner is still lacking.

The components of the signaling pathways are similar among the different plant species and require dozens of intensively regulated genes (for a review, see [26]). Besides complex proteins, peptides also play an important role in establishing plant–bacterial symbiosis. In *Alnus glutinosa*

### Glossary

#### Arbuscular mycorrhizal fungi (AMFs):

a type of endomycorrhizal fungi that penetrate and enter the root cells of host plants. The AMF symbiosis is intracellular and formed by a monophyletic group of fungi from the phylum Glomeromycota and the roots of 70–90% of land plant species. In an arbuscular mycorrhiza, a symbiont fungus penetrates the cortical cells of the roots of a vascular plant and forms arbuscules. AMFs survive without symbiosis by using nutrients released by saprotrophic microbes.

#### Ascorbate peroxidase (APX):

a hydrogen-peroxide-scavenging enzyme that is specific to plants and algae and is indispensable to protect chloroplasts and other cell organelles from damage by hydrogen peroxide ( $H_2O_2$ ) and the hydroxyl radicals produced from it.

#### Biological $N_2$ fixation (BNF):

a process performed by a specialized group of prokaryotes. These organisms utilize the enzyme nitrogenase to catalyze the conversion of  $N_2$  to  $NH_3$ . Plants can readily assimilate  $NH_3$  to produce amino acids. Prokaryotes capable of BNF include aquatic organisms, such as cyanobacteria, free-living soil bacteria, such as *Azotobacter*, bacteria that form associative relationships with plants, such as *Azospirillum*, and, most importantly, bacteria, such as *Rhizobium*, *Bradyrhizobium*, and *Frankia*, that form symbioses with legumes and other plant species.

**Carbon sequestration:** a natural or artificial process by which carbon dioxide is removed from the atmosphere and held in solid or liquid form.

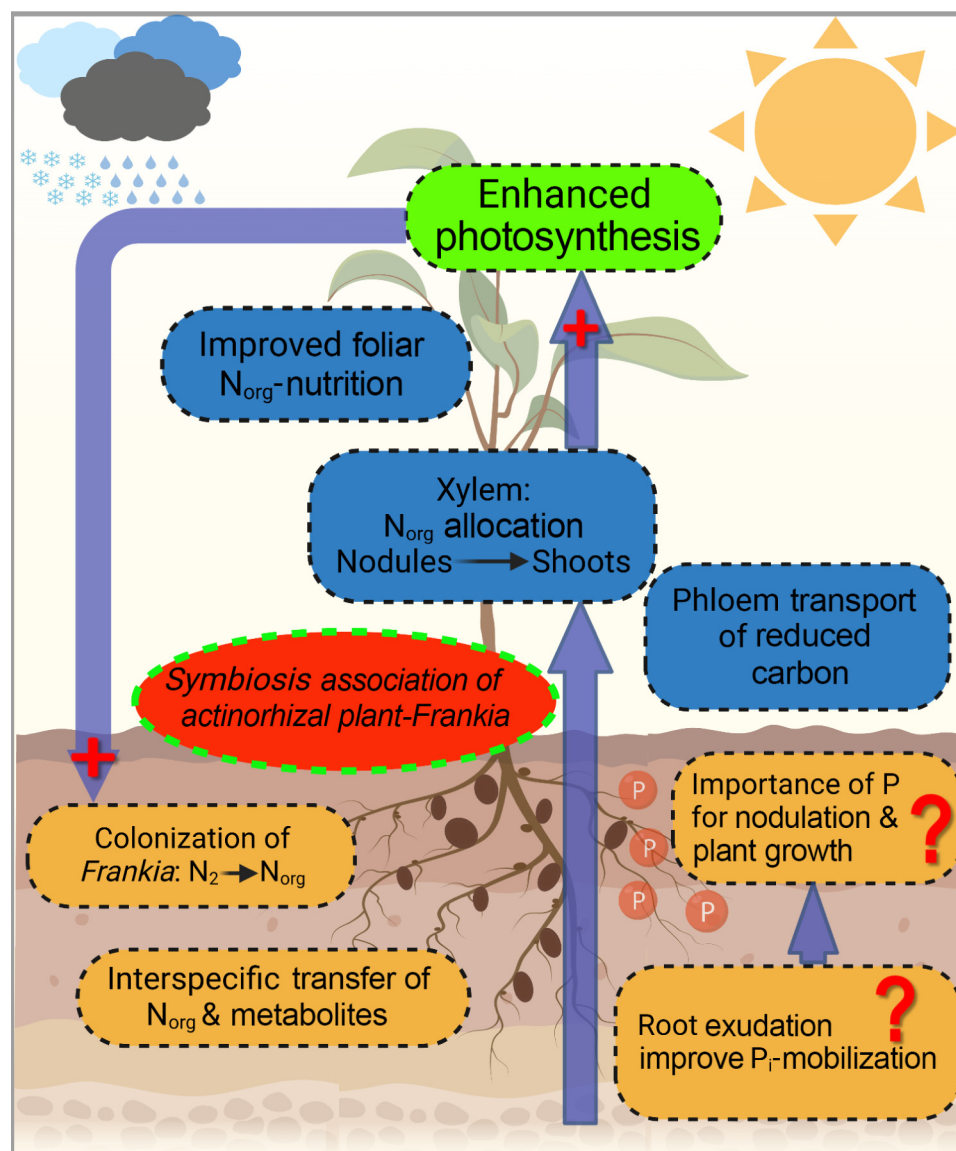
**Catalase (CAT):** a ubiquitous heme enzyme that catalyzes the detoxification of  $H_2O_2$ .

