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(54) Title: METHOD FOR IMPROVING SOIL MICROORGANISM POPULATION

(57) Abstract

The invention relates to compositions and method for the treatment of arable soil, for the improvement of the growth of plants and harvest–results, method for the preparation of the compositions, the pesticide resistance microorganism strains forming the base of the compositions, and method for their preparation. The cells of one of the microoganisms according to the invention or their mixtures are applied on the arable land, or bound to the seeds of the plant. In the method according to the invention a composition is introduced into and/or onto the arable soil, said composition containing at least one of the 12 soil bacterium strains, isolated from the environment of differents plants and from different soils, made pesticide resistant, which convert the nitrogen of the atmosphere to compounds utilizable by the plants, solubilize the mineralised phosphate compounds and biosynthesize substances promoting the development of the plants, produce polysaccharides, thus advantageously transforming the structure of the arable soil, and resulting in acceptable harvest results, when pratically abolishing the use of the expensive fertilizers.

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# Method for improving soil microorganism population

## Technical field

The present invention relates to compositions containing microorganism(s), the microorganisms and process for preparing of the compositions, and method for the treatment of the soil and the plants.

More particularly the present invention relates to the production of the compositions, using any of the microorganisms detailed hereunder, or their mixtures.

Furthermore, the present invention relates to the preparation of the cultures of microorganisms to be used and to the microorganisms.

More particularly, the present invention relates to a method for the treatment of the soil and the plants with the composition containing at least one of the following microorganisms: Azospirillum lipoferum ssp. lip7R 885 (NCAIM P(B) 001253), Azospirillum amazonense ssp.: K21R 887 (NCAIM P (B) 001256), Azospirillum irakense ssp. 5041R 889 (NCAIM P (B) 0011255), Azospirillum brasilense ssp. A41R 879 (NCAIM P (B)001254), Azotobacter vinelandii ssp. ESZ 2132 (NCAIM P (B) 001257), Pseudomonas sp. Szeged-344 O.P. 14 (NCAIM P (B) 001251), Pseudomonas fluorescens var. MOB24, Res24 (NCAIM P (B) 001252), Bacillus circulans var. Res. 97 (NCAIM P (B) 001261), Bacillus megaterium var. Res. 54 (NCAIM P (B) 001250), Rhizobium meliloti var. PolRes. 7 (NCAIM P (B) 001259), Alcaligenes faecalis var. Res36 (NCAIM P (B) 001260) and Phyl106-R+324 (NCAIM P (B) 001258), which are resistant against plant protection agents, seed-dresser agents and other chemicals generally used in plant cultivation, as well as to the treatment with compositions containing the pesticide resistant microorganisms mentioned above, which live and multiply in the immediate environment of the plants.

## Background Art

Cropland is a natural medium of the plants and microorganisms, it is a self-controlling ecosystem, and in natural conditions the existence of one of them determines the other. When humans, to improve harvest results, with their activity (deep ploughing,

use of farmyard manure and fertilizers, use of plant protection agents, etc.) destroy the balance formed during evolution in structure and function, there are changes, which have non-predictable effects may occur. Humans cultivate relatively few plant species, but in order to successfully cultivate these plants, the presence of many other different living and active microorganisms is necessary. In order to form a microorganism population which is optimal for the cultivation of a crop plant, many years', decades' selection time is necessary. However, the determinative type microorganisms of the advantageous microorganism population can be introduced into the cropland, and the conditions necessary for the optimal growth can be formed in a few days. This results in higher harvest results, without disturbing the natural ecosystem. The useful and dominant microorganisms living in the environment of an economically important plant, can be determined by laboratory experiments, and can be cultivated and prepared individually in industrial scale, and introduced into the soil.

There are very few scientific data available, regarding the question, how the microorganism system of the soil can be regulated. Accordingly, besides the prevention of the hardly recoverable damages, investigations are necessary for the discovery of environmentally and economically advantageous intervention possibilities.

The ecosystem of the soil microorganisms has important regularities. In the immediate vicinity of the root of living plants (rhisosphere) and the germinating seeds (spermatosphere) the number of microorganisms is different and different species can be identified than in greater distance. The roots and the germinating, swelling seeds excrete substances (proteins, polysaccharides, non-living plant cells detaching from the roots, biologically active compounds, chemoattractants, etc.) which can be used by the microorganisms as nutritives. The multiplication of the bacteria around the root can be influenced by many different factors. These factors depend on the region, the quality of the soil, the composition of the microorganism population, etc. The ability of spore formation, the ability of siderophore, bacteriocin and phytohormone production, mobility, chemotactic responses to the effect of the root exudates, the physiological condition of the plant, the seasons, the quality of plant are all important factors in the formation and effectivity of the association.

The microorganism culture of the ecosystem is characterized by homeostasis, i.e. the effort for the preservation or restoration of the balance formed. This regulation force makes difficult to keep the microorganisms important for us alive in the soil, but it is possible, by carefully planned selection work.

To investigate and understand the role of the soil microorganisms in plant production, the circulation of the most important chemical elements, and the energy and elements necessary for the life-functions must be known.

The carbon source in the soil is provided basically and in overwhelming majority by the use of the energy of the sun, by photosynthesis, and depending on the conditions 30-80% of the carbon assimilated gets into the soil, where it mineralizes, transforming into organic substances.

In the decomposition of the plant parts containing the assimilated carbon, microorganisms are taking part in 85-90%. During the decomposition, carbon dioxide, new biomass, metabolites, and finally, humus is forming.

The nitrogen cycle is more complicated than the carbon cycle. In the transformation of the nitrogen, biological and chemical processes are playing role. In the nature the majority of the nitrogen gas is present in the so called inert form, and the so called fixed nitrogen, which can be used by the living organisms (nitrate, ammonia) is available only in limited amount.

