# UTILIZATION OF ROOT-COLONIZING FUNGI FOR IMPROVED PERFORMANCE OF AGRICULTURAL CROPS

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**Abstract**

Soil is a non-renewable natural resource which forms all ecosystems on the earth and provides the basis for food production for heterotrophic organisms, including men. The increase in the world population requires also an increase in agricultural production which was and is mainly achieved by massive use of mineral nutrients. However, the experience of the last century has demonstrated that the high mineral input has severe consequences for the ecosystems. An alternative more environmentally friendly strategy for agricultural production is provided by the nature itself: Beneficial root-colonizing fungi and bacteria have tremendous impact on the performance of agricultural plants (crops). Understanding of these symbioses requires knowledge about the communication between the partners. The microbes often release bioactive compounds into the rhizosphere which activate signaling or transport processes and thus promote plant performance. A general new concept for fertilizers in the agriculture could be to utilize microbe-derived bio-effectors in combination with appropriate nutrient supplies to promote biomass and yield production of agricultural plants while simultaneously reducing the input of agrochemicals. Here, we describe some concepts for the identification and utilization of microbial preparations and microbe-derived bio-effectors for the improvement of the performance of agricultural plants, using the root-colonizing endophytic fungus *Piriformospora indica*.

**Key words:** bio-fertilizer, microbial bio-effectors, *Piriformospora indica*, root-colonizing fungi

## 1. Introduction

Beneficial root-colonizing microorganisms and their metabolites are powerful tools to promote plant growth and performance, in particular under biotic and abiotic stress. The microorganisms form symbiotic/mutualistic interactions with the roots, propagate together with the roots in the rhizosphere and deliver bioactive compounds and nutrients to the host. In contrast to the living microbes, single bioactive compounds activate only a limited spectrum of plant responses and do not deliver nutrients to the roots. However, for agricultural application with the goal of stimulating biomass production and resistance against pests, the combination of root-colonizing microbes, microbe-derived bio-effectors and sufficient nutrient supply has huge advantages compared to the application of any of these compounds alone. Applicable bio-effectors have to fulfill a number of criteria: their isolation/production should be cost effective, they should be stable under different conditions (heat, cold, pH, drought, pests, diseases etc.) and highly effective for a larger host spectrum. Microbe-derived bio-effectors activate plant signaling pathways in the root cells, which ultimately promote growth and biomass production, or confers resistance against biotic and abiotic stress. Depending on the specificity, nature and origin of these bio-effectors, their application may be restricted, e.g. to specific plant species (such as nod factors for legumes). Therefore, the use of bio-effectors of root-colonizing microbes which have a wide host range is desirable. These microbe-derived effectors should activate general and not host-specific processes in plant cells.

Among the downstream signaling events in the roots, plant components realize programs which are induced by the microbial bio-effector(s). This often starts with the activation of a (membrane-associated) receptor, followed by downstream signaling events including phosphorylation and calcium changes. Typical examples of those processes provide mycorrhizal systems, for which a lot of components of the signaling pathway have been elucidated in the past.

A central player downstream of receptor activation in beneficial plant/microbe interactions is $Ca^{2+}$ [9, 31]. In mycorrhizal interactions, the phosphorylation cascade at the plasma membrane induces $Ca^{2+}$ changes in the cytoplasm and nucleus.

This probably involves a secondary messenger that might be the product of phospholipases C and D. These phospholipases could be regulated by phosphorylation and the activity of the cation channel formed by DMI1/POLLUX and CASTOR [1, 10]. The $Ca^{2+}$ spikes in the nucleoplasm and nuclear-associated cytoplasm activate a $Ca^{2+}$/calmodulin-dependent kinase (CCaMK), which is located in the nucleus [20, 17, 12]. This $Ca^{2+}$-activated kinase regulates nodulation-induced (nodulin) gene expression *via* the transcriptional regulators NSP1 and NSP2 [12, 28], GRAS proteins as well as ERN, an ERF transcription factor. Thus, $Ca^{2+}$ signaling starts at the plasma membrane and might end in the nucleus with the activation of specific transcription factors leading to a beneficial symbiosis between the symbionts. The involvement of $Ca^{2+}$ spiking and $Ca^{2+}$/calmodulin-dependent protein kinases in beneficial plant/microbe interactions appear to be a general phenomenon [17, 22, 10, 11, 32]. Ivashuta et al. [11] used RNA-interference-mediated knockdown of CDPK1 in Medicago and demonstrated that the plants have defects in root development and do not form mycorrhiza. Their actin skeleton is disrupted and defense genes become activated.