#### Differentially expressed proteins (DEPs):

indicates the relative abundances (molar ratios) of proteins in samples under different conditions (groups) (e.g., control vs. treated).

**Ectomycorrhizal fungi (EMFs):** a type of mycorrhizal fungi that enclose the root cells of the host plants but usually do not penetrate the root cells. These fungi are extracellular and belong to a polyphyletic group. EMFs usually survive without symbiosis through mineralizing nutrients from organic matter.

**Hydrosphere:** the aqueous envelope of the Earth including bodies of water and aqueous vapor in the soil and atmosphere.



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**Figure 1. Benefit of actinorhizal symbioses for plant growth.** The interplay of *Frankia* and its host plant can improve carbon (C) assimilation and atmospheric nitrogen ( $N_2$ ) fixation. The importance of phosphorus (P) nutrition for nodule initiation or the growth of nodules and host plants is controversial in the literature (highlighted by the question marks). At present, there is no information about the effects of root exudation on pedosphere inorganic P mobilization (highlighted by the question marks). Rectangular frames represent the various nutritional and metabolic pathways between *Frankia* strains and their host plants. Elliptical frame represents the symbiotic association of actinorhizal plants with *Frankia*. Plus signs indicate significantly enhanced N and C transportation and allocation via xylem and phloem. Figure created using BioRender (<https://biorender.com/>).

(L.) Gaertn. nodules, the defensin-like (DFL) peptide Ag5 helps to exchange N-containing metabolites by increasing their membrane permeability [27]. Similar results have not been reported for legume–rhizobia symbioses.

Because many actinorhizal plants are also capable of forming mycorrhizal associations, similar to legume–rhizobia–mycorrhizal fungi associations, a tripartite symbiosis (host actinorhizal plant–

**Legume:** any species of a large family (Leguminosae synonym Fabaceae, the legume family) of dicotyledonous herbs, shrubs, and trees having fruits that are legumes or loments, bearing nodules on the roots that contain  $N_2$ -fixing bacteria, and including important food and forage plants (e.g., peas, beans, clovers).

**Mycorrhizal symbioses:** intimate associations between plant roots and mycorrhizal fungi.

**Nitrogenase:** an enzyme complex that catalyzes the ATP-dependent reduction of dinitrogen ( $N_2$ ) to  $NH_3$  in biological N fixation.

**Nodule/nodulation:** differentiated organs in the roots of legume species that provide appropriate conditions for N fixation/the process of forming nodules and especially root nodules containing symbiotic bacteria.

**Organic hydrocarbon:** an organic compound comprising hydrogen and carbon found, for example, in crude oil, natural gas, and coal.

**Organogenesis:** the origin and development of bodily organs.

**Phytoremediation:** the treatment of pollutants or waste (as in contaminated soil or groundwater) using green plants that remove, degrade, or stabilize the undesirable substances (e.g., toxic metals, organic hydrocarbons).

**Polyphosphate (polyP) kinase:** the major enzyme that catalyzes the reversible transfer of the terminal phosphate of ATP to form a long-chain inorganic polyP.

**Polyphyletic group:** a group comprising a collection of organisms in which the most recent common ancestor of all of the included organisms is not included, usually because the common ancestor lacks the characteristics of the group.

**Provenance/ecotype:** a population of a species that survives as a distinct group through environmental selection and isolation and that is comparable with a taxonomic subspecies.

**Rhizobia:** bacteria that establish mutualistic symbioses with plant hosts in which the bacteria fix  $N_2$ , provide this to the plant, and receive carbon.

**Rhizosphere:** soil that surrounds and is influenced by the roots of a plant.

**Salinization:** an increase of the salt concentration in soil that, in most cases, is caused by dissolved salts in the water supply. It can be caused by flooding of the land by seawater or seepage of seawater or brackish groundwater through the soil from below.

*Frankia*–mycorrhizal fungi) is often established that allows plant growth on marginal soils [28–30]. Most prominent examples are species of the genus *Casuarina* spp., which are able to develop a symbiosis with *Frankia casuarinae* together with **ectomycorrhizal fungi (EMFs)** and/or AMFs for a better P supply. These plants are highly tolerant to drought, salinity, flooding, and heavy-metal pollution and are used extensively for the rehabilitation of degraded sites and to prevent soil erosion [31,32]. Moreover, these associations are particularly important in high-latitude regions, where legumes are absent or rare while actinorhizal plants are abundant and capable of vigorous growth [25].

### Efficiency of N<sub>2</sub> fixation by actinorhizal symbioses and its interaction with associated non-N<sub>2</sub>-fixing plant species

Global estimates assume that BNF adds 139–175 million tons of N per annum to terrestrial ecosystems. BNF by actinorhizal plants is thought to contribute 25% to total terrestrial BNF on a global scale and thus constitutes an extremely important ecosystem N source, particularly in forests and marginal terrestrial ecosystems [33].