Primarily it is the biological nitrogen fixation which is responsible for the mineralisation of the nitrogen gas. Since over a hectare the amount of molecular nitrogen is 6-7 x $10^8$ tons, this represents an unlimited source for the biological nitrogen fixation. The interest of the experts turned to the nitrogen-fixing organisms, i.e. is to those, which are able to reduce the inert, molecular nitrogen to ammonia, since among others the cognition of these microorganisms and the proper use of their properties can assure the worldwide disappearance of hunger, in an environmental-friendly way.

During the reduction of the molecular nitrogen in microorganisms the nitrogenase enzyme complex catalyses the reduction of nitrogen to ammonia. The key enzymes of the enzyme system include nitrogenase, containing a molibdene-iron cofactor, and the nitrogenase reductase.

Some of the nitrogen-fixing microorganisms bind the nitrogen in free-living state, but many bacteria can fix the nitrogen only in association with an other, higher living creature (associative and symbiotic nitrogen fixation).

The free-living nitrogen-fixing bacteria (i.e. the Azotobacters) live in the soil and are able to bind nitrogen by themselves, the associative nitrogen-fixers bind significant amount of nitrogen only in the presence of the immediate vicinity of an other living creature. These are the Rhizobium and Azospirillum species.

The phosphorous cycle, contrary to the nitrogen cycle, is practically closed in natural conditions. The input and the output is the same, the leakage is little, phosphorous does not get into the air. This element finally accumulates in the waters, seas, and a little amount gets back to the land (in the form of guano).

In the living cells of the soil the phosphorous accumulates in organic substances, and the mineralisation threof goes with high speed (3-8 $g/m^2$/year). The solubility of the forming phosphorous substances - i.e. their availability for the plants- is different, only 5% of the 400-1200 mg phosphorous detectable in 1 kg average soil is available. The turnover of some phosphorous compounds is 500-2000 years.

It is known that by the introduction of some phosphonolytic microorganism strains into the soil, and providing the nutrients necessary for their multiplication, the complex phosphorous compounds, which otherwise are not available for the plant, can be brought into solution. If the introduced microorganisms "work", and the mineral content of the soil is suitable, the application of the phosphorous-containing fertilizers can be abandoned or reduced. This latter is advantageous as well, because the phosphorous-based fertilizers introduced transform very quickly and are lost for the plant, while in the presence of the microorganisms capable of mobilizing the phosphorous these become assimilable.

The microorganisms multiplying in the soil biosynthesize compounds which are physiologically active from the point of view of the plants. From these the most important ones are the phythohormones, the auxins (indol-acetic acid), ethylene, gibberellins, kinetins, etc. Some Pseudomonas strains produce siderophores in the presence of a little amount of iron. The siderophores are able to collect the iron. As a consequence other

bacteria and fungi, among other the potential potato-pathogenic Erwinia caratovora, multiplying in the rhysosphere - since they can not use the iron from the siderophores - are inhibited, because of the iron-deficiency. On the other hand, these siderophores significantly stimulate the growth of the plants (potato, sugar-beet, radish) on iron-deficient soils, as they directly supply the iron fixed to the plant.

For the solution of the problems mentioned above many different technical processes were elaborated [Coppola, S. et al.: Annali di Microbiologia and Enzimologia 21, 45 (1971); Brown, M.E. et al.: Journal of General Microbiology 53, 135 (1968); Brown, M.E. et al.: Microbiol. 139, 327 (1987); Döbereiner, J. and Pedrosa, F.O.: Brock/Springer Series in Contemporary Bioscience. Science Tech. Publishers. Madison, Springer Verlag, Berlin (1987); Döbereiner J. et al.: Proc. of the 1st International Symp. on Nitrogen Fixation, Newton and Nyman eds.. Washington State Univ. Press (1976); Dommerques, Y. et al.: Soil Biol. Biochem. 5, 83 (1973); Elmerich, C.: Mol. Gen. of the Bacteria-Plant Interaction. Pühler ed., Berlin: Springer Verlag, 367 (1983); Soil Microbiology, Ed.: N. Walker, N., Butterworths. London and Boston, 67 (1978); Varma, S. and Mathur, R.S.: Current Science 58(19). 1099 (1989); Adams, F. and Conrad, J.P., Soil Science 75, 361 (1953); Eklund E. and Sinda E.: Plant and Soil 35, 495 (1971); Hayman, D.S.: Soil Microbiology, Ed.: Walker. N.. Butterworths, London and Boston, 67 (1978)].

Hungarian inventors have patent protection for powder-form nitrification cultures (Hungarian Patent No. HU 143.391), and the preparation of Azotobacter chroococcum and Rhizobium cultures (Hungarian Patent No. HU 195.068), and again Azotobacter chroococcum and Bacillus megaterium microorganism cultures (Hungarian Patent No. HU 207.752). The Azotobacter chroococcum was deposited on No. 00238, the Bacillus megaterium was deposited on NCAIM (P) B 1140. More particularly, the authors of the HU 188.434 and HU 207.751 claim the fermentative production of the mixture of the deposited microorganisms mentioned. According to HU 213.163, the authors supplement the cultures of the microorganisms of the HU 207.751 Patent with carboxymethyl-cellulose. Patent application No. HU 1671/96 claims cultures containing Azospirillum

lipoferum ssp., Azotobacter vinelandii sp., Pseudomonas fluorescens ssp. and Bacillus megaterium microorganisms.

The application of the microorganisms used in the procedures mentioned is limited by the fact that these microorganisms in different production conditions, in soils of different composition, in the presence of the generally used chemical agents are alive only for a short time, the environment and rhisosphere of the different plants does not always represent optimal living conditions for them.

