NANO-FERTILIZERS

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Strategic role of nanotechnology for fertilizers: potential and limitations

The ability of people to construct and manipulate materials at nano-scale has increased tremendously during the last decade building the fundamentals of the interdisciplinary science nanotechnology. Nanomaterials behave differently than the same material at non-nano scale; they have high surface area to volume ratio, high solubility, and specific targeting due to small size, high mobility, and low toxicity. They can be engineered for surface reactivity or other desired characteristics - unique behavior that can be both useful and profitable. As of March 2011, over 1300 commercially available products contain nanomaterials. Nanotechnology was a $1 trillion industry in 2015.

According to the National Nanotechnology Initiative (NNI) (https://www.nano.gov/about-nni), *"Nanotechnology research and development is directed towards understanding and creating improved materials, devices and systems that exploit nanoscale properties"*. Following the definition of Royal Society, *"Nanotechnologies are the design, characterization, production and application of structures, devices and systems by controlling shape and size at nanometer scale"*.

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Recently nanotechnology has emerged as the sixth revolutionary technology after the green revolution of the 1960s and the biotechnology revolution of the 1990s. Nanotechnology is a novel scientific approach that involves the use of materials and equipment capable of manipulating physical and chemical properties of a substance at molecular levels. It merges science and technology leading to revolutionary breakthrough in electronics, energy, remediation, automobile, space technology, and life sciences. The potential uses and benefits of nanotechnology are enormous. Nowadays, nanotechnology is progressively moved away from the experimental into the practical areas. Among others, it promises significant contribution to agricultural research in solving important agricultural problems, such as detection of pollutants, plant diseases, pests, and pathogens; controlled delivery of pesticide, fertilizers, nutrients, and genetic material; formation and binding of soil structure. Today, when agricultural scientists are facing major challenges such as reduced crop production, nutrient deficiency and climate change, nanotechnology has offered promising applications for precision farming. This innovative technology embraces wide applications such as plant disease control, enhanced nutrient uptake, improved plant growth and sustained release of agrochemicals. Interestingly, a nanoparticle (NP)-based strategy has gained momentum and become increasingly popular in the agricultural sector as a result of its unique properties compared with that of the biopesticides. The application of nanotechnology to agriculture (the so called agri-nanotechnology, Fig. 1) is getting significant attention, primary in the following several categories:

- Increase production rates and yield;
- Increase efficiency of resource utilization;
- Minimize waste production;
- Specific applications that include nano-fertilizers and nano-pesticides;
- Nano-based treatment of agricultural waste;
- Nano-sensors.

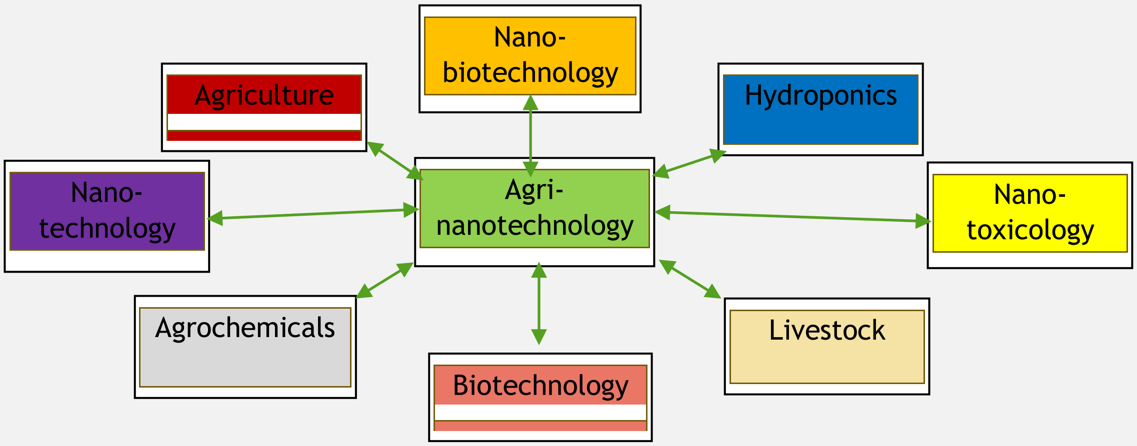

Fig. 1. Multidisciplinary nature of agri-nanotechnology.

Currently, nanotechnology potential in sustainable agriculture management is clearly recognized. It occupies a prominent position in transforming agriculture and food production. The development of nano-devices and nanomaterials could forward novel applications in plant biotechnology and agriculture. Thus, the development of slow/controlled release fertilizers on the basis of nanotechnology has now become crucial for promoting the development of environment friendly and sustainable agriculture. Applying nanoscale or nanostructured materials as fertilizer carriers leads to the development of the so-called "smart fertilizer" - new facilities that enhance nutrient use efficiency and reduce costs of environmental protection.

Nano-fertilizers *vs.* conventional fertilizers - formulation and delivery of nano-fertilizers

Outburst of world population in the last 10 – 15 years has imposed the necessity for higher agriculture productivity to satisfy the food needs of billions of people. The increasing nutrient deficiency in soils causes significant economic losses for farmers on the one hand and considerable decreases in nutritional quality of grain for food and feed. The crop productivity can be enhanced through application of fertilizers, although they have an additional role in enhancing the food production especially after the introduction of high yielding and fertilizer responsive crop varieties. Conventional fertilizers are generally applied on the crops by either spraying or broadcasting. An important factor, on which the mode of application depends, is the real final concentration of the fertilizers in the plants. Conventional fertilizers offer nutrients in chemical forms that are not fully accessible to plants. Additionally, the inversion of these chemicals to insoluble form in soil is the reason for the very low utilization of most of the macronutrients. A concentration much below the minimal desired one reaches to the targeted site due to leaching of chemicals, drift, runoff, evaporation, hydrolysis by soil moisture, and photolytic and microbial degradation. It has been estimated that around 40–70 % of nitrogen, 80–90 % of phosphorus, and 50–90 % of potassium content of applied fertilizers are lost in the environment and never reach the plant. These problems superimpose repeated use of fertilizers. According to the International Fertilizer Industry Association, world fertilizer consumption sharply picked up in 2009–2010 and 2010–2011 with growth rates of 5–6 %. World demand is estimated to reach 192.8 Mt by 2016–2017. The repeated use on its turn adversely affects the inherent nutrient balance of the soil and results in environmental pollution affecting normal flora and fauna. It is reported that excess use of fertilizers increases pathogen and pest resistance, reduces soil microflora, diminishes nitrogen fixation, contributes to bioaccumulation of pesticides, and destroys habitats for birds. This vicious circle causes sustainable and economic losses.