The rates of symbiotic N<sub>2</sub> fixation by actinorhizal plants and legumes have been assessed in numerous terrestrial ecosystems (e.g., [34]). These estimates suggest that BNF by legumes can achieve very high rates, commonly more than 150 kg N ha<sup>-1</sup> year<sup>-1</sup> in many unmanaged ecosystems [34]. Legumes represent the third-largest family of flowering plants, with more than 18 000 species. Many of these plant species constitute important grain and forage crops for humankind, including, for example, soybean [*Glycine max* (L.) Merr.], common bean (*Phaseolus vulgaris* L.), chickpea (*Cicer arietinum* L.), and alfalfa (*Medicago sativa* L.) [35]. Legumes can fix approximately 25 kg N t<sup>-1</sup> of aboveground dry mass and can easily reach 300–400 kg N ha<sup>-1</sup> year<sup>-1</sup> when root biomass is included [36]. By comparison, chemical N fertilizer is added often at 100 kg N ha<sup>-1</sup> year<sup>-1</sup> to agricultural systems, but in some parts of the world N fertilizer application amounts to more than 500 kg N ha<sup>-1</sup> year<sup>-1</sup> [37]. The rates of symbiotic N<sub>2</sub> fixation by actinorhizal plants are usually lower and characterized by high intraspecific variation. Published estimates suggest rates of 30–50 g N tree<sup>-1</sup> season<sup>-1</sup> by actinorhizal plants, but for extrapolation to the ecosystem scale, a wide range of values for the major taxa has to be considered [38]. Published estimates include: red alder trees (*Alnus rubra* Bong.) ranging from 22 to 300 kg N ha<sup>-1</sup> year<sup>-1</sup> [39]; snow-brush (*Ceanothus velutinus* Dougl.) ranging from 24 to 101 kg N ha<sup>-1</sup> year<sup>-1</sup> [28]; *Myrica faya* Ait. in Hawaii at 18 kg N ha<sup>-1</sup> year<sup>-1</sup> [40]; and *Casuarina equisetifolia* L. ranging from 15 to 94 kg N ha<sup>-1</sup> year<sup>-1</sup> [41]. Both precise spatial and temporal estimates of rates of BNF by actinorhizal plants at the ecosystem scale and the quantification of specific amounts of N originating from N<sub>2</sub> fixation by actinorhizal plants transferred to associated non-N<sub>2</sub>-fixing plants remain largely unexplored (Box 1).

Although actinorhizal plants seem to positively facilitate establishment and growth of associated non-actinorhizal plant species, *in situ* such benefits will be determined by the state of succession of the ecosystem, its species composition, and the functional groups of non-actinorhizal species and their specific N-acquisition strategies, as well as the growth rate and stand density of the actinorhizal species [38,42]. Ultimately, the putative effects of N<sub>2</sub> fixation by actinorhizal plants at the ecosystem level will depend on the changes of soil N cycling and the responses of associated non-N<sub>2</sub>-fixing plant/tree species to these changes. In this context, N<sub>2</sub> fixation can affect the input of litter, the senescence of roots, and organic N composition and content in the **rhizosphere**, and hence the bacterial communities involved in N cycling [42,43]. However, large knowledge gaps on the significance of N<sub>2</sub> fixation by actinorhizal species for N, P, and C cycling in the rhizosphere require future research, as does a comparison with existing information on the consequences of N<sub>2</sub> fixation by legumes for these processes (e.g., [44]).

**Soil reclamation:** usually accompanies land reclamation, which is the process of improving lands to make them suitable for more intensive use. It may include reclaiming the soil's lost fertility, lack of minerals, nutrients, or moisture.

**Superoxide dismutase (SOD):** a metal-containing antioxidant enzyme that reduces harmful free radicals of oxygen formed during normal metabolic processes to oxygen and H<sub>2</sub>O<sub>2</sub>.

**Zonobiome:** the group of biomes constituting a particular ecozone.



### Box 1. Challenges in precise estimates for $N_2$ fixation by actinorhizal plants at the ecosystem or landscape scale and the transfer of fixed N to associated non- $N_2$ -fixing plants

Many estimates of BNF rates by actinorhizal plants are from studies under artificial and controlled conditions (e.g., laboratory, growth chamber, and greenhouse studies) [38], whereas information at the ecosystem level under field conditions is scarce and mostly remains to be elucidated [15]. Considerable limits inherent in large-scale ecological studies with actinorhizal plants for precise estimates of annual rates of BNF are also a consequence of the great complexity and heterogeneity of the soils and associated ecosystems inhabited by this group of species. In addition, many large-scale estimates of  $N_2$  fixation in natural ecosystems are biased, since they frequently represent the maximum potential rates of BNF from a few point measurements rather than the actual BNF representative under a given set of field conditions during an entire year. Thus, more realistic, explicit estimates of the ecosystem-scale N inputs by BNF of actinorhizal plants in space and time need to be obtained in future studies [34].