During our investigations and experiments it has been found, that the multiplication and survival of the microorganisms cultivated in the laboratories - which are advantageous for the development of the plants, fix nitrogen, mobilize phosphorous, promote the development of the plants, improving the structure of the soil - in the immediate environment of the plant, surprisingly depend on the type of the soil and the plant. Different microorganisms exert their effect in the chernozyom, low humus-content, loess, fields, or clay soils. During the investigations it was recognized, that surprisingly, distinct microorganism strains are able to multiply and exert their effects for a long time in the rhisosphere of different plants or in its environment. That is why experiments were made to for the isolation of microorganisms, which, from the point of view of the yield, have advantageous effects in the environment of an agriculturally important plant. It was determined, that the effectiveness and survival of the microorganisms depend on the quality of the inocula, the time of inoculation, and according to the facts mentioned above, depend on the soil and plant host.

Furthermore, we determined that certain polysaccharide-producing microorganisms, surprisingly enough even for the experts, advantageously change the structure of the soil from agricultural point of view.

Generally the croplands and the plants are treated with plant protecting agents, the seeds are treated with caustics. These substances inhibit the development of the microorganisms, in extreme cases kill them. We determined the sensitivity of our microorganisms, isolated from the soil, which have advantageous effects to the development of the plants, and have a long lifetime in the soil, i.e. the response to the

generally used plant protection agents, and isolated those, which were not killed by the compounds, used in normal concentration.

The aim of the patent invention is to provide preparations which contain microorganism(s) resistant to the plant protection agents, the microorganism content of which, in the environment of a plant, in the rhisosphere, or directly among the plant cells, promote the development of a plant or plant family in a given plant environment, by fixing or mobilizing the elements of vital importance, producing plant growth factors and polysaccharides, thus, minimizing the need of using fertilizers.

Best Method for Carrying out the Invention

The present invention is based on the recognition, that for the production of the targeted preparation, the following microorganisms, resistant to the plant protection agents are the most suitable: Azospirillum lipoferum ssp. lip7R 885 (NCAIM P(B) 001253), Azospirillum amazonense ssp.: K21R 887 (NCAIM P (B) 001256), Azospirillum irakense ssp. 5041R 889 (NCAIM P (B) 0011255), Azospirillum brasilense ssp. A41R 879 (NCAIM P (B)001254), Azotobacter vinelandii ssp. ESZ 2132 (NCAIM P (B) 001257), Pseudomonas sp. Szeged-344 O.P. 14 (NCAIM P (B) 001251), Pseudomonas fluorescens var. MOB24, Res24 (NCAIM P (B) 001252), Bacillus circulans var. Res. 97 (NCAIM P (B) 001261), Bacillus megaterium var. Res. 54 (NCAIM P (B) 001250), Rhizobium meliloti var. PolRes. 7 (NCAIM P (B) 001259), Alcaligenes faecalis var. Res36 (NCAIM P (B) 001260) and Phyl106-R+324 (NCAIM P (B) 001258). These microorganisms were isolated, and cultivation procedure was elaborated for these microorganisms.

The present invention relates to the cultivation procedures of the microorganisms mentioned above, preparations containing the same, the use of the preparation(s) containing the microorganism(s) and the microorganisms.

According to the description hereinabove microorganisms were isolated from European soils, or from the root environment of some plants, and the nitrogen-fixing, phosphate-solubilizing, polysaccharide-producing and phytohormone biosynthesizing abilities thereof were tested, their resistance was improved against different chemicals

generally used in the agriculture, the selected microorganisms were taxonomically identified, technology developed for their cultivation and preparations were produced from their cultures, and finally their effect to the development of the plants and the harvest results was determined by greenhouse experiments.

Azospirillum, Pseudomonas, Rhizobium, Alcaligenes, Azotobacter and Bacillus species and sub-species were isolated.

The least known Azospirillum species are the Gram-negative variable bacteria living in the soil, which in microaerophylic (i.e. in the presence of 1-2% oxygen) conditions, in close connection with the root of the plants are able to reduce the nitrogen content of the air to ammonia.

The Azospirillum strains were isolated from the root environment of corn, wheat, barley, rye and grassland, grown in different regions, in different soils. The bacterium suspension originating from the soil sample is plated to the MM medium (the composition of the medium will be given later), and cultivated under micro-aerophylic conditions. 72 hours later the Azospirillum colonies were identified. The Azospirillum colonies, unlike the other small bacterial and fungal colonies grow to about 3 mm diameter.

The morphology characteristic of the Azospirillum cells is shown by the Azospirillum sp. cultures, growing exponentially in liquid TA and soft agar Nfb(ll) medium (the composition of the medium will be given later). The form of the cells is vibroid and S, their size is 1-2x2-4 μm. They need biotin for their growth. On the basis of microscopic observation they can move fast. Their mobility is due to their polar flagella. They accumulate in their cells poly-beta-hydroxy-butyrate granula and carotenes. The reddish coloration of the aging cultures is due to their carotene content. For their growth, they can utilize organic acids, i.e. malic acid, lactic acid, pyruvic acid and succinic acid. Fixation of the molecular nitrogen is happening under microaerophylic conditions. Under extreme conditions, i.e. at desiccation, or at low or high pH values, in the absence of nitrogen or carbon sources, the cells form cysts, which have no flagella, but they contain poly-beta-hydroxy-butyrate granulae, and are surrounded by capsular polysaccharide. The carbon source utilization spectra of the microorganisms is different, depending on the

species: the A. amazonense is glucose +, saccharose +, inositol +, the A. brasilense is glucose ±, saccharose and inositol -, the A. irakense utilizes glucose (+)and saccharose, but can not utilize inositol (-), and finally A. lipoferum can utilize only glucose (+). On nitrogen free medium the carbon source utilization spectrum is even more different, and thus, the four species mentioned above can be distinguished from each other. The carbon source utilization spectrum of the microorganisms isolated by us is partly different from that of the ATCC 29.731 A. lipoferrum, A. amazonense, A. brasilense and A. irakense neotype [Holt, G. and Co., Bergey's Manual of Determinative Bacteriology, 9th edition (1994)]. Contrary to the typic strains they grow well at 3,5% NaCl, their microscopic picture is different in soft agar (this will be described later) in different phases of cultivation, for example their coloring pigment production is more intensive on potato-extract agar.