It is well known that yields of many crops have begun to drop down as a result of imbalanced fertilization and decrease in soil organic matter. Moreover, excessive applications of nitrogen and phosphorus fertilizers affect the groundwater and also lead to eutrophication in aquatic ecosystems. The remaining minerals may either leach down and/or leak and become fixed in soil or contribute to air pollution. Considering these facts, the large-scale application of chemical fertilizers to increase the crop productivity is not an acceptable option for sustainability. Especially in a long term perspective, although the conventional fertilizers increase the crop production they disturb the soil mineral balance and decrease soil fertility. In addition to the irreparable damage that the excess use of chemical fertilizers causes to the soil structure and mineral cycles, it spoils the soil microflora, plants, and consequently - the food chains across ecosystems leading to heritable mutations in future generations of consumers. Thus, there is an urgent need to optimize the use of chemical fertilization to fulfill the crop nutrient requirements and to minimize the risk of environmental pollution. Accordingly, it is very important to develop smart materials that can systematically release chemicals to specific targeted sites in plants which could be beneficial in controlling nutrition deficiency in agriculture, while keeping the natural soil structure and contributing to clean environment. The nano-fertilizers are promising alternative in this context.

A nano-fertilizer refers to a product in nanometer scale that delivers nutrients to crops. Nano-fertilizer technology is recent innovation. Substituting traditional methods of fertilizer application by nano-fertilizers is an approach to release nutrients into the soil both gradually and in a controlled way. Nano-fertilizers show controlled release of agrochemicals through site targeted delivery, reduction in toxicity, and enhanced nutrient utilization of delivered fertilizers. They possess unique features that enhance plants’ performance in terms of ultrahigh absorption, increase in production, rise in photosynthesis, and significant expansion in the leaves’ surface area. Besides, the controlled release of nutrients contributes to preventing eutrophication and pollution of water resources.

In nano-fertilizers, nutrients can be encapsulated by nanomaterials, coated with a thin protective film, or delivered as emulsions or nanoparticles. There are many throughput examples of nano-fertilizers application. Thus, treatment with TiO_2 nanoparticles on maize had a considerable effect on growth, whereas the effect of TiO_2 bulk treatment was negligible. Titanium nanoparticles increased light absorption and photo energy transmission. In another experiment, a compound of SiO_2 and TiO_2 nanoparticles increased the activity of nitrate reductase in soybeans and intensified plant absorption capacity, making its use of water and fertilizer more efficient. Nano-organic iron-chelated fertilizer is proved to be environmentally sustainable. The positive effect from the uptake and penetration of ZnO_2 nanoparticles on tomato plants leaves supports its potential use as a future nano-fertilizer. Nano-fertilizers that ensure slow, targeted, efficient release have the potential to increase the efficiency of nutrient uptake. Engineered nano-particles are useful for mitigating the chronic problem of moisture retention in arid soils and enhancing crop production by increasing the availability of nutrients in the rhizosphere. Coating and binding of nano-particles help to regulate the release of nutrients from the fertilizer capsule. Application of a nano-composite consisting of nitrogen, phosphorus, potassium, micronutrients, mannose, and amino acids enhanced the uptake and use of nutrients by grain crops. Zn–Al layered double-hydroxide nano-composites have been employed for the controlled release of chemical compounds that act as plant growth regulators. Nano-porous zeolite based on nitrogen fertilizer can be used as alternate strategy to improve the efficiency of nitrogen use in crop production systems. As super-fertilizer, carbon nanotubes were found to penetrate tomato seeds and affect their germination and growth rates. Analytical methods indicated that the carbon nanotubes penetrated the thick seed coat and supported water uptake inside seeds.

These facts support the statement that fertilizers based on nanotechnology have the potential to surpass conventional fertilizers following several important indices (as showed in Table 1).

Table 1. Conventional fertilizers vs. nano-fertilizers

Index	Nano-fertilizer	Conventional fertilizer
Solubility	High	Low
Dispersion of mineral micronutrients	Improved dispersion of insoluble nutrients	Lower solubility due to large particle size
Soil adsorption and fixation	Reduced	High
Bioavailability	High	Low
Efficiency of nutrients' uptake	Increased uptake ratio; saves fertilizer resource	Conventional fertilizer is not available to roots and nutrients' uptake efficiency is low
Controlled release	Release rate and pattern precisely controlled	Excess release leading to toxicity and soil imbalance
Effective duration of release	Extended effective duration	Used by the plant at the site and time of application; the rest is converted in insoluble form
Loss rate	Reduced loss of fertilizer nutrients	High loss rate due to leachi8ng, drifting, run off

The nano-fertilizers should be formulated in a way that they retain important properties such as high solubility, stability, effectiveness, time-controlled release, enhanced targeted activity with effective concentration, and less eco-toxicity due to the safe, easy mode of delivery and disposal.

A great potential in targeted delivery of nutrients to living systems possess the nanoparticles. They can be loaded by nutrients most commonly through one of the following ways:

- absorption on the nanoparticles;
- attachment on the nanoparticles mediated by ligands;
- encapsulation in nanoparticulate polymeric shell;
- entrapment in nanoparticles.

Thus, it has been shown that chitosan nanoparticles suspensions containing N, P, and K fertilizers can be useful for agricultural applications. Similarly, urea-modified hydroxyapatite (HA) nanoparticles are exploited for slow and sustained release of nitrogen over time with the crop growth. The large surface area of HA facilitates the large amount of urea attachment on the HA surface and the strong interaction between HA nanoparticles and urea contributes to the slow and controlled release of urea. Polymer-based mesoporous nanoparticles can also provide efficient carrier system to agrochemical compounds. Mesoporous silica nanoparticles (150 nm) have been reported to entrap urea and to release it in a controlled manner in soil and water.

The efficiency of the nano-fertilizers and their impact on plant systems is influenced by the method of their application. The nano-fertilizers' delivery to plants can be realized through the listed below methods. The approaches include either *in vitro* or *in vivo* application, as shown in Table 2.