Downstream signaling programs initiated by microbe-derived bio-effectors also include phytohormones and components which promote cell division or elongation, cell wall biosynthesis genes etc., and processes which transfer the "beneficial" information from the roots to the shoots (e.g. through the induced systemic resistance, ISR). ISR is a state of enhanced defense capacity of a plant. ISR is the result of colonization of the roots by beneficial microorganisms, such as plant-growth promoting rhizobacteria, mycorrhizal fungi or beneficial endophytes [36]. The root cells recognize microbe-associated molecular patterns (MAMPs), i.e. small biomolecules from the microbes, which induce an activation of the plant immune responses in systemic manners. ISR is characterized by the fact that it confer a broad spectrum resistance in systemic tissues against many types of plant pathogens, including viruses, bacteria, fungi, and oomycetes and in some cases even insect herbivores. The signaling events from the roots to the leaves also include phytohormones, such as ethylene and jasmonate [35].

Limitations in either microbe-derived bio-effectors or endogenous plant signal components restrict plant performance. Finally, realization of the growth-promoting programs is only successful if nutrients are not limiting. They can be delivered to the roots *via* the hyphae of beneficial fungi, but also *via* microbial preparations, or activation of uptake systems by microbe-derived bio-effectors.

Figure 1 summarizes our three-step concept, which needs to be considered during improvement of agricultural plant species with fungal/microbial tools.

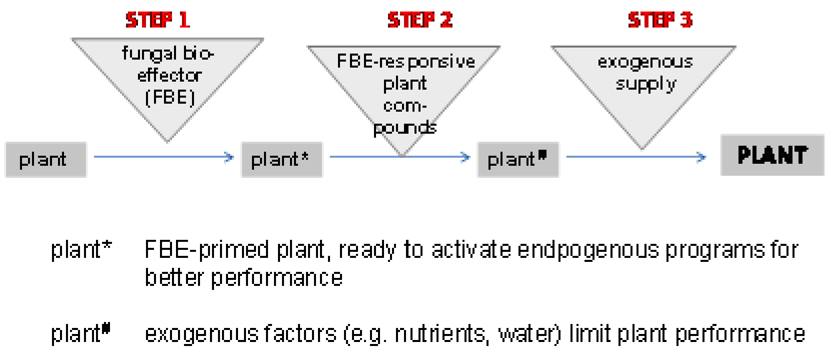


**Figure 1**: Scheme describing the needs for the improvement of agricultural plant species with fungal/microbial tools.

Microbe-derived bio-effectors activate (early) plant signaling events. Therefore, the identification, purification and chemically synthesis of novel bio-effectors which promote plant performance (biomass production and resistance against pathogens) is an essential task for the future. They should be combined with microbial extracts or microbes because they contain additional bio-effectors and provide optimal macro- and micronutrient combinations for the plants. The new bio-effectors can be applied to biofertilizers which are already commercialized with the goal to reduce the overall amount of agrochemicals.

Realization of programs beneficial for the plants is only successful if nutrients and water are not limited. The next goal in the design of optimal fertilizers with low chemical input is to supply sufficient nutrients for growth. With this respect, it is important to have the adequate combination of the nutrients in stoichiometric relations. To realize this concept, commercially available chemical biofertilizers can be used as a basis. The agrochemicals can be reduced to amounts which are absolutely required for optimal plant growth and performance, and supplemented with microbes, microbial preparations and microbe-derived bio-effectors.