Some of the Azospirillae isolated by us are close to the Azospirillum lipoferum, Azospirillum amazonense, Azospirillum brasilense or Azospirillum irakense species, our experiments were conducted with these.

In the soil samples collected screening was performed for phosphate-mobilizing microorganisms, described hereunder. The selected microorganisms were investigated taxonomically. Some of the microorganisms turned out to be Pseudomonas, the others Bacillus species.

The Pseudomonas strains are thin rod form, Gram-negative, aerobic cells, on most of the media produce coloring substances, they are saprophytes. Carotene production can not be observed, they do not grow over 40 °C. On gelatin agar liquifying can be observed. Our strains utilize glucose, and do not utilize starch. Although the variants of the fluorescent Pseudomonas strains are in close relation taxonomically, certain taxonomic heterogeneity can be observed between our microorganism and the typic strains: our microorganism - characteristically to the Pseudomonae - utilizes well the glucose, galactose, D-arabinose, maltose, lactose, starch and inuline. Contrary to the type-strains, they grow well on xylose, saccharose and to some extent on sorbitol. As a sole carbon source, they can utilize glycine.

On the basis of the facts mentioned above, one of our microorganisms could be identified as Pseudomonas fluorescens, and found to be close to the variants of the Biovar III group. The strain was named Pseudomonas fluorescens ssp. Further experiments were conducted with the Pseudomonas sp. Szeged-344 O.P.14 strain, isolated from a Szeged soil.

From among the Bacillus strains capable of dissolving the insoluble phosphate, on the basis of taxonomic markers some strains proved to be Bacillus circulans, and some others proved to be Bacillus megaterium. Two strains were selected for further experiments.

The Rhizobium meliloti strain was isolated in September 8, 1996, from the alfalfa field of the Subasa farm, close to Szeged-Kiskundorozsma. From the alfalfa growing in the loess soil a well developing plant was selected, and the well developed nodules were separated from its roots. The nodules were washed by sterile distilled water, triturated, and the particles were suspended in physiological saline solution. Sterile dilution was made from the suspension, and plated on complete medium. The nitrogen-fixation capability of the colonies grown following the 48 hours' incubation was tested in the so-called symbiotic plant test, according to the following protocol: the surface of the commercially available alfalfa seeds (Medicago sativa) was sterilized by 2 hours' heat treatment at 72 °C with 20% hypochlorite solution, followed by careful washing, and finally germinated on 1% distilled water agar. The seedlings were put to 1,5% Gibson agar slants (see later) and cultivated in greenhouse for one week. The one week old seedlings were inoculated with the cells of a bacterial colony, and cultivated for additional 8 weeks in greenhouse. From the strains belonging to the plants showing the three best development (dry weight of the parts over the root is 22-26 mg, compared to the 3-5 mg weight of the control plants) those were selected which produced the most polysaccharide, and were named Rhizobium meliloti var.

The Alcaligenes species were isolated from soil samples collected from the fields in the region of Komárom, Szeged, Esztergom and Kiskőrös, in summertime, by plating on Tag agar, containing 0,05% aniline-blue. From the strains isolated on the basis of color-change, producing significant amount of polysaccharide one was named Alcaligenes

faecalis var. on the basis of the taxonomic markers, and was stored for further experiments. Our strain is a Gram-negative microorganism of 1,5-1 μm size, it does not move, or moves very slowly, can be classified into the Achromobacteraceae family. It does not produce indole, and does not liquefy gelatine.

An other microorganism proved to be Micrococcus roseus on the basis of the taxonomic, morphologic and ribosomal DNA investigations was also isolated and showed very advantageous effect during the plant tests. The strain of this microorganism made resistant to the plant protection agents was deposited as Phyl 106-R+324.

From the soil sample mentioned above microorganisms were isolated, which belong to the Azotobacter genus, using selective cultivation on Nfb(II) soft agar and plating on MM medium. One of the subspecies, which proved to be Azotobacter vinelandii on basis of the taxonomic markers, was due to its nitrogen fixation properties and stored for further experiments. The cells of the strain are pleiomorphous, they have a coccoid shape. In the presence of oxygen they move fast. They are Gram-negative. On nitrogen-free medium they produce fluorescing, yellowish-green pigment, and utilize well ramnose and meso-inositol.

One aim of the present invention is that the microorganism with advantageous effects, and introduced into the cropland, should save their viability as long as possible, and multiply as fast as possible. The bactericide or bacteriostatic effect of the chemicals, plant protection agents, fungicides, herbicides generally used in the agriculture inhibit the achievement of this result. That is why the microorganisms isolated and maintained according to the description above, should have been made resistant against the plant protection agents, pesticides and fungicides generally used in the agriculture, preferably according to Example 4, and were deposited in the National Collection of Agricultural and Industrial Microorganisms.

The present invention relates to compositions and methods, which result in the improvement of the harvest results of plant cultures. The compositions are produced according to the followings: the microorganisms tested and isolated from different croplands and from the environment of different plants, surviving for a long time in the given soil, in the environment of a defined plant family, made resistant against the

chemicals generally used in the agriculture, are cultivated, and the preparation containing the culture are introduced into the cropland or to the seeds of the adequate plants. According to the present invention the preparations can be used in such a way, that the soil, the plants or the plant seeds are treated with the preparation containing at least one of the following microorganisms or a kind of mixture of these: Azospirillum lipoferum ssp. lip7R 885 (NCAIM P(B) 001253), Azospirillum amazonense ssp.: K21R 887 (NCAIM P (B) 001256), Azospirillum irakense ssp. 5041R 889 (NCAIM P (B) 0011255), Azospirillum brasilense ssp. A41R 879 (NCAIM P (B)001254), Azotobacter vinelandii ssp. ESZ 2132 (NCAIM P (B) 001257), Pseudomonas sp. Szeged-344 O.P. 14 (NCAIM P (B) 001251), Pseudomonas fluorescens var. MOB24, Res24 (NCAIM P (B) 001252), Bacillus circulans var. Res. 97 (NCAIM P (B) 001261), Bacillus megaterium var. Res. 54 (NCAIM P (B) 001250), Rhizobium meliloti var. PolRes. 7 (NCAIM P (B) 001259), Alcaligenes faecalis var. Res36 (NCAIM P (B) 001260) and Phyl106-R+324 (NCAIM P (B) 001258).