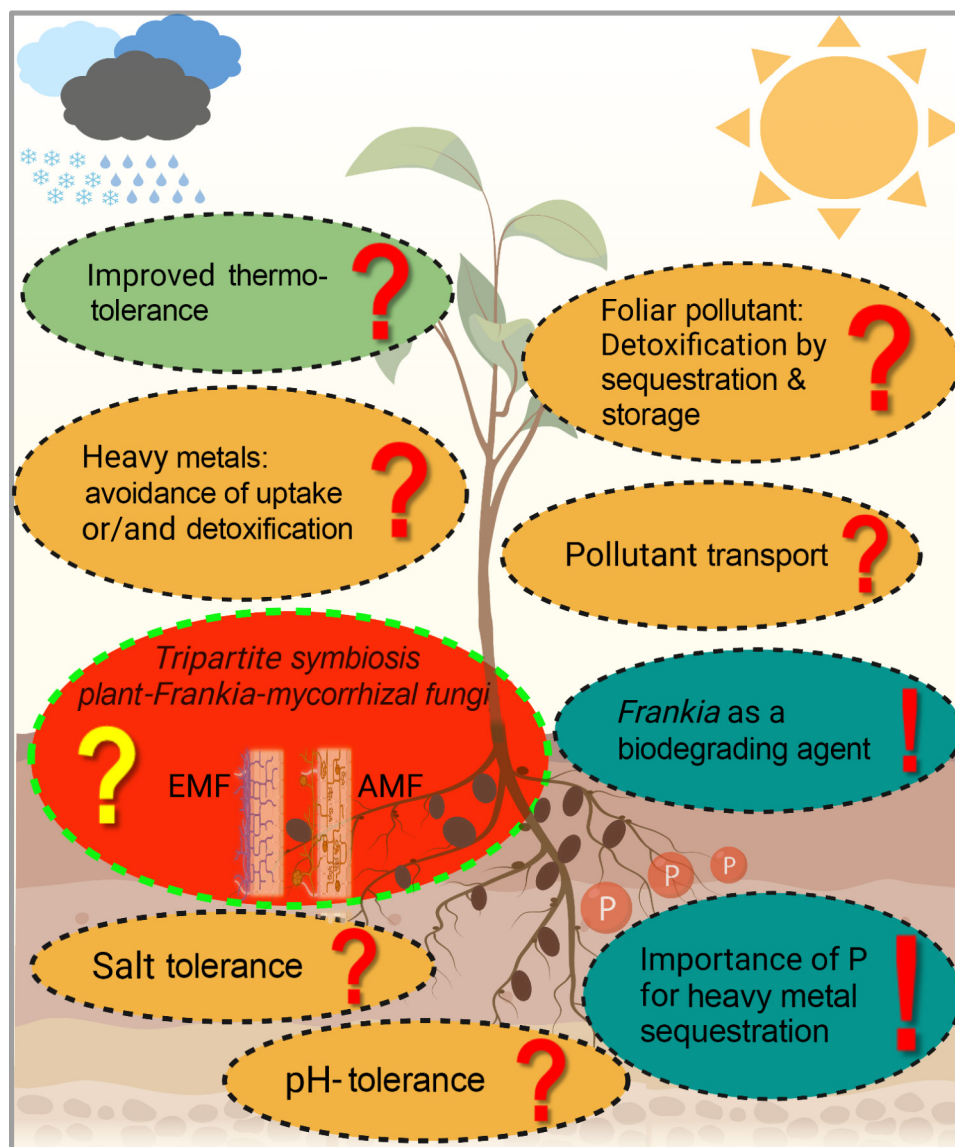
By the application of advanced technological approaches (e.g., the  $^{15}N$  natural abundance technique), some studies have further investigated the interactions between actinorhizal plants and associated non-actinorhizal (non- $N_2$  fixing) plant species [38,42,99]. The results showed that the introduction of exogenous actinorhizal plants could replace endogenous plant species and induce alterations of local ecological processes by contributing to the development of novel terrestrial ecosystems and to successional processes [38]. For instance, in a mixed conifer forest in the arid environment of Nevada, USA, *Ribes* shrubs associated with snowbrush showed depleted  $\delta^{15}N$  values compared with a *Ribes* stand without snowbrush, indicating that additional N input from the actinorhizal snowbrush affected the surrounding non- $N_2$ -fixing plant species at the community scale [99]. In the same region, [42] reported elevated soil N and C status (for N% and C%, ca +2.5 times), depleted foliar  $\delta^{15}N$  values (ranging from ca -5‰ to -1‰), and enhanced foliar N concentrations for several non- $N_2$ -fixing shrubs (ca +23%; e.g., low sagebrush, yellow rabbitbrush, wax currant) and forbs in the presence of actinorhizal plants compared with reference plots. These results suggest that the active  $N_2$  fixation of actinorhizal plants can positively influence the N status of the surrounding vegetation and ecosystems. However, quantitative information about the amount of N originating from  $N_2$  fixation by actinorhizal plants that is transferred to associated non- $N_2$ -fixing plants remains largely unexplored. This lack of information is due to the limited application of qualitative methods at the ecosystem or landscape level and requires attention in future studies.

### $N_2$ fixation and stress tolerance in actinorhizal symbioses

Efficient adaptation of  $N_2$ -fixing plants to harsh environments such as poor, degraded, and/or contaminated soils largely depends on their ability to establish symbiotic interactions with soil microorganisms (e.g.,  $N_2$ -fixing bacteria, mycorrhizal fungi), a key physiological mechanism that has been widely accepted because N represents the scarcest nutrients in these environments [45]. Among other  $N_2$ -fixing symbiotic plant species, actinorhizal plants have been traditionally considered pioneer species for the reclamation of poor, degraded, and/or contaminated soils and harsh environments due to their ability to successfully adapt to a wide range of abiotic stresses [46]. The environmental stress tolerance of actinorhizal plants is a complex trait involving the ability of both symbiotic partners to tolerate and function effectively under these conditions [47].

During the past years, several studies clearly demonstrated that inoculation with selected *Frankia* species confers or increases the capacity of the plant partner to tolerate abiotic stresses (Figure 2 and Table 1) including extreme temperature and pH, heavy metals, high salinity, and various organic pollutants, but the mechanisms involved are largely unknown [48,49]. Similar to legume-rhizobia symbioses, both the *Frankia* symbiont and its host plant show high variation in stress adaptation and  $N_2$ -fixation efficiency between ecotypes. Therefore, extensive screening and characterization of both *Frankia* strains and plant ecotypes with optimum  $N_2$  fixation performance and abiotic stress tolerance are needed to establish the best combinations of symbiotic partners for specific environments [47]. Generally, symbiotic microorganism diversity seems to be limited in poor and stressed soils, highlighting the need for external inoculation of *Frankia* symbionts during **soil reclamation** with actinorhizal plants [46]. Moreover, even closely related plant species can contribute differently to the development of mine soils, indicated, for example, by a more positive effect of black alder than green alder [50].