## 2. A source of new bio-effectors: e.g. Sebacinales

The effects of arbuscular mycorrhizal fungi (AMF) and plant-growth promoting rhizobacteria on the performance of plants, in particular crop plants have been summarized in many review articles [cf. 29, 2] and will not be repeated here. Here, we focus on the identification of bio-effectors from the well studied fungus *Piriformospora indica* with a large host range, which avoids host-specific effects.

Like many other members of Sebacinales, such as *Sebacina vermifera*, *Piriformospora indica* is a wide-host root-colonizing endophytic fungus, which promotes plant growth and performance, in particular under physical, biological and nutrient stress [4, 21]. The fungus can be easily cultivated on complex and minimal substrates. It produces spores, which germinate and can grow in a large (extreme) temperature, humidity and pH range. Cultivation conditions have been established in different laboratories around the world, including large-scale fermention techniques with biomass production of up to 500 litre. *P. indica* belongs to the Sebacinales in Basidiomycota. Together with other beneficial members of this family, such as *Sebacina vermifera*, *P. indica* has a vast geographical distribution and has been reported from Asia, Europe, South America and Australia.

*P. indica* colonizes the roots of all tested plant species (> 140), among them are the major species with economic importance in agriculture (e.g. barley, wheat, rice, tomato, corn, sugar beet, potato, tomato, sorghum, Chinese cabbage, *Brassica napus, etc.*). The entire genome of *P. indica* has been sequenced and the sequence is publically available since 2011 [38, 39]. Several cDNA libraries of the fungus, grown under different conditions, are available. More importantly, two groups have established efficient transformation systems [38, 37] with which RNAi and overexpression cassettes can be introduced into *P. indica* [37, 14]. They have been used to manipulate macronutrient uptake, e.g. phosphate [13, 15]. The use of *P. indica* as a growth- and biomass-stimulating fungus in agriculture, in particular in nutrient-deficient soils, as well as its effect on early flowering, enhanced seed production, stimulation of active ingredients such as medicinal-relevant metabolites and hardening of tissue-culture-raised plants is interesting because of (a) the absence of any obvious host specificity. (b) A single infection processes (such as imbedding seeds into a fungal medium or infection of roots prior to transfer of seedlings to soil) is sufficient to allow efficient root colonization and propagation of the fungal hyphae in a growing root environment. (c) The fungus survives under quite different agricultural-important (soil and environmental) conditions, such as cold (< 0°C), extreme heat (45°C), salt- and heavy metal-polluted soils, and soil with various pH values (3.3-9.2). (d) It promotes micro (e.g. Fe)- and macro (such as P, S and N)- nutrient uptake from the soil. (e) The fungus confers resistance to root and leaf pathogen infections by activation root-to-shoot signaling events similar to those known as ISR. Increased insect and nematode resistance has also been observed for *P. indica*-colonized plants. (f) Stimulation of plant growth and biomass can also be achieved by an exudate component [32, 16]. The compound has been purified, is water-soluble and heat-stable, but only synthesized in extremely low amounts by the fungus. Taken together, *P. indica* (compounds) can be used for improving the performance of agricultural plants under quite different environmental conditions.

Besides stimulation of plant biomass production, laboratory studies have particularly focused on the role of *P. indica* in phosphate (**P**), nitrate (**N**), sulfur (**S**) and iron (**Fe)** mobilization for plants, for improving drought resistance, and for conferring resistance against leaf and root pathogens.

(**P**): Yadav et al. [37] and Kumar et al. [13] have shown that the fungal P transporter PiPT is actively involved in the P transportation to maize plants and, in turn, *P. indica* helps improve the nutritional status of the host plant and this is required for growth promotion and higher biomass/seed yield. Also, the growth promoting effect of *P. indica* on barley, wheat and tomato is dramatically stimulated under P limitations. Therefore, this fungus is particularly interesting for the application on soil with P-limitations.

(**N**): A long-known targets of *P. indica* is the nitrate assimilation [24]. This demonstrates the potential of the fungus to promote nitrate uptake and metabolism in crop plants, especially under N limitations.