As a result of the treatment, the development of the plants is faster, they become more resistant against the pathogens, the structure of the soil and the water supply of the plants improve, and high yields can be reached with reduced amount, or by total abandonment of fertilizers.

One of the greatest advantages of the method according to the present invention is that during plant cultivation the use of nitrogen- and phosphate-base fertilizers, the environmental polluting effects of which is obvious becomes needless. The compounds biosynthesizing in the microorganism cells and promoting the development of the plants accelerate the development of the plant treated, increases the development of the root and as a consequence of this, the water supply of the plants, the distributed microorganisms interfere with the development of the plant- pathogenic microorganisms, the polysaccharides biosynthesizing in the cells of certain microorganisms are especially improving the structure of the soil, the water balance of the soil and the life of the soil. The compositions according to the present invention, the production and use of the same, contrary to the already known compositions and preparation processes with the same aim and application, are based on the use of different microorganisms with different effect,

isolated from different soils, having specific effect to some, economically important field crops.

The microorganisms according to the present invention can be cultivated on a medium containing as carbon source for example glucose, starch, saccharose or molasses, as nitrogen source for example corn steep liquor, casein, yeast extract or ammonium salts, and other inorganic salts, salts dissociating to ions of trace elements, but obviously, any usable carbon- and nitrogen-sources can be used, which make possible the propagation of the microorganisms according to the present invention.

The cultures containing the microorganisms according to the present invention can be distributed directly to the cropland in the medium used for the cultivation, but preparations can be produced which save the viability of the microorganisms, among others preparations which contain vehicle which binds the bacteria to the seeds with adhesive forces. The amount of bacteria distributed to the locus can be between about 5 x $10^{11}$ and 5 x $10^{15}$ cells per hectare, advantageously between about $10^{12}$ and $10^{13}$ cells per hectare.

The microorganisms isolated from different plant environments, identified and made resistant were deposited according to the Budapest Treaty in the National Collection of Agricultural and Industrial Microorganism, under the following deposition numbers:

Azospirillum lipoferum ssp.lip7R 885(NCAIM P(B) 001253),
Azospirillum amazonense ssp.: K21R 887 (NCAIM P (B) 001256),
Azospirillum irakense ssp. 5041R 889 (NCAIM P (B) 0011255),
Azospirillum brasilense ssp. A41R 879 (NCAIM P (B)001254),
Azotobacter vinelandii ssp. ESZ 2132 (NCAIM P (B) 001257),
Pseudomonas sp. Szeged-344 O.P. 14 (NCAIM P (B) 001251),
Pseudomonas fluorescens var. MOB24, Res24 (NCAIM P (B) 001252),
Bacillus circulans var. Res.97 (NCAIM P(B) 001261),
Bacillus megaterium var. Res.54 (NCAIM P(B) 001250),
Rhizobium meliloti var. PolRes.7 (NCAIM P(B) 001259),
Alcaligenes faecalis var. Res36 (NCAIM P (B) 001260),

Phyl106-R+324 (NCAIM P (B) 001258).

The claims of the present invention are valid for the deposited strains and for their natural and artificial mutants, variants, or any other cell line of the microorganisms, which was gained by any known method.

The present invention is illustrated by the following examples, without limiting the scope of the protection.

The percents are given in weight percents, except it is mentioned otherwise.

Example 1.

Isolation of associative microorganisms fixing the nitrogen of the air, from different soils and from the environment of different plants, and demonstration of their nitrogen fixing properties

The Azospirillum species are Gram-negative variable bacteria, living in the soil, which in microaerofil (in the presence of 1-2% oxygen) conditions, in close connection with the roots of the plants, are able to reduce the nitrogen of the air to ammonia, and supply it to the plants.

The Azospirillum species were isolated between February 5, 1993, and December 20, 1996, from different regions of Europe, from different soils (humus, loess, sodic, brown and black), from the root environment of different plants (cereals, sunflower, corn, grass). The chemical attractants excreted by the roots of the plants, for example the organic acids and sugars attract the Azospirillum strains by chemotaxis. The bacteria move to the roots by their flagella, and by reaching them they colonize.

From the 10x, 100x, 1000x and 10000x dilution of the actual soil sample 100 µl suspension was diluted to Petri dishes containing MM and Nfb(II) soft agar. The composition of the soft agar is the following:

| | | |
|---|---|---|
| $K_2HPO_4$ | 1,65 | g/l |
| $KH_2PO_4$ | 0,87 | g/l |
| $MgSO_4 \times 7\ H_2O$ | 0,29 | g/l |
| NaCl | 0,18 | g/l |
| $CaCl_2 \times 2\ H_2O$ | 0,07 | g/l |

| | |
|---|---|
| $FeCl_3$ x 6 $H_2O$ | 0,01 g/l |
| $NaMoO_4$ x 2 $H_2O$ | 0,005 g/l |
| $MnSO_4$ x $H_2O$ | 0,00014 g/l |
| $ZnSO_4$ x 7 $H_2O$ | 0,007 g/l |
| $CuSO_4$ x 5 $H_2O$ | 0,000125 g/l |
| $CoSO_4$ x 7 $H_2O$ | 0,00014 g/l |
| $H_3BO_3$ | 0,00003 g/l |
| Glucose | 20,0 g/l |
| Bacto agar | 20,0 g/l |

The glucose was sterilized separately from the other components of the MM medium by autoclaving (121 °C, 30 minutes), and after cooling to 60 °C they were combined. The pH of the sterile medium was adjusted to 7,4 by sterile 1 mole/l NaOH.