Table 2. Modes of nano-fertilizers' application

***In vitro* methods**	***In vivo* methods**
Aeroponics: ➢ Principle: the technique, first reported in 1992, consists of continuously spraying of a nutrient solution on suspended in air roots; ➢ Advantages: the technique allows strict control of the gaseous environment around the roots; ➢ Disadvantages: the techniques requires a high level of nutrients to sustain rapid plant growth, thus its application is restricted.	**Soil Application:** ➢ Principle: direct delivery to soli; ➢ Requirements: careful choose of the persistent time of the fertilizer in the soil; special attention to the soil texture, salinity, plant sensitivities to salts, and pH of the amendment. Negative soil particles affect the adsorption of mineral nutrients. The anion exchange capacity of most agricultural soils is small compared to cation one. Among anions, NO_3^- remains mobile in the soil solution and is susceptible to leaching by water, PO_4^{3-} binds to soil particles containing Al or Fe because the positively charged $Fe^{2+}/^{3+}$ and Al^{3+} exchanges OH^- group with phosphates, resulting in tightly bounding of the latter, which mobility and availability in soil can limit plant growth. ➢ Advantages: the most common method of nutrient supplement using chemical and organic fertilizers.
Hydroponics: ➢ Principle: the plants are grown with their roots immersed in a liquid nutrient solution (without soil), introduced in 1937 for dissolved inorganic salts, known as well as the so called "solution culture"; ➢ Requirements: careful choose of the volumes of nutrient solution, maintenance of oxygen demands and pH. ➢ Advantages: application of supporting materials (e.g. sand) that allow nutrient solution to be flushed from one end and old solution to be removed from the other end. ➢ Disadvantages: frequent pathogen attack and high moisture rates which may cause over wilting of soil-based plants.	**Foliar Application** ➢ Principle: liquid fertilizers are directly sprayed onto leaves, generally used for the supply of trace elements; ➢ Advantages: reduces the time lag between application and uptake by plant during the rapid growth phase; circumvent the problem of restricted uptake of a nutrient from soil; agronomic advantage of foliar application since stomata and leaf epidermal cells are majorly involved in nutrient uptake ➢ Disadvantages: further needs for standardization of application protocol to avoid damage to the leaves; need of specific time (morning and evening) of spraying because the stomata open during these time periods only; possibility of plant damage if incorrect concentration of fertilizer is applied.

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Technology expansion has improved ways for large-scale production of nanoparticles of physiologically important metals, which are now used as **"smart delivery systems"** in order to improve fertilizer formulation by minimizing nutrient loss and increasing the uptake in plant cell. "Smart delivery system" means combination of specifically targeted, highly controlled, remotely regulated, and multifunctional characteristic to avoid biological barriers for successful targeting. The specific properties of nano-fertilizers, i.e. their high surface area, sorption capacity, and controlled-release kinetics to targeted sites, attribute them as smart delivery system.

Smart fertilizers are becoming reality through transformed formulation of conventional products using nanotechnology. The nanostructured formulation allows a fertilizer to intelligently control the release speed of nutrients in order to match the uptake pattern of a specific crop. It improves solubility and dispersion of insoluble nutrients in soil, reduces soil absorption and fixation and increases the bioavailability, hence the nutrient uptake efficiency.

Biosynthesis of nanoparticles my microorganisms

Mediated synthesis of metal nanoparticles by microorganisms

Recently**,** the use of biological entities has emerged as a novel method for the synthesis of nanoparticles. Biotechnological way for the synthesis of nanoparticles possess many advantages, such as use of known microbial technologies and processes for scale up the obtaining of biomass. This is leading to economic viability, possibility of readily covering large surface areas by suitable growth of the microbes, which is of major advantage in the field of agriculture for easier production of bio-fertilizers.

The disadvantages of the convention methods for obtaining of metal nanoparticles like high energy and cost fabrication demands, as well as toxic by-products production makes the implementation of such approaches at large scale very complicated. Using of microbial cell factories like bacteria, fungi, algae, viruses and actinomycetes provide a smart alternative way of synthesising metallic nanoparticles. The biosynthesis of metallic nanoparticles in these microorganisms is a costly and eco friendly technology. The use of broad number of microorganisms belonging to prokaryotic as well as eukaryotic types takes part in the synthesis of long range of metal nanoparticles as gold (Au), silver (Ag), lead (Pb), platinum (Pt), copper (Cu), iron (Fe), cadmium (Cd) and metal oxides such as titanium oxide (TiO), zinc oxide (ZnO), etc. These microorganisms represent a varied ambience for the nanoparticles production. The nanoparticles produced are highly useful, safe and environmental friendly in nature with a lot of applications ((Syed, PhD Thesis). In agriculture, the most used nanoparticles as bioeffectors are coper (Cu), iron (Fe), silver (Ag), gold (Au). The future challenges in this respect comprise optimal biosynthesis of nanoparticles with defined size and shape as well as optimal duration of the fermentation process in order to enhance their stability.

Microbiological synthesis is a new approach for manufacture of nanoparticles and realization of the so called bio-nanofactories. The major characteristics of nanoparticles are revealed by the researchers, who prepared nanoparticles of desirable shape and size.

The principal flow chart for microbiological synthesis of metallic nanoparticles is presented in Fig. 2.