Past reports have focused on the tolerance, nodulation capacity, and  $N_2$ -fixing efficiency of various *Frankia* isolates under various medium and soil-pH conditions (Table 1). Typical *Frankia*



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Figure 2. Perspectives for future studies on actinorhizal symbioses with respect to plant growth on depleted, degraded, and/or contaminated soil. Question marks highlight topics of interest. There is some information available about resistance to heavy metals, salt exposure, temperature changes, and pH and about biodegradation of toxic organic compounds by *Frankia*. However, the interplays of the plant–*Frankia* symbiosis and the tripartite symbiotic association of plant–*Frankia*–mycorrhizal fungi [particularly arbuscular mycorrhizal fungus (AMF)] are mostly unknown. The exclamation marks represent decisive conclusions that can be drawn from reported references (i.e., *Frankia* as an efficient biodegrading agent and the importance of P for heavy-metal sequestration). Abbreviation: EMF, ectomycorrhizal fungus. Figure created using BioRender (<https://biorender.com/>).

strains isolated from several plant species belonging to the genera *Alnus*, *Casuarina*, *Coriaria*, and *Componia* showed that optimum pH values and tolerance were mainly strain and culture-condition dependent [51,52]. For most of the isolates, optimum pH values for growth ranged between 6 and 8, except for the BMG5.1 strain recently isolated from *Coriaria japonica*, belonging to the newly established *Frankia coriariae* species, a member of the elusive *Frankia* cluster-2, which

Table 1. Recent studies on the response of *Frankia* strains, actinorhizal plants, and their association to various environmental stresses

Feature	Plant	<i>Frankia</i> strain	Related environmental stress <sup>a</sup>	Refs
P	<i>Alnus glutinosa</i>	ACN14a	Organic acids and TCA metabolism of <i>Frankia</i>	[75]
	<i>Casuarina equisetifolia</i>	Isolated <i>Frankia</i> from root nodules	Metabolite patterns in root nodules of <i>C. equisetifolia</i>	[76]
	<i>Discaria trinervis</i>	BCU110501	Influence of soil P levels on nodulation and plant growth, metabolism	[24]
	Various actinorhizal plants	Various <i>Frankia</i> strains	Nutrient metabolism between <i>Frankia</i> nodules and host plants	Reviewed in [77,78]
Sulfur (S)	<i>A. glutinosa</i>	ACN14a	Organic acids and TCA metabolism of <i>Frankia</i>	[75]
	<i>C. equisetifolia</i>	Isolated <i>Frankia</i> from root nodules	Metabolic patterns in root nodules of <i>C. equisetifolia</i>	[76]
	Various actinorhizal plants	Various <i>Frankia</i> strains	Nutrient metabolism between <i>Frankia</i> nodules and host plants	Reviewed in [77,78]
Heavy-metal toxicity and resistance	<i>A. glutinosa</i>	Crushed nodule suspension of Ag1.1.8Bu	Ni <sup>2+</sup> toxicity	[79]
	<i>Alnus incana</i>	Crushed nodule suspension	Heavy-metal mine tailings including Cu <sup>2+</sup> , Mn <sup>2+</sup> , Bi <sup>3+</sup> , Br <sup>-</sup> , and Zn <sup>2+</sup>	[80]
	<i>A. glutinosa</i>	ACN14a	Al <sup>2+</sup> , Cd <sup>2+</sup> , Co <sup>2+</sup> , Cu <sup>2+</sup> , Ni <sup>2+</sup> , Mg <sup>2+</sup> , Mn <sup>2+</sup> , Zn <sup>2+</sup> , Pb <sup>2+</sup> and CrO <sub>4</sub> <sup>2-</sup> resistance	[59,81,82]
	<i>A. glutinosa</i> <i>Alnus cordata</i> <i>Elaeagnus angustifolia</i>	Ag1.1.8Bu Ac4 E38	Ni <sup>2+</sup> resistance	[79]
	<i>C. equisetifolia</i> <i>C. equisetifolia</i> Hybrid of <i>Casuarina junghuhniana</i> and <i>C. equisetifolia</i>	UGL020602q UGL020603q ORS021001	Al <sup>2+</sup> , Co <sup>2+</sup> , and Pb <sup>2+</sup> resistance	[83,84]
	<i>Casuarina glauca</i> <i>Casuarina cunninghamiana</i>	CglS1N1 CglS1N2 CglS3N1 CglS3N2 CglS3N5 CglT5L3 Ccl9 CglT3L2 CglT7N2 G5 Ccl13	AsO <sub>4</sub> <sup>3-</sup> , CrO <sub>4</sub> <sup>2-</sup> , SeO <sub>3</sub> <sup>2-</sup> , Pb <sup>2+</sup> , and Cu <sup>2+</sup> resistance; all of isolated <i>Frankia</i> strains can resist Cu, Co, and Zn at low concentrations except Pb, which has a highly toxic effect at the same concentration; G5 was the most resistant strain for tested heavy metals	[56]
	<i>Alnus crispa</i> <i>C. cunninghamiana</i> <i>C. cunninghamiana</i> <i>Coriaria nepalensis</i> <i>Comptonia peregrina</i> <i>C. peregrina</i> <i>Datisca cannabina</i> <i>E. angustifolia</i> <i>E. angustifolia</i> <i>Elaeagnus umbellata</i> <i>E. umbellata</i> <i>Alnus nitida</i>	ACN1 AG Cc1.17 Ccl3 CN3 Cpl1-P Cpl1-S DC12 EAN1pec El5c Eul1c EUN1f QA3	Ag <sup>+</sup> , AsO <sub>4</sub> <sup>3-</sup> , CrO <sub>4</sub> <sup>2-</sup> , SbO <sub>3</sub> <sup>-</sup> , SeO <sub>3</sub> <sup>2-</sup> , Pb <sup>2+</sup> , Cd <sup>2+</sup> , Co <sup>2+</sup> , Ni <sup>2+</sup> , and Cu <sup>2+</sup> resistance	[85]
	<i>A. crispa</i> <i>A. crispa</i> <i>Alnus viridis</i> <i>C. cunninghamiana</i>	can12canACN10a Avcl1 Ccl3	Al <sup>2+</sup> , Cd <sup>2+</sup> , Co <sup>2+</sup> Cu <sup>2+</sup> , Ni <sup>2+</sup> , Zn <sup>2+</sup> , and Pb <sup>2+</sup> resistance	[81]
	<i>C. nepalensis</i> <i>D. cannabina</i>	CN3 DC12	Cu <sup>2+</sup> and SeO <sub>3</sub> <sup>2-</sup> resistance	[55,86]