(**S**): Maruyama-Nakashita et al. [19] identified Sulfur Limitation1 (SLIM1) as a central regulator of plant S responses and metabolism in Arabidopsis. *Slim1* is also a target of *P. indica* in Arabidopsis. Sulfate uptake and plant growth under S starvation were significantly reduced in *slim1* and SLIM1 controlled both the activation of sulfate acquisition and degradation of glucosinolates under S limitations. *P. indica* also establishes a reduced atmosphere in the cells [3, 31, 14] which is directly linked to sufficient S supply. Furthermore, SLIM1 is required for ISR in Arabidopsis. This suggests that the *P. indica* target SLIM1 regulates important aspects of the plant S metabolism, the redox status in the cell and the ISR which primes the aerial parts to respond more efficiently to pathogen attack.

(**Fe**): Higher plant sulfite and nitrite reductases contain the Fe-harbouring siroheme as essential prosthetic group [6, 7, 18]. Thus, assimilation of all inorganic S and the majority of N in the biosphere depend on the availability of siroheme and without it, there would be no life on earth. The two enzymes uroporphyrinogen III methyltransferase and sirohydrochlorin ferrochelatase which mediate siroheme biosynthesis [23] as well as the nitrite and sulphite reductases are targets of *P. indica* under Fe limitations (Sun et al. submitted). This demonstrates that *P. indica* regulates key proteins controlling S and N metabolism *via* Fe (Fig. 2) and siroheme.

The fungus plays an important role for survival of the seedlings under Fe limitations.

(**Drought tolerance**): *P. indica* confers drought tolerance to leaves of colonized plants. When drought-exposed seedlings are transferred to soil, seedlings colonized by *P. indica* perform much better than the uncolonized controls [25, 30]. The same effect can be achieved with a *P. indica*-derived exudate preparation, suggesting that fungal components are sufficient to protect leaves against drought stress. Since drought is a severe problem in many agricultural areas, both the living fungus as well as exudates from the fungus may be a powerful tool to confer drought tolerance in leaves.

(**Resistance against leaf and root pathogens**): *P. indica* confers resistance against root and leaf pathogens. The resistance in the roots is mainly caused by competition between the beneficial and pathogenic microbes. Therefore, *P. indica* is a good candidate for a biocontrol agent against root pathogens in agriculture. In the leaves, an ISR-type signaling pathway from the roots primes defense processes and therefore promotes resistance against pathogens. Although the mechanism is unknown, *P. indica* can also be used in agricultural plants to protect the aerial parts against pathogen infections.

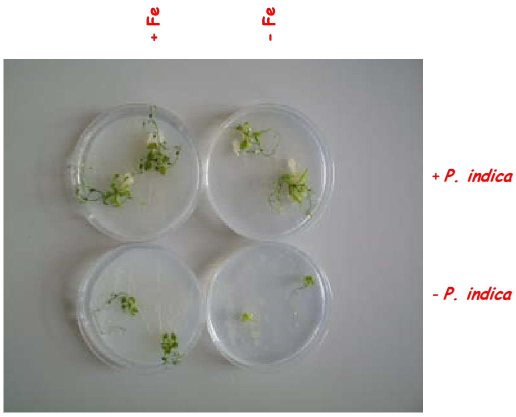


**Figure 2**: Arabidopsis seedlings grown on PNM medium with/without Fe and with/without P. indica.

*P. indica* contains bio-effector(s) released into the medium and into its cell wall which is/are sufficient to trigger plant growth. The large host range of the fungus and the observation that exudates and lyophilized *P. indica* fractions are capable of promoting growth of many different plant species makes this fungus an ideal source for the isolation of bioactive fractions, which can be used on a wide range of agriculturally important plant species. We have recently identified a *P. indica*-derived growth-promoting compound (Fig. 3) that is heat, acid, basis and pH stable, and - besides growth promotion – confers other benefits to the plants.