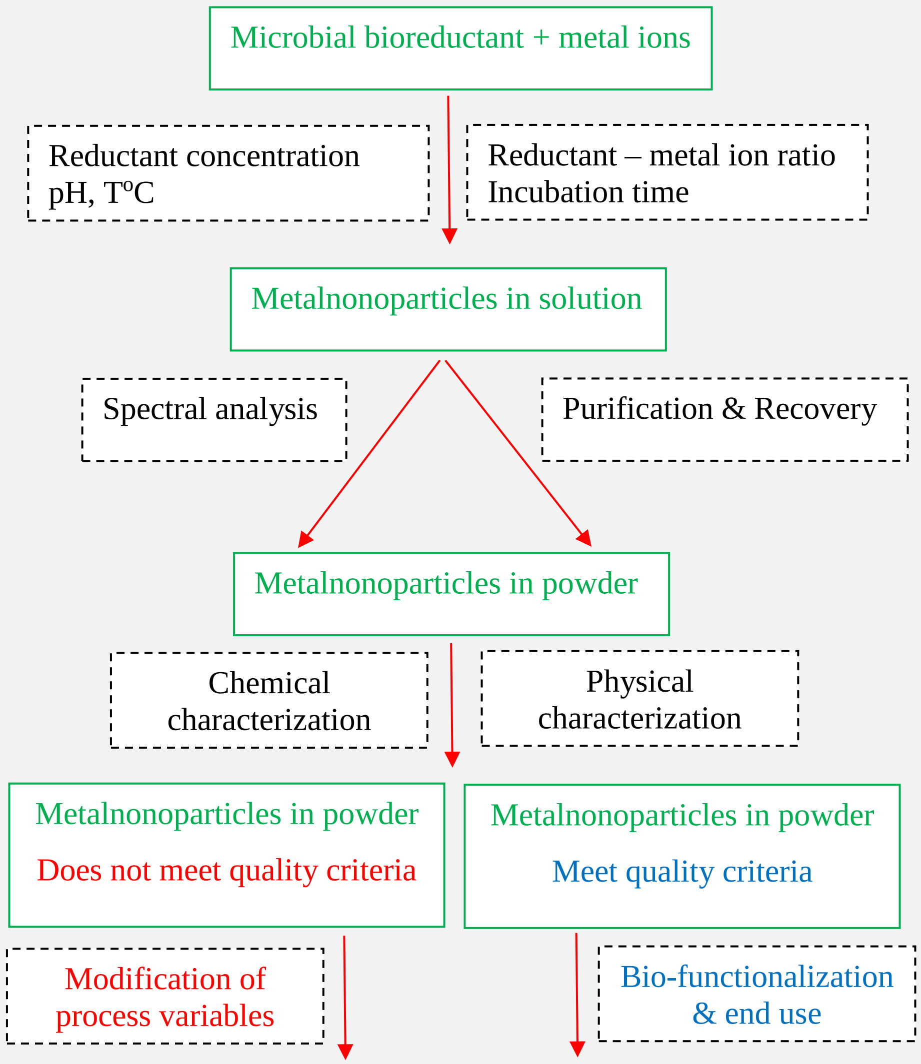

Fig.2. Principal flow chart for microbiological synthesis of metallic nanoparticles

The following important parameters play a significant role in biosynthesis of nanoparticles.

1 Bioresources used for nanoparticles biosynthesis: The synthesis of nanoparticles is characterized by choice of the most convenient microorganism in respect to: growth rate, enzyme production and the respective metabolic pathways. Some of the microorganisms like bacteria, viruses, fungi, yeasts and algae are used for the biosynthesis of metallic nanoparticles and are an object of specific research.

2. Cellular metabolites involved in biosynthesis: molecules like enzymes, proteins, polysaccharides etc. are acting as a reducing and stabilizing agents in the biosynthesis of nanoparticles. They can be utilized in the process as a whole cells of microorganisms, crude cell preparations, and crude or purified enzymes obtained from the microorganisms. The obtained nanoparticles are resulted mainly from bioreduction, which is realized by co-enzymes such as NADH, NADPH, FAD, etc. It is found that nanoparticles synthesis with the help of whole cell of fungi is much cheaper as compare to purified enzymes from the same fungus strain (Syed, PhD Thesis).

3. Reactions facilitating nanoparticles biosynthesis: the process of this biosynthesis is initiated with harvesting of microbial biomass, which is related with residual nutrients and metabolites to avoid wrong by product reactions. During the processes of scalling up the production rate and product yield are of special interest and optimization is necessary (e.g. production time, pH, temperature etc.). The process of optimization of these factors can influence the particles morphology and their properties. Thus, currently researchers have directed their investigations on arranging the optimal reaction conditions as well as the equipment used in the bioreduction process (Syed, PhD Thesis).

4 Growth of inoculum for biosynthesis of nanoparticles: biosynthesis of nanoparticles depends on growth conditions of microorganisms-producers like: nutrients, pH, temperature, etc. These factors need to be optimized. They are also important in case of using whole cells and crude enzymes. Another important parameter for optimization of the inoculums is the harvesting time, so that it is necessary to monitor the enzyme activities during the time course of growth (Syed, PhD Thesis).

Microbial nanoformulations: exploring potential for nano-farming

Nanoparticles, synthesized by microbes are highly stable and could offer a non-toxic, cost-effective and eco-friendly approach for synthesis over chemical ones. This green synthesis has a great advantage over the chemical methods, causing toxic effect on environment. Thus, the use of agriculturally important microorganisms for nanoparticles biosynthesis and their further role in agriculture is of substantial significance. The use of nanoformulations may enhance the stability of bio-fertilizers and bio-stimulators with respect to desiccation, heat, and UV inactivation.

Nano-fertilizers uptake, translocation, and fate in plants

The uptake and fate of nano-fertilizers in plant is an emerging field of research interest. The uptake, translocation, and accumulation of nanoparticles depend on the plant itself, more specifically on the plant species, age, and growth environment. Also these processes are linked to the physicochemical properties, functionalization, stability, and mode of delivery of the nanoparticles. A schematic representation of the uptake, translocation, and biotransformation pathway of various nanoparticles is proposed by Rico et al. (2011) along with possible modes of cellular uptake in the plant system. According to this presentation the root system uptakes and translocates to the foliar part of a plant, regardless it species appurtenance, ZnO^{2+}, Cu^{2+}, Al^{3+}, Ag^{2+} and Fe_3O_4 Nano-Particle (NP). In addition, indicatives for species dependence are available for translocation of Cu NP, ZnO NP, Al NP, Ag NP, (all in leaves), $Ni(OH)_2$ NP in the stem, and CeO_2 NP in both stem and leaves. A translocation of the Fe_3O_4 NP in the stem is also speculated.

The probable differential nanoparticle interaction on exposure in the root absorption zone can be summarized in Table 3.

Table 3. Localization and interaction of different nano-particles in the root absorption zone.

Nano-particle	Localization / interaction
Fe_3O_4 NP	Cambium
ZnO NP	Endodermis, metaxylem; Zn^{2+} - in the metaxylem
CeO_2 NP	Cortex
Al NP	Cortex Al^{3+} - in the metaxylem
Ag NP	Cortex; Ag^{2+} - in the metaxylem
Cu NP	Cortex; Cu^{2+} - in the cambium and metaxylem
TiO_2 NP	Cortex
Ni $(OH)_2$ NP	Metaxylem

The entry of the nanoparticles through the cell wall depends on the cell wall pore diameter (5–20 nm). Because of this, nanoparticles or nanoparticle aggregates with diameter less than the pore size of plant cell wall can easily enter through the cell wall and reach up to the plasma membrane. Functionalized nanoparticles can facilitate the enlargement of the pore size or the induction of new cell wall pore formation to enhance the nanoparticles uptake. Research discussions are going on about the uptake of nanoparticles into plant cell mediated by binding to carrier proteins through aquaporin, ion channels or endocytosis. Additionally, nanoparticles can also be transported into the plant by forming complexes with membrane transporter proteins or root exudates. Other studies reported that nanoparticles could enter through stomata or the base of trichome in leaf. Studies on the uptake and translocation of TiO_2-alizarin red S complex in *Arabidopsis thaliana* seedling have revealed that mucilage released by the roots develops pectin hydrogel complex around the root which is most probably responsible for the entry of the nanoparticle-dye complex.