The composition of the Nfb(II) medium is the following:

| | |
|---|---|
| L-malic acid | 5,0 g |
| $K_2HPO_4$ | 0,5 g |
| $MgSO_4$ x 7 $H_2O$ | 0,2 g |
| NaCl | 0,1 g |
| $CaCl_2$ | 0,02 g |
| Trace-element solution* | 2,0 ml |
| Bromo-thymol blue (5% solution of the substance dissolved in 0,2 mole/l KOH) | 2,0 ml |
| 1,564 solution of Fe-EDTA | 4,0 ml |
| Vitamin solution** | 1,0 ml |
| Agar | 1,75 g |

The pH of the solution was adjusted to 6,8 with 1 mole/l KOH, dissolved in water.

The composition of the trace-element solution is the following:

| | |
|---|---|
| Fe(II)-sulfate x 7 $H_2O$ | 200 mg |
| Fe(III)-chloride x 6 $H_2O$ | 10 mg |

| | |
|---|---|
| $MnSO_4$ x $H_2O$ | 1 mg |
| $CuSO_4$ x 5 $H_2O$ | 2 mg |
| $NaMoO_4$ x 2 $H_2O$ | 1 mg |
| $CoCl_2$ x 6 $H_2O$ | 2 mg |
| $ZnSO_4$ 7 $H_2O$ | 2 mg |
| Sodium-tetraborate x 10 $H_2O$ | 1 mg |
| $P_2O_5$ x 24 $WO_3$ x $H_2O$ | 0,5 mg |
| Bismuth-nitrate x $H_2O$ | 0,1 mg |
| $SnCl_2$ | 0,01 mg |
| Selenium-chloride | 0.01 mg |
| KI | 1 mg |
| Citric acid | 100 mg |
| Water distilled | 1000 ml |

** the composition of the vitamin solution is the following:

| | |
|---|---|
| Vitamin C | 50 mg |
| Vitamin B1 | 5 mg |
| Vitamin E | 2 mg |
| Vitamin A | 2 mg |
| Biotin | 4 mg |
| Water distilled | 100 ml |

The bacteria introduced in the MM media were incubated in anaerobic thermostats, the atmosphere of the thermostats was exchanged to nitrogen, and the oxygen concentration was adjusted to 1,6%, by introducing the necessary amount of air. The plates were incubated at 32 °C, and after 72 hours the Azospirillum colonies are identified. According to the dilution, on the plate made from the 10x diluted suspension continuous bacterium layer developed, and from the 10000x diluted suspension generally 30-50 colonies developed. The Azospirillum colonies grew to 3 mm of diameter,

differently from the other small bacterial or fungal colonies. Some colonies were morphologically similar to the Azospirillum colonies. Some of these were further studied.

The Nfb(II) soft agar cultures were put into anaerobic thermostat, using the medium and cultural conditions mentioned above. In these conditions mainly the Azospirillums multiplied, with recognizable, characteristic morphology.

The different Azospirillum strains isolated from the MM and Nfb(II) media were purified according to general microbiological practice, the primary bacterium colony plated twice to one colony on complete Tag medium. The Azospirillum bacterium strains purified twice to one colony were propagated in liquid Tag medium, and the stock cultures were stored. The bacterium suspension stored at -80 °C was considered to be the stock culture, and every experiments started from this culture. The composition of the Tag medium is the following:

| | |
|---|---|
| Bacto Trypton (Difco) | 1,0% |
| Yeast Extract (Difco) | 0,1% |
| NaCl | 0,5% |
| Agar | 2,5% |

After sterilization the aqueous solutions of the following substances were added, in the following final concentration:

0,1% 0,1 mole/l $CaCl_2$ x 6 $H_2O$

0,1% 0,1 mole/l $MgCl_2$ x 6 $H_2O$

0,2% glucose (sterilized separately).

The pH of the medium was adjusted to 7,2 after sterilization.

The root colonization could be demonstrated by the following simple experiment. On the root of the maize and wheat plants, planted into sterile perlit treated with MM medium (pot of 15 cm diameter) and treated with equal number ($10^{10}$ cells per pot) of Azospirillum bacteria, on basis of microscopic counting significantly more Azospirillum cells were detectable than on the root of the controls. Root colonization occurs on the basis of specific recognition mechanism. During colonization the Azospirillum cells

penetrate in the intercellular space of the plants, and with their active nitrogen fixation they can provide part of the nitrogen need of the host plant (associative nitrogen fixation). This was demonstrated in our laboratory experiments by the production of higher amount of green mass by the maize and wheat treated with the Azospirillum strains, the results of which are presented hereunder. The Azospirillum cells produced phythohormones and growth promoting substances. The production and beneficial results of these substances could be demonstrated by improved germination effectiveness and more intensive plant development, with experiments conducted under laboratory conditions.

The morphologic characteristics of the Azospirillum species are given in the general part of this description.

Microorganisms belonging to the genus Azotobacter were isolated from field soil samples collected in the Esztergom region. by selective cultivation on Nbf(II) soft agar and nitrogen-free MM medium. One of our strains which, on basis of the taxonomic markers and carbon source utilization spectrum was identified as Azotobacter vinelandii, and intensively fixed the molecular nitrogen of the atmosphere, was named ESZ 2132, and was made resistant to different plant protection agents (herbicides), defined hereunder, and deposited.

The nitrogen fixing ability of the Azospirillum and Azotobacter strains was determined by acetylene-reduction method also. According to the method [Dilworth, M.J.: J. Biochem. Biophys. Acta 127, 285 (1966)] to a culture in a closed container acetylene was added with injection syringe, and after a 12 hour incubation, 0,25 ml gas mixture was injected in the Propak N column of a Perkin-Elmer gas chromatograph. The acetylene and ethylene concentration of the gas mixture was determined by flame-ionization detector. From the height of the acetylene and ethylene peaks the activity of the nitrogenase enzyme-complex unanimously could be determined. Our Azospirillum and Azotobacter strains reduced 5-55 nmole acetylene to ethylene per hour.