Table 1. (continued)

Feature	Plant	<i>Frankia</i> strain	Related environmental stress <sup>a</sup>	Refs
Heavy-metal toxicity and resistance	<i>E. umbellata</i>	Eul1c		
	<i>E. angustifolia</i>	EaN1pec	Proteomic mechanism of Pb <sup>2+</sup> resistance	[58]
	<i>E. angustifolia</i> <i>D. cannabina</i> <i>A. glutinosa</i> <i>E. umbellata</i> <i>A. nitida</i> <i>C. nepalensis</i>	Ccl3 ACN14a QA3 EAN1pec Eul1c EUN1f DC12 CN3	Bioinformatic analysis for Co <sup>2+</sup> , Cu <sup>2+</sup> , Fe <sup>2+</sup> , Mo <sup>2+</sup> , Mn <sup>2+</sup> , Ni <sup>2+</sup> , and Zn <sup>2+</sup> resistance	[87]
	<i>Discaria americana</i>	Natural field soil containing <i>Frankia</i>	Zn <sup>2+</sup> toxicity and resistance	[88]
	<i>A. glutinosa</i> <i>A. incana</i>	Natural field soil containing <i>Frankia</i>	Cu <sup>2+</sup> and Pb <sup>2+</sup> resistance	[89]
	<i>C. equisetifolia</i>	Ceq1	<i>Frankia</i> salinity resistance levels are strain dependent; <i>C. equisetifolia</i> and <i>Frankia</i> (strain Ceq1) symbiotic system is highly resistant to severe salt stress (~300–500 mM NaCl) Free-living <i>Frankia</i> strain Ceq1 salinity resistance up to 300 mM NaCl	[90]
Salinity toxicity and resistance	<i>C. glauca</i> <i>C. cunninghamiana</i>	Ccl156 CglM4 ThR Ccl6	<i>C. glauca</i> – <i>Frankia</i> symbiosis can tolerate salt stress up to 200 mM NaCl; at lower NaCl concentration (~50 mM), inoculated <i>C. glauca</i> seedlings even showed higher growth rate and percentage of nodulation; <i>C. glauca</i> – <i>Frankia</i> ThR symbiosis can tolerate salt stress up to 600 mM NaCl	[91–93]
	Casuarinaceae spp.	Ccl6 Allo2 Ccl3 CeD	Ccl6 and Allo2 can withstand up to 1 M NaCl whereas Ccl3 cannot withstand more than 475 mM NaCl; some other strains exhibited NaCl resistance ranging from 650 to 750 mM Ccl6 and CeD developed mechanisms to maintain N metabolism activities under saline conditions (NaCl >500 mM) Genome analysis revealed increased salinity tolerance with certain gene expression (e.g., Ccl6-RS22605)	[47,62,94–97]
	<i>Elaeagnus macrophylla</i>	Ema1	Salinity tolerance of <i>E. macrophylla</i> – <i>Frankia</i> symbiosis ranging from 50 to 100 mM NaCl concentration Free-living <i>Frankia</i> strain Ema1 up to 200 mM NaCl	[63]
	<i>Hippophae salicifolia</i>	Hsli10 Hsli9 Hsli8 Hsli2 Cpl2	Salinity-resistant strain Hsli10 showed more efficient regulation of antioxidant, N, and protein metabolism and decreases in enzyme activity compared with the salinity-sensitive strain Hsli8	[64,65,98]

<sup>a</sup>Abbreviations: Ag, silver; Al, aluminum; AsO<sub>4</sub><sup>3−</sup>, arsenate ion; Bi, bismuth; Br, bromine; Cd, cadmium; Co, cobalt; CrO<sub>4</sub><sup>2−</sup>, chromate ion; Cu, copper; Mg, magnesium; Mn, manganese; Mo, molybdenum; NaCl, sodium chloride; Ni, nickel; Pb, lead; SbO<sub>3</sub><sup>−</sup>, antimony ion; SeO<sub>3</sub><sup>2−</sup>, selenium trioxide; TCA, tricarboxylic acid; Zn, zinc.

exhibited a mildly alkaline pH optimum [52,53]. By contrast, [54] reports that of twenty *Frankia* strains investigated, only three strains were resistant to pH 4.6 and none of these *Frankia* strains remained viable at pH 4.2. The molecular mechanisms of this pH sensitivity remain unexplored. Moreover, the potential of various *Frankia* strains and actinorhizal symbioses for tolerance to wide temperature regimes and for **organic hydrocarbon** detoxification has been documented (Box 2).