Recent studies on the mechanism of nanoparticle uptake and translocation have exploited fluorescently labeled monodispersed mesoporous silica nanoparticles which were shown to penetrate the roots via symplastic and apoplastic pathways and translocate via xylem tissue to the aerial parts of the plants including the stem and leaves. However, the exact mechanism of nanoparticle uptake by plants is still not fully elucidated.

In the cytoplasm, nanoparticles are targeted to different cytoplasmic organelles and interfere with different metabolic processes of the cell (Table 3). It is shown that the uptake of TiO_2 nanoparticles in wheat include localization in parenchyma and vascular tissues of the root. The cell internalization and upward translocation of ZnO nanoparticles in *Lolium perenne* (ryegrasses) is realized through the root cells and then - move up to the vascular tissues.

The uptake and accumulation of ZnO nanoparticles when applied at higher concentration is straitened since the nanoparticles get agglomerated which inhibits their entry through the cell wall pores. Moreover, X-ray absorption spectroscopy of ZnO-treated seedlings revealed presence of Zn^{2+} ions instead of ZnO suggesting the role of the roots in ZnO ionization on its surface.

Another class of nanoparticles – the magnetite NP, behave in a way that their presence in root, stem and leaves is reported, and the extent of the nanoparticles uptake is proven to be affected by the type of the growth medium. A higher uptake was achieved in hydroponic medium as compared to the plant grown in sand, whereas no uptake was observed in plants grown in soil which might be due to the adherence of magnetite nanoparticles to soil and sand grains.

Finally, it should be mentioned that besides some conclusive studies on TiO_2 and ZnO nanoparticles, most of the uptake, translocation, and accumulation studies in plants are reported only up to the germination stage. Hence, the fate of nanoparticles in the plant system is still largely unknown.

Nano-fertilizers effect on plant physiology and metabolism

The majority of recent studies support the idea that nanoparticles exercise some adverse effects on plants. However, there are few studies that have suggested that nanoparticles, when delivered in controlled safe dose, may contribute to promotion of plant growth and yield. In this respect, multi-walled carbon nanoparticles (MWCNP) have been shown to promote seed germination and growth of tomato and enhance the growth of tobacco cells. The same phenomenon was observed in MWCNTs in mustard plant. Using the so called germination index and relative time of root elongation as etalon parameters it was shown that oxidized MWCNPs exercise better effect at lower concentration than the non-oxidized ones.

Comparative studies for evaluation of the seed yield and prevention of leaf abscission in borage plant, made with nanosilver and silver nitrate, have shown that the former was performing better. It is known that the plant hormone ethylene plays a key role in leaf abscission, and silver ions inhibit ethylene by replacing copper ions from the receptors. When the both compounds were applied on the plants through the foliar spray method it was observed that nanosilver was effective at a lower concentration than silver nitrate. Similar promoting effect of biosynthesized silver nanoparticles on emergence of seedling and various plant growth parameters of many economically important plant species were reported.

Various studies have been performed to clarify the effect of ZnO nanoparticles on the growth of different plants. Thus, it was shown a stimulatory effect on the growth of *Vigna radiata* and *Cicer arietinum*; ZnO nanoparticles adsorption on the root surface was observed through correlative light and scanning electron microscopy and such by the seedlings through inductively coupled plasma/atomic emission spectroscopy. The effect of ZnO nanoparticles on plant cell physiology was investigated using cellular antioxidant system as a model. Applying the foliar spray method on chickpea seedlings it was shown that low concentrations of ZnO nanoparticles has positive effect on the plant growth and the seedlings biomass accumulation has improved which may be due to lower reactive oxygen species (ROS) levels (evidenced by the lower malondialdehyde content). Field experiments confirmed that usage of 15 times lower dose of ZnO nanoparticles compared to the recommended dose of ZnSO4 led to 29.5 % higher pod yield.

Comparable positive effects of ZnO and CeO2 nanoparticles on *Cucumis sativus* fruit quality were observed. The application of both nanoparticles resulted in increased starch content and possibly – in altered carbohydrate pattern.

Stimulation of the antioxidant activity and nitrate reductase by a mixture of SiO2 and TiO2 nanoparticles in G. max was found, in addition to the better productive effect and increase in water and fertilizer uptake capacity of the model plant. The application of TiO2 nanoparticles was demonstrated to promote photosynthesis under both visible and ultraviolet light and growth in spinach. An increase of 73 % in dry weight, threefold higher photosynthetic rate, and 45 % increment in chlorophyll after seed treatment in spinach were observed. The authors speculate that the reason of increment in photosynthetic rate may be due to the increase in absorption of inorganic nutrients which enhanced the utilization of organic substance and quenching of oxygen-free radicals.

Unlike most of the nanoparticles, for which application at high concentration are not recommended due to the observed negative impact, TiO2 nanoparticles applied at concentrations as high as 2,000 ppm increased seed germination and seedling vigor in *Brassica napus*.

Hence, it is clear that different metal nanoparticles showed positive influence at various concentration range, e.g. Pd and Au at lower concentration, Si and Cu at higher concentration, and Au and Cu in combined mixture. This behavioral patter was confirmed by field studies with *G. max* and *Brassica juncea*: nanocrystalline powder of iron, cobalt, and copper at an extra low concentration promoted seed germination rate, and a marked increase in the chlorophyll index, number of nodules, and crop yield was observed. Similarly, foliar spray of gold on plant in field experiments showed positive effect resulting in increased plant height, stem diameter, number of branches, number of pods, seed yield, and – interestingly, improved the redox status of treated plants.

Ethical and safety issues of nano-fertilizers application

Undoubtedly nanotechnology has incredible potential to revolutionize many aspects of human life. However, the advancement of this multidisciplinary branch of science, especially the benefits from their practical application have to be considered with some precautions.

The major concern at world scale is whether the unknown risks of nanoparticles involving their environmental and health impact prevail over their potential benefits. Thus, the risks associated with the application of nanoparticles are yet to be evaluated before nanoparticles application is fully accepted and implemented. Hence, "nanotoxicology," has been developed which is responsible for assessing toxicological potential and promoting safe design and use of nanoparticles. Due to the thorough quantitative analysis of the potential health impacts, environmental clearance, and safe disposal of nanoparticles improvements in designing further applications of nanotechnology can be anticipated.

No direct human disease has been linked to nanoparticles so far. Nanoparticles which constitute a part of ultrafine particulate matter can enter in the human/animal system through oral, respiratory, or intradermal routes. Currently, there is a common assumption that the small size of nanoparticles allows them to easily enter tissues, cells, and organelles and interact with functional biomolecular structures (i.e., DNA, ribosomes) since the actual physical size of an engineered nanostructure is similar to many biological molecules (e.g., antibodies, proteins) and structures (e.g., viruses).