Example 2.

The isolation of microorganisms solubilizing phosphate and producing phythohormones

In different regions of Hungary soil samples were collected, the aqueous suspensions of the samples were plated on Tag medium (see above), and the isolates having Pseudomonas and Bacillus colony- and cell-morphology were investigated.

The different Pseudomonas and Bacillus bacterium strains were purified according to general microbiological practice, the primary bacterium colony plated many times to one colony on complete Tag medium. The bacterium strains purified by plating were propagated in liquid and solid Tag medium, and stored.

The phosphate mobilizing abilities of the microorganisms were determined on modified Pikovskaya (HP) medium, containing 1% hydroxyapatite, and Nutrient Agar (Oxoid), supplemented with 10% tricalcium-phosphate and 0,2% glucose.

The composition of the media mentioned above was the following:

Modified Pikovskaya medium:

| | |
|---|---|
| Hydroxyapatit | 1,0% |
| $(NH_4)_2SO_4$ | 0,05% |
| NaCl | 0,02% |
| KCl | 0,02% |
| $MgSO_4 \times 7\ H_2O$ | 0,01% |
| Yeast Extract (Difco) | 0,05% |
| Agar | 1,5% |
| Glucose (sterilized separately) | 1,0% |

The solutions of the following compounds were supplied to the medium after sterilizing:

| | |
|---|---|
| 1% | 20 mg/100 ml $FeSO_4 \times 7\ H_2O$ |
| 1% | 40 mg/100 ml $MnSO_4 \times H_2O$ |

Before sterilization the pH of the medium was adjusted to 7,2.

Naoxg: Nutrient Agar (Oxoid) prepared according to the description of the manufacturer + 0,2% glucose.

When testing the phosphate-solubilizing ability of the strains on Pikovskaya (HP) medium, selected during the pilot experiments, the colonies grown for 3-4 days at 30 °C developed 27 and 34 mm clearing ring. The suspensions of these same strains, prepared from TAg slants with 1 ml distilled water, were put into the holes made in Naoxg plates, supplemented with 10% tricalcium-phosphate (0,1 ml/hole). Incubating the cultures at 30 °C, in 2-3 days well visible clearing rings could be observed. In case of the thoroughly tested Pseudomonas strains the sizes of these rings were 21 and 23 mm, in case of the Bacillus the size was 27 mm.

Each of the two Pseudomonas strains (named mob4 and mob21), and the Bacillus strain proved to have strong phosphate solubilizing effect. The strains dissolved both inorganic phosphate form well detectably, so it can be determined, that they have good phosphate-solubilizing properties.

The siderophore and hormone producing abilities of the strains were tested on the King B medium, generally used for the cultivation of the Pseudomonae. The composition of the medium was the following:

| | |
|---|---|
| Peptone | 2% |
| Glycerol | 1% |
| $KH_2PO_4$ | 0,15% |
| $MgSO_4$ x 7 $H_2O$ | 0.15% |
| Agar | 2% |

Before sterilization the pH of the medium was adjusted to 7,2 by NaOH.

The Pseudomonas strains producing siderophores bound the iron ions, which were transferred to the plants even in iron-depleted soils. Besides, they hindered the propagation of some plant pathogen strains, for example the Erwinia caratovora, as they can not utilize the fixed iron. The siderophore production was tested by the inhibition of the growth of *Escherichia coli* MC1061. The cell suspensions of the two strains were prepared from King B agar culture, and then dripped (30-50 µl) to King B plate, not containing or containing iron (1 µmole/l $FeCl_3$ x 7 $H_2O$) for 48 hours, cultured at 28 °C, then the plate was sprayed by the *Escherichia coli* MC1061 culture harvested from TAg

agar, and the incubation was continued for another 28 hours at 28 °C. Different inhibition zones were observed around the cultures. The results of the average of four experiments are given in Table 1 (Bacillus megaterium wa used as negative control, and Pseudomonas fluorescens is used as positive control).

Table 1.

| Microorganism | King B inhibition zone | King B + 1 μmole/l $FeCl_3$ Inhibition zone |
|---|---|---|
| mob4 | +++* | - |
| mob21 | + | - |
| P. fluorescens | ++ | - |
| B. megaterium | + | - |

*Degree of inhibition: - no inhibition

\+ weak inhibition

++ strong inhibition

+++ very strong inhibition

During the investigation of the siderophore production significant inhibition zone was observed at the mob4 and the positive control strain, which disappeared in the presence of iron ions (in the presence of sufficient amount of iron the siderophores produced by Pseudomonae did not inhibit the iron uptake, and thus, did not inhibit the growth of the bacteria).

It was noted earlier that some Pseudomonas strains produced plant hormones. Our selected microorganisms were tested for the capability, whether they are able to biosynthesize gibberellic acid on TAg medium, the composition of which was given above. The investigations were performed by silicagel thin layer chromatography (Merck), using gibberellic acid A as standard (40 μg/ml, produced by Phylaxia Pharma, Budapest), after the extraction of the cultures by ethylacetate, the extract was extracted by twice the volume of sodium-hydrogen-carbonate, and extracted again in a solution of pH 2,5 with ethyl-acetate. In the evaporation residue of the extract a spot was found with Rf value close to that of the standard, at our following strains:

Table 2.

| Strain | $R_f$ | Size of the spot ($mm^2$) |
|---|---|---|
| standard | 0,41 | 24 |
| mob4 | 0,46 | 17 |
| mob21 | 0,42 | 27 |
| P. fluorescens | 0,49 | 14 |
| B. megaterium | 0,57 | 7 |

The mob4 and mob21 strains were selected (they produced about 3-15 μg hormone per milliliter), and they were named Pseudomonas ssp.