Similarly, *Frankia* isolates are reported to exhibit a high degree of diversity in their ability to tolerate high concentrations of various heavy metals in the soil (Table 1). Early studies demonstrated that several *Frankia* isolates are resistant to different heavy metals, including Cu, As, Pb, Se, and Cr [47,55,56]. At least in the case of Pb and Cu, this resistance seems to involve heavy-metal sequestration and/or binding mechanisms [55], which are investigated in more detail using bioinformatics and proteomic approaches. The significance of avoidance strategies, however, is largely unexplored for both heavy-metal and salt stress. The Cu resistance of *Frankia inefficax* strain



### Box 2. Potential of *Frankia* strains and actinorhizal symbioses to tolerate temperature stress and organic hydrocarbon toxification

The ability of various *Frankia* isolates to effectively grow and nodulate under various temperature regimes has been well documented [47]. These studies revealed that temperature affects the ability of various *Frankia* strains to both: (i) survive and grow long term in various environments; and (ii) efficiently fix nitrogen. In most of the cases, *Frankia* could grow effectively at temperatures ranging from 25 to 37°C, depending greatly on the strains under study [100]. In rare cases, slow growth could be observed at temperatures below 18°C, while higher temperatures greatly impaired the growth of the strains studied. In addition, N<sub>2</sub> fixation appears to be temperature dependent, with most of the strains isolated so far exhibiting optimum nitrogenase activity at soil temperatures ranging between 25 and 30°C, depending on the *Frankia* isolate's origin [47,51,101]. However, studies with Siberian alder growing in permafrost environments indicate that woody plants associated with *Frankia* are also able to perform BNF at low soil temperatures [39,102,103]. In the case of rhizobia-mediated BNF, a wide range of cold tolerance has been observed, depending mainly on the rhizobia strain and the host legume, including studies with arctic strains that were able to grow at temperatures as low as 0°C [104]. Rhizobia-like bacteria are known to adapt to freezing environments by utilizing several biochemical and molecular mechanisms, including the production of cold-shock proteins (CSPs) [105], and by modulating their membrane fluidity by shifting fatty acid composition [106]. By contrast, little is known about the efficiency of actinorhizal BNF under freezing conditions, the *Frankia* strains involved, or the physiological and molecular processes responsible for cold adaptation. Previous studies have shown that *Frankia* nodulation of *A. incana* (L.) Moench under freezing conditions results in major shifts in the nodule-forming *Frankia* populations, indicating that at least partially efficient nodulation under these extreme conditions is dependent on specifically adapted *Frankia* strains [107].

The potential of *Frankia* strains and actinorhizal symbiosis for the detoxification of organic hydrocarbons have also been evaluated. Strain QA3 isolated from *Alnus nitida* (Spach) Endl. was able to use naphthalene as a sole source of carbon and energy. Bioinformatic analysis of the genome identified a potential operon for aromatic compound degradation as well as several ring-hydroxylating dioxygenases [108]. Besides aromatic compounds, *Frankia*–alder symbiosis increases the mineralization of S-triazines. Therefore, *Frankia* is discussed as an underutilized tool for the phytoremediation of organic contaminations [109].

Eul1c involves surface binding and transport proteins CopA and CopCD transferring the metal out of the bacterium by forming unusual globular structures on the cell surface, which is strongly dependent on external phosphate (summarized in [57]). Pb-stressed *Frankia* sp. strain EAN1pec undergoes membrane surface modifications and shows binding of high Pb quantities at the cell surface by an upregulated **polyphosphate (polyP) kinase** [58]. Like other symbiotic interactions such as EMF and AMF symbioses, the role of actinorhizal symbioses in significantly enhancing the revegetation and phytoremediation of heavy-metal-contaminated sites has been well documented for alder trees [59]. However, for other actinorhizal symbioses, the mechanisms and extent of resistance remain unexplored.

As **salinization** becomes a major threat to soil fertility on a global scale [60,61], the use of actinorhizal plants is emerging as a viable option for the utilization and reclamation of saline land. Several *Frankia* strains are very tolerant to high-saline conditions, including isolates from *Casuarina* (e.g., strains Ccl6 and Allo2) that could withstand *in vitro* up to 1000 mM NaCl [62]. However, as for other abiotic stressors, the ability to tolerate high NaCl concentrations is strongly strain dependent. For example, the growth of *Frankia* strains isolated from *Elaeagnus macrophylla* (Thunb.) is already strongly inhibited at 100 mM NaCl [63]. Several studies have also highlighted the biochemical mechanisms responsible for the ability of *Frankia* to grow in high-saline environments. These mechanisms include the regulation of Na<sup>+</sup> influx/efflux and intracellular macronutrient content, such as K<sup>+</sup>, Mg<sup>2+</sup>, and Ca<sup>2+</sup> accumulation [63,64]. Recent studies with the highly salt-tolerant *Frankia* strain Hsli10, isolated from *Hippophae salicifolia* (D. Don), showed that its ability to grow at NaCl concentrations of up to 750 mM is linked to the modulation of several molecular, biochemical, and physiological mechanisms, including the upregulation of antioxidative enzyme activities such as **superoxide dismutase (SOD)**, **catalase (CAT)**, and **ascorbate peroxidase (APX)**, as well as a decline in the ratio of monounsaturated to polyunsaturated fatty acids in its membranes [65]. In addition, genetic differences between salt-tolerant and salt-sensitive *Frankia* strains isolated from *Casuarina* have been identified, including adaptations

at both the transcriptional and the translational level that mediate cell-envelope remodeling and the induction of novel, previously uncharacterized proteins [62]. Proteome comparison between nodulated and non-nodulated *Casuarina glauca* Sieb. ex Spreng. plants exposed to high NaCl levels revealed several **differentially expressed proteins (DEPs)**, mainly involved in carbohydrate metabolism and metabolic processes such as regulation of antioxidant status, as well as the biosynthesis of secondary metabolites [66] that may also be involved in antioxidative metabolism [67].