### 3. Proposal for new fertilizers with low agrochemical inputs

As shown in Figure 4, commercially available biofertilizers can be used as a basis for optimizing their functions and for reducing the amount of agrochemicals. They can be complemented with beneficial fungi and/or plant-growth promoting bacteria, for instance as hyphae or spores. In many cases it is also possible to use exudates and/or lyophylates from these microbes, because they contain sufficient nutrients and also (so far unidentified) bio-effectors. Finally, if bio-effectors are available and successfully tested in laboratory studies, they can be chemically synthesized in huge amounts for agricultural applications, by adding them to

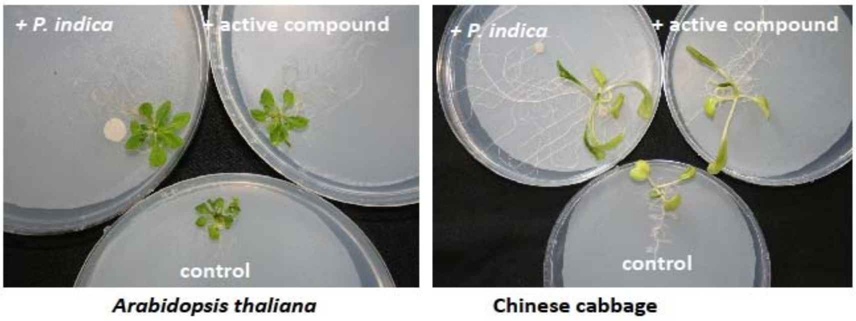


**Figure 3:** A growth-promoting compound from P. indica stimulates growth of Arabidopsis (left) and Chinese cabbage (right) seedlings.

biofertilizers or formulations via the seed coat technique. In particular, analogs of these compounds which are more effective than the bio-effectors can be synthesized and tested. This combinatory strategy provides a novel concept for optimizing available biofertilizers or formulations.

The beneficial function of the new products has to be first tested in laboratory experiments, followed by field experiments with important crop plants. It is also required to test them under different agriculturally important field conditions, e.g. in different geographic areas with different climates. A cost/benefit analysis is required to compare the production costs of the new products with those of the conventional products available on the market.

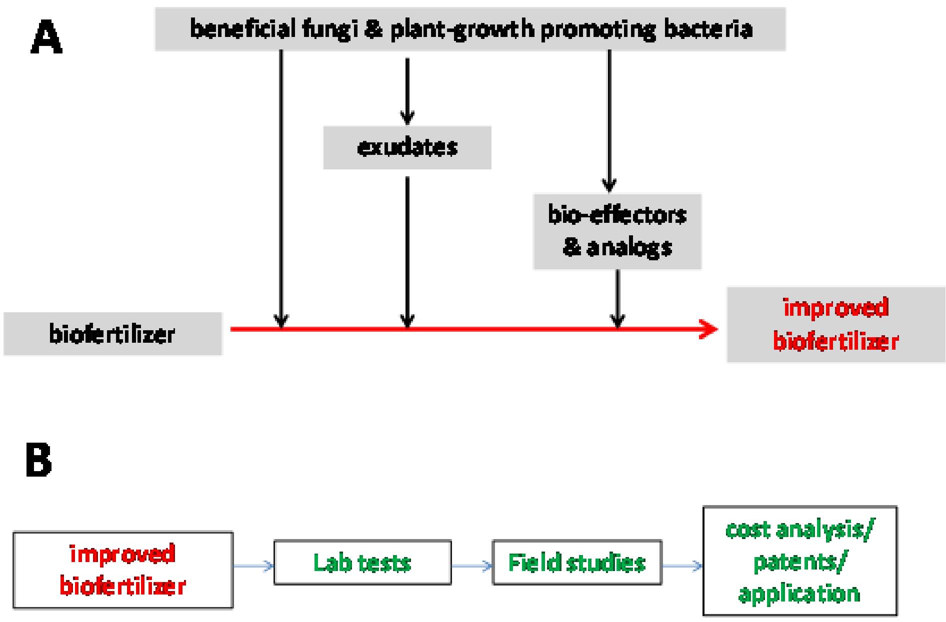


**Figure 4**: Beneficial fungi and/or plant-growth promoting bacteria can be used to improve commercially available biofertilizers (A). The beneficial function of the new products has to be first tested in laboratory experiments, followed by field experiments with important crop plants (B).

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