Of course there is still a need for proper physicochemical characterization and determination of appropriate exposure protocols and reliable methods for assessing nanoparticles outcome in the environment, their internalization, and their kinetics in living organisms. These are the prerequisites for establishment of optimal experimental conditions that will allow precise determination if a particular nanoparticle poses a threat to human health. However, the interdisciplinary research of materials scientists, environmentalists, and life scientists is contributing to identification of the true, if any, hazards of nanotechnology. The heterogeneous and developmental nature of nanotechnology is making risk assessment quite subjective. The absence of standardized methodologies and guidelines makes it difficult to compare the safety/toxicity assessments from different research groups. It is most likely that different types of nanoparticles vary as to their toxicological properties. To interpret correctly any toxicological data, it is essential to calculate and determine the expected concentrations of nanoparticles that may be exposed to the biological system or present in the ecosystem. The risk assessment of nanoparticles has to be performed on a case-by case basis. Thus the ethical issues must be specific for a specified product at a given time, and alternative assessments are needed to take into consideration ethical, social, and political values that relate policies such as those involving nanotechnology.

The use of nanotechnology in agriculture is very important as it directly affects humans. Nano-fertilizers enable nanoparticles to enter in the food chain allowing their distribution in every organism related to the food chain. Literally all substances can be toxic to plants, animals, or humans at some exposure level. However, this does not limit their use in various applications which are formulated minding the critical exposure concentration. As mentioned above the promoting effect of the nanoparticles on plant growth and physiology is expressed at very low concentrations, hence is hardly to believe that these concentrations will pose significant health and environmental damage.

Many countries have identified the potential of nanotechnology in the food and agriculture sectors. Meanwhile they recognize the need for assessment of the food safety implications of nanotechnology. As suggested by the scientific committee of the European Food Security Authority (EFSA), *"the risk assessment paradigm (hazard identification, hazard characterization*, *exposure assessment and risk characterization) is applicable for nanoparticles (EFSA Scientific Committee 2011). However, risk assessment of these nanoparticles in the food and feed area should consider the specific properties of the subject nanoparticles in addition to those common to the equivalent non-nanoforms.”*

Deciding the risk associated with the use of a particular nanoparticle in food and feed means taking into consideration various parameters, among which physicochemical characterization of nanoparticles, their stability in the food and feed, toxicokinetics (absorption, distribution, metabolism/biotransformation, excretion/elimination) within the human and animal systems.

GENETICALLY ENGINEERED MICROBES

Genetically modified bacteria for agricultural purposes

There are numerous bacterial genera which representatives can influence plant growth and production. Among these representatives there are plant pathogens that can suppress plant diseases and they are used as biocontrol strains. Another group or bacterial species can contribute to increased plant growth by enhancing the availability of nutrients. These bacteria constitute the bio-fertilizers and are known as well as growth-promoting rhizobacteria (PGPR). The name of PGPR is associated with their ability to grow well at the interface between soil and plant root (the rhizosphere). PGPR can be applied either as seed coating or directly to soil. However, to exert their growth-promoting effect sufficient numbers of the introduced PGPR have to survive in soil and rhizosphere, which not always happens. Consequently, the efficacy of PGPR is not always sufficient for commercial applications and there is a need to improve their performance. One of the possible decisions is to apply genetic modifications to facilitate their survival efficiency.

Survival of genetically modified bacteria in soil

Any microbial cell introduced into the environment will encounter a large number of biotic and abiotic factors affecting its survival. Both biotic and abiotic factors are equally important. Thus, high clay content, high pH, and relatively high moisture content can have a positive effect on bacterial survival. On the contrary, dry periods, presence of competing microorganisms, predation by protozoa, and lysis by bacteriophages negatively affect the number of introduced bacteria. Speaking about biotic factors affecting the activity and survival of introduced bacteria, the presence of plant roots that provide nutrients to the microorganisms living in their vicinity is very important. Among the microorganisms that are well adapted to the rhizosphere are members of the genera *Agrobacterium*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Erwinia*. *Pseudomonas*, *Rhizobium*, and *Xanthomonas*.

Microbial survival depends on the interrelation between the environmental conditions and the physiological state of the bacteria. As a result of this interactions bacterial cells can switch their metabolism to different physiological states. For instance, cells can become more stress resistant or form dwarf cells, they can produce exopolysaccharides for protection, they can enter a viable but non-culturable state, and some are able to form spores or associations with plants.

One can speculate that the survival pattern of the GM bacteria will follow the one of their wild-type parents. In fact, this extrapolation should be applied with some precautions. Firstly, the expression of the inserted genes requires an extra amount of energy, which could reduce their environmental fitness. In addition, the insertion could have disrupted unknown functions weakening the competitiveness of the strains. Secondly, it is possible the GMMs to evolve and adapt to the prevailing environmental conditions via natural selection. This last statement is supported by evidence for evolutionary adaptation of bacteria to degrade the herbicide 2,4-dichlorophenoxyacetic acid resulting in increased competitive fitness to use succinate as a substrate. Similarly, it is reported that environmental stresses could alleviate the debilitating effects of mutations - organisms may become more tolerant to genetic perturbations under certain environmental stresses.

GMMs have been shown to survive even better than the wild-type strain in studies with artificial growth conditions. However, enhanced survival of GMMs has rarely been observed under field conditions. Often, the population of introduced bacterial cells declines rapidly in soil, and the GM species survive in a mode similar to that of non-modified bacteria. There are a lot of experimental studies in which no difference in survival between GMM and parent strain could be detected (for *Pseudomonas chlororaphis, P. fiuorescens, Sinorhizobium meliloti).* Furthermore, some GMMs were reported to be out-competed by the parent strains. It is speculated that the presence of a number of constitutively expressed marker genes in a GMM had a negative effect on its survival in competition with the wild- type strain. Most probably it is the metabolic load that is responsible for the decreased fitness, since this effect does not occur under nutrient-rich conditions.

To correctly interpret bacterial survival data of crucial importance is to use a reliable method for detection, since cells that enter a non-culturable state cannot be detected with standard cultivation-based techniques. And various studies have shown that GMMs introduced into soil become non-cultuable. The presence of viable but non-culturable cells, dead cells, or naked DNA, detected with molecular techniques contributes to the complexity and the ecological significance of GMMs and their fitness in the context of the effect of the genetic modification introduced. The reliable way in which the effect of small differences in fitness will be measurable is to co-inoculate GMM and its parental strain placing them in direct competition. However, results from such direct competition experiments have to be interpreted with care as well, since commercial application of GMMs does not include direct competition between GMM and wild-type strain.