The ability of *Frankia* strains to survive and successfully nodulate actinorhizal plants under the earlier-described abiotic stress conditions has been proven to be beneficial for the ability of these plants to tolerate abiotic stresses under field conditions [47]. In highly degraded bauxite-mine soils in India, *C. equisetifolia* plantlets co-inoculated with *Frankia*, AMF, and *Phosphobacterium* showed 90–100% survival over control seedlings. Moreover, growth was significantly higher than for control seedlings and nutrient uptake (N, P, and K) was increased in inoculated plants [68]. Nodulation significantly enhanced the ability of alder plants to grow in the presence of heavy metals [59], resulting in significantly lower accumulation of toxic ions in the shoots compared with un-inoculated plants. Also, in a greenhouse experiment, *Frankia*-inoculated alder species were successfully used for reclamation of gold-mine waste rock, where actinorhizal plants improved soil quality by, for example, restoring soil pH [69].

### Specific benefits of actinorhizal N<sub>2</sub>-fixing plants

Recent studies demonstrated that inoculation with *Frankia* can improve the growth of *Casuarina* species and enhance the diversity of herbaceous vegetation in saline environments [70]. However, as in all bioreclamation applications, the success of the employed strategy is mainly dependent on the compatibility of the two symbiotic partners used, their mutual resistance characteristics towards the abiotic stress at hand, and their adaptation to the respective environment. Thus, future studies should be focused on the extensive exploitation of the natural biodiversity and the isolation of novel *Frankia* strains from extreme environments and in-depth characterization of the molecular and biochemical mechanisms governing the adaptation of these strains to various abiotic stresses and the performance of nodulation and N<sub>2</sub> fixation under these conditions.

Actinorhizal plants have rarely been used in practice for the regeneration and decontamination of degraded soils by phytoremediation approaches. Positive examples include *C. equisetifolia* [46] and the two alder species *Alnus viridis* (Chaix) DC. ssp. *crispa* and *Alnus incana* (L.) Moench ssp. *rugosa* [71]. The latter were used to revegetate impacted landscapes due to Canadian oil sand extraction procedures, which generate material that has high pH, high salt, and low nutrient residues and contains phytotoxic hydrocarbons. In these approaches, pre-inoculation of alder seedlings in greenhouses prior to their out-planting on affected reclamation sites significantly accelerated their growth and development.

The present literature survey shows (Table 1) that actinorhizal plants possess a high potential for applications in a broad range of polluted environments that cannot be covered by commonly used black locust. Successful application requires careful selection of the symbiosis partners (i.e., the plant species **provenance/ecotype** and the compatible *Frankia* strain). The capacity for N<sub>2</sub> fixation by actinorhizal plants may be somewhat lower and hence the duration required for soil restoration by phytoremediation slower than with N<sub>2</sub>-fixing legume trees such as black locust. However, actinorhizal plants may be particularly useful for soil restoration in extreme environments that do not allow the application of legume plants for phytoremediation, such as boreal **zonobiomes**. In addition, the diversity of plant species capable of actinorhizal symbiosis

will not only allow soil restoration under a broad range of environmental conditions, but will also increase the diversity of commercial products derived by such applications, ranging from wood with differing characteristics and quality (e.g., different *Alnus* or *Casuarina* species,) up to human food (e.g., fruits of sea buckthorn). Moreover, planting *A. viridis* accelerated the rate of succession by stimulating the growth of established *Salix alaxensis* (Andersson) Coville and *Populus balsamifera* L. due to increased BNF [72]. In a similar way, *Elaeagnus angustifolia* L. in mixed plantations with non-N<sub>2</sub>-fixing *Populus euphratica* Oliv. and *Ulmus pumila* L. improved soil fertility and afforestation as a sustainable land-use option for degraded croplands [73].

## Concluding remarks and future perspectives

As most natural and agricultural environments are under increasing pressure from climate change and human population growth, actinorhizal plants represent an interesting and important tool for the mitigation of negative effects on soil fertility by phytoremediation, in parallel to their well-established economic uses in local communities [49,74]. Thus, new opportunities and avenues arise from both ecological and economic perspectives for phytoremediation based on actinorhizal research that until recently has been focused mainly on studies of the molecular evolution of N<sub>2</sub>-fixation symbiosis rather than the molecular ecophysiology of stress compensation and tolerance (see Outstanding questions).

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## Declaration of interests

No interests are declared.

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## Outstanding questions

Which environmental and genetic plant and microbial features determine the efficiency of N<sub>2</sub> fixation in actinorhizal symbioses? How can they be characterized?

What is the quantitative significance of actinorhizal symbioses for the phytoremediation of heavy metals and organic pollutants in various zonobiomes under field conditions? How do legume species and actinorhizal plants differ in this context?

What is the qualitative benefit of tripartite symbioses (host plant–*Frankia*–AMF and/or –EMF mycorrhizal fungi) formed by actinorhizal plant species? Which mechanisms and processes of such tripartite symbioses mediate improved N and P nutrition and high tolerance to drought, salinity, flooding, and heavy-metal and organic pollution?

Which plant–microbial interactions mediate enhanced mobilization of soil P resources on N<sub>2</sub> fixation by actinorhizal symbiosis?

Which features of participating processes allow efficient N<sub>2</sub> fixation at close to 0°C (i.e., under permafrost climate conditions) by actinorhizal symbioses?

Do N<sub>2</sub>-fixing actinorhizal plants improve or inhibit their association with non-N<sub>2</sub>-fixing plants? How much N can be transferred to associated non-N<sub>2</sub>-fixing plants growing in the vicinity of N<sub>2</sub>-fixing actinorhizal plants? Which advanced approaches or techniques should be developed and applied to address these research questions?

How can we precisely evaluate the contribution of actinorhizal plants to soil restoration and phytoremediation as well as subsequent vegetation succession at the ecosystem and landscape level?

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