All these data, contradictory to some extend show that conclusion regarding survival of GMMs as compared to their parental strains cannot be definitely drawn. In each case where colonizing ability and survival of the GMM are of importance, these parameters will have to be determined.

Environmental impact of GMMs inoculated into soil

Possible effects of the release of GMMs in natural microbial ecosystems are quite diverse. The range encompasses events such as input of organic substrate, displacement of species, changes in population structure, and possible loss of certain functions; production of toxic metabolites, which might lead to disturbance of key ecological processes. It should be taken into consideration that small changes in community composition are difficult or even impossible to determine, and the relationship between microbial diversity and ecosystem functioning is not quite clear. Undoubtedly, soil microbial diversity is enormous with a high redundancy of functions. Disappearance of a few species with certain functions will be difficult to detect, since many functions can be performed by a large number of different microbes. In this sense, only extreme disturbances might affect soil microbial communities to the extent that certain functions will be negatively influenced.

The limited culturability of the indigenous soil microflora is one of the major problems in microbial ecology. DNA- and RNA-based techniques, which do not involve cultivation of the microorganisms, are currently used to detect the impact of GMMs on the indigenous microbial community. Methods that are suitable to analyze shifts in community structures are denaturing gradient gel electrophoresis (DGGE), amplified ribosomal DNA restriction analysis (ARDRA), terminal restriction fragment length polymorphisms (T-RFLP), and single-strand conformation polymorphism (SSCP).

Fate and effect of bio-fertilizer strains – field release

GM derivatives of bacteria that contribute to an enhanced nutrient availability for plants, and thereby increase plant growth.

The most important bio-fertilizers are bacteria, such as *Azospirillum* and *Rhizobium* that can fix nitrogen. *Rhizobium*, *Bradyrhizobium*, and *Sinorhizobium* are plant symbionts, which form root nodules in leguminous plants and fix atmospheric nitrogen. These bacteria have been used widely as plant inoculants to increase yield of leguminous crops. There is a long history of safe use of non-modified rhizobia as inoculants to increase yields of crops. However, yield increase is variable, and the success of inoculants seems to be dependent on competition with indigenous strains that are usually less effective. *Rhizobium*, *Bradyrhizobium*, and *Sinorhizobium* have been reported to survive in soil for years, in some cases even without the presence of their specific host. *Rhizobium* was shown to be able to form nodules when its host plant was planted again after several years. This shows that presence of the host plant is not strictly necessary for their survival, but also characteristics of the strain not related to symbiosis play a role in its survival in bulk soil for years. Fast-growing *Rhizobium* species were found to be more susceptible to desiccation than the slower-growing *Bradyrhizobium*.

Genetically modified *Azospirillum* and *Rhizobium* strains

Except for carbon dioxide (CO2) which plants obtain from the atmosphere, plants get all their nutrients from soil. Nature has developed various mechanisms to supply plant nutrients by means of renewable resources, and the best example of this principle is biological nitrogen fixation in leguminous plants. Nitrogen-fixing bacteria can be regarded as a self-propagating source of nitrogen for plants. Unfortunately, not all plants are able to perform such interaction with N_2-fixing bacteria. That is why at present plant production yields still largely depend on input of chemical fertilizers. Most of these fertilizers are very mobile in the soil and are supplied in greater quantities than required for optimal plant growth. The loss of valuable compounds is not only of economic importance; this also causes serious problems for the environment, through leakage in surface and ground water and accumulation of in the atmosphere.

Different strategies have been developed that aim at better uptake of fertilizers by plant roots. These include other formulations of fertilizer (e.g. slow-release fertilizer) and the use of Plant Growth Promoting Rhizobacteria (PGPR).

PGPR can exert their effect in both direct and indirect way. The indirect pattern comprises exercise of biocontrol of pathogens and deleterious microorganisms. The best documented example of PGPR acting in a direct plant growth promoting way is phytostimulation. Various bacteria genera are capable of producing plant growth stimulating factors (auxins, cytokinins, etc.) and when colonizing the roots of plants, they promote root growth. This assures a better uptake of water and nutrients by the plants and can result in higher crop yields.

<u>GM *Azospirillum* increases nitrogen uptake</u>

It is known that *Azospirillum* strains can promote plant root development and increase nitrogen uptake through the produced by them phytohormones. However, the mechanisms by which, and the conditions under which, these bacteria produce phytohormones as well as the interaction between bacteria and plant roots, are still not defined and require a better understanding.

To elucidate these mechanisms several important questions/approaches should be addressed:

- The genetic and biochemical grounds of the synthesis of indole-3-acetic acid (IM, the plant growth promoting hormone produced by *Azospirillum*;
- The construction of genetically modified *Azospirillum* stains with known production levels of IAA (i.e. IAA-minus , IAA-attenuated, IAA-over producers;
- Testing the effect of these genetically modified bacteria on plants (growth promotion, nitrogen uptake) and on the environment (interaction with resident microbial flora, survival and spread) under field conditions.

At present GM *Azospirillum* strains with these basic features are available. Research studies with these strains are focused on their impact on resident microbial populations, plant growth and nitrogen uptake rates from soil. These studies are being conducted in lab experiments (i.e. growth cabinet and glasshouse studies) in order to gain vital information on the way GM strains are likely to behave under field conditions. The experiments are conducted with a range of crops, soil types and climate conditions, representing the agricultural parameters existing within

Europe. Despite of the advancement of these research studies extensive and careful testing under containment is required before the GM *Azospirillum* can be considered for field release,

GM *Rhizobium* strains with increased competitiveness

Legume inoculation with highly efficient nitrogen fixing bacteria is a widely used approach to increase productivity of leguminous crops. This inoculation is not always successful since native soil bacteria with low nitrogen-fixing efficiency can out-compete the introduced strains in terms of nodulation initiation. Critical for the successful use of rhizobial inoculants is their competitiveness, i.e. the ability to dominate nodulation. Thus, inoculant strains are modified in a way that they occupy a sufficient number of root nodules to provide high rates of nitrogen fixation for the plant host.

Experiments with *Sinorhizobium meliloti* strains from diverse geographical origins regarding their competitiveness for alfalfa roots have shown that in all cases this property has been enhanced by genetic manipulation. The said genetic manipulation comprises modification of the expression of the *nifA* gene which is responsible for the control of all the rest nitrogen-fixation (*nif*) genes. When thus GM *S. meliloti* strains were mixed with wild-type ones, the former occupy most of the nodules on the alfalfa roots. The precise mechanism of this improvement is not understood yet but it is speculated that *nifA* regulates the expression of genes different from the *nif* cluster resulting in an advantage during nodule formation and development.

The ability of *Rhizobium* strains to efficiently recognize the plant root is another feature that contributes to their nodulation competitiveness. This is very important because the efficient inoculation means lower doses of the bacterial strain. Furthermore, the movement of the inoculation strain towards the plant roots is another factor influencing competitiveness. Experiments with GM *Rhizobium leguminosarum* strains, engineered to express β-glucuronidase, reporter gene (*gusA)*, showed that the percentage of the nodules induced by the GM *gusA*-labeled strain compared to the nodules induced by a flagella-deficient non-motile strain is higher. In this way it was proven that the functional flagella are required for effective competition for nodulation.

All these data provides valuable information regarding the mechanism of root attraction allowing the development of *Rhizobium* strains with enhanced nodulation competitiveness and increased host specificity.

Impact of GM *Rhizobium* strains on arbuscular mycorrhizal fungi

Arbuscular micorrhizal fungi are important group of fungi that form symbiotic relationships with plants. A major question is whether the application of GM *Rhizobium* strains with increased competitiveness leads to increase of the colonization and nodulation of the plant root or it interferes the beneficial symbiotic relationship.

In lab and green-house experiments it has been established that GM *Sinorhizobium meliloti* strain, with improved nodulation ability, did not interfere with any aspect of mycorrhiza formation by the representative AM fungi *Glomus mosseae.* On the contrary, GM S. *meliloti* increased the number of AM colonization units and the nutrient acquisition ability of the mycorrhizal plant.

GM *Rhizobium* strains: field release

Several *Rhizobium* species have been GM either to improve nitrogen fixation, or to study their survival making use of marker genes through field trials.

Thus, a Tn5-marked R. leguminosarum strain introduced into a field as an inoculant for peas and cereals persisted for 5 years in the plots where peas were grown. The persistence of the strain was attributed to the soil type, the cultivation of the proper host plants, and the climate conditions. Potential non-target effects on the microbial ecosystem were not studied.

The use of an improved R. meliloti strain, with additional copies of nifA and dctABC, resulted in an increase of alfalfa yield of 12.9% in a field study. However, at sites with high nitrogen concentrations or native rhizobial populations alfalfa yield did not increase.

The fate of a Tn903-marked R. meliloti strain introduced into alfalfa-planted field plots was studied and it was found that the cell numbers decreased rapidly after inoculation. One year after introduction, numbers of introduced cells had dropped to below the numbers of indigenous rhizobia.

In a contained field experiment a GM S. meliloti strain with enhanced competitiveness for nodule occupancy was released in the rhizosphere of alfalfa. Effects of the GMM and the wild type on the indigenous microbial communities were studied by restriction fragment length polymorphism (RFLP) and temperature gradient gel electrophoresis (TGGE). Inoculation of wild type and GMM had only limited effects. It appeared that alfalfa plants had a greater influence on the microbial community than the inoculated strains.

Both the fate and ecosystem effects of a Luc-marked S. meliloti in a field experiment with Medicago sativa were studied. The bacteria were detected up to 12 weeks after introduction. No effects of the strains on carbon and nitrogen concentrations in the soil could be detected, and there were no differences in the total number of colony forming units of indigenous microorganisms. Over a thousand bacterial isolates obtained from the plots were further studied by ARDRA, and the dominant groups were identified by 16S rRNA sequencing. In the rhizosphere of M. sativa numbers of Alcaligenes and Pseudomonas were reduced as a result of the inoculation. Molecular analysis by studying SSCP banding profiles revealed shifts confirming the effect of the inoculum on the native microbial population.

In China wild type and GM Alcaligenes faecalis isolates have been introduced into rice fields at a large scale to improve crop productivity. A. faecalis, a non-nodule-forming nitrogen-fixing isolate, was GM by insertion of a constitutively expressed nifA regulatory gene. Nitrogen fixation appeared to be 15-20% higher and yield was 5-12% higher compared to the non-treated fields. The possible ecosystem effects of the introduction of this GM strain by DGGE of amplified 16S rDNA in a microcosm experiment was studied. The introduced GM strain survived well in the rhizosphere. DGGE banding profiles of samples treated with the modified strain closely resembled profiles of untreated samples throughout the 40 days of the experiment, suggesting that there are no obvious effects on the bacterial community. Overall, the survival of the strain and the increase in crop yield indicate that this derivative of A. faecalis is a good candidate for commercial application, since its ecosystem effects seem very limited.

The impact and fate under field conditions of GM *Rhizobium* strains were investigated in a field trial with a model system comprising different GM *Rhizobium leguminosarum* v. *viciae* strains, marked with the *lacZ* gene and HgCb resistance genes *(mer* genes) inoculated in the rhizosphere of pea plants. Three modified strains were used:

- 1110 strain containing plasmid pDG3 carrying genes for resistance to HgCb *(mer* genes) and *lacZ* whose expression is under the control of the l*acZ -lacO* system
- 1111 strain carrying the plasmid pDG4 in which the *lacZ* gene is constitutively expressed at high levels;
- 1112 strain containing a copy of *mer* genes and a regulated *lacZ* gene inserted into the chromosome.

Wild-type *R. leguminosarum* v. *viciae* 1003 was used as a control.

These strains were monitored according to the reporter *lacZ/mer* system along with the soil metabolic activity plus nitrogen transforming capacity.

The field experiments showed that all tested strains colonized the rhizosphere to the same extent; similar values were determined for the respiration rate and soil metabolic activity as well as for the nitrogen transforming capacity of all tested strains. These results indicate that although the presence of the plant had a considerable impact on carbon mineralization in soil, the impact of GM *Rhizobium* strains is indistinguishable from the impact of the wild-type strain and also suggest that the impact of the plant on microbial activity is considerably greater than the impact of GM inoculants compared with wild-type strains.

In spite of the fact that the field trials with GM bio-fertilizers are limited the initial results about their use are promising in respect to the improved performance in agricultural applications. GM bio-fertilizers have been introduced with an encouraging success regarding the survival and the activity of the inoculants, which is dependent on the environmental conditions. So far, non-target effects of GM bio-fertilizer strains that have been reported are small and insignificant compared to natural variations, such as differences between populations of different plant species..

However, our knowledge on the benefits, fate and effects of GM strains in the environment is still quite limited and partial.

Questions that have to be solved include: how and when (at what physiological state) bacteria survive best in soil; what is their effect on the natural microflora; how can be mix microbial community structured and optimized for use in agriculture. And last but not least – what is the ecosystem effects of GM strains, especially on non-target organisms.

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