The strains were investigated and identified according to the procedure described above.

Example 3.

Isolation of polysaccharide producing microorganisms

3A. Isolation of Rhizobium strain

The Rhizobium meliloti strain was isolated in September 8, 1996, from the alfalfa field of the Subasa farm, close to Szeged-Kiskundorozsma. From the alfalfa growing in the loess soil a well developing plant was selected, and the well developed nodules were separated from its roots. The nodules were washed by sterile distilled water, triturated, and the particles were suspended in physiological saline solution. Sterile dilution was made from the suspension, and plated on complete medium. The nitrogen-fixation ability of the colonies obtained in 48 hours' incubation was tested in the so called symbiotic commercially available plant test, according to the following protocol: the surface of the alfalfa seeds (Medicago sativa) was sterilized for 2 hours with heat treatment at 72 °C, with 20% hypochlorite solution, and with careful washing, and finally germinated on 1% distilled water agar. The seedlings were put to 1,5% Gibson agar slants (see later) and cultivated in greenhouse for one week. The one week old seedling were inoculated with the cells of a bacterial colony, and cultivated for another 8 weeks in greenhouse. From the strains belonging to the plants showing the three best development (dry weight of the

parts over the root is 22-26 mg, compared to the 3-5 mg weight of the control plants) those were selected which produced the most polysaccharide, and were named Rhizobium meliloti var.

The composition of the Gibson slant agar:

| | |
|---|---|
| $KH_2PO_4$ | 0,6 g |
| $K_2HPO_4$ | 0,6 g |
| $CaCl_2 \times 2\ H_2O$ | 0,171 g |
| NaCl | 0,01 g |
| $MgSO_4 \times 7\ H_2O$ | 0,2 g |
| $FeCl_3 \times 6\ H_2O$ | 0,27 g |
| Trace element solution* | 1,0 ml |
| Water distilled to | 1000 ml |

The pH of the solution is adjusted to 7,0 by 1 mole/l aqueous KOH solution

*The composition of the Gibson trace-element solution:

| | |
|---|---|
| $H_3BO_3$ | 3,0 g |
| $MnSO_4 \times 4\ H_2O$ | 2,23 g |
| $ZnSO_4 \times 7\ H_2O$ | 0,287 g |
| $CuSO_4 \times 5\ H_2O$ | 0,125 g |
| $CoCl_2$ | 0,065 g |
| $NaMoO_4 \times 2\ H_2O$ | 0,242 g |
| Water distilled to | 1000 ml. |

3B. Isolation of Alcaligenes and Bacillius strains

Soil samples were collected in different regions of Hungary, the aqueous suspension of the samples was plated on TAg medium, and the isolates having Alcaligenes and Bacillus colony- and cell-morphology were investigated. The exopolysaccharide producing cell lines were selected.

The different Alcaligenes and Bacillus strains were purified according to general microbiological practice, the primary bacterium colony being plated many times to one

colony on complete Tag medium. The bacterium strains purified by plating were propagated in liquid and solid Tag medium, and stored.

According to our investigations the Alcaligenes strain selected biosynthesized water soluble, succinoglycane type polysaccharide of known structure. The microorganism cultivated under the name Alcaligenes sp. 67-91 was investigated taxonomically (see above) and it proved to be Alcaligenes faecalis.

The selected strain, named Bacillus sp. spore+25, producing polysaccharide, was investigated taxonomically (see above), and it proved to be Bacillus circulans.

The strains were made resistant against the plant protection agents and were deposited under the deposit numbers given above.

Pesticide resistant variants were produced from the polysaccharide producing Rhizobium, Bacillus and Alcaligenes strains, and the experiments described later were conducted with these variants, the purpose of which was the improvement of the structure of the soil.

Example 4.

Formation of resistance against seed-dresser substances in microorganisms

Solutions are made from the following plant protection agents:

Counte 5 G: O,O-diethyl-S-(tert-butyl-thiomethyl)-phosphorodithionate

Chinufur 40 FW: 2,3-dihydro-2,2,-dimethyl-7-benzofuranoil-N-methyl-carbamate

Thimeth 10 G: O,O-diethyl-S-ethyl-thiomethyl-ditiophosphate

Dursbán 5 G: O,O-diethyl-O-(3,5,6-trichloro-2-pyridil)-thiophosphate

Pyrinex 48 EC: O,O-diethyl-O-(3,5,6-trichloro-2-pyridil)-thiophosphate

Abelda plus 80 EC: S-ethyl-diisobutyl-thio-carbamate

Acetin A 500 EC: N-(ethoxy-methyl)-2-ethyl-6-methyl-chloro-acetanilide

Atrazin 500 FW: 2-chloro-4-ethyl-amino-6-isopropyl-amino-triazine

Dikamin D: 2,4-dichloro-fenoxy-acetic acid

Bladex 500 SC: 2-(4-chloro-6-ethylamino-1,3,5-triazine-2-yl-amino)-2-methyl-propionitrile

Igran 500 FW: 4-ethylamino-2-tert-butylamino-6-methyl—trio-triazine

Dual 960 EC: 2-ethyl-6-methyl-N-(1-methyl-2-methoxy-ethyl)-chloro-acetanilide

Olitref: 2,6-dinitro-N,N-dipropil-4-trifluoro-methyl-aniline

Flubalex: N-ethyl-N-(n-butyl)-2,6-dinitro-4-trifluoro-methyl-aniline

Trophy: N-(ethoxy-methyl)-2-ethyl-6-methyl-chloro-acetanilide

Stomp 330: N-(1-ethyl-propyl)-3,4-dimethyl-2,6-dinitro-aniline

Fundazol (benomyl): 1-butyl-carbamoil-benzimidazol-2-methyl-carbamate

The first four compounds are insecticides, seed-dressers, the Fundazol is an antifungal agent. The other substances on the list are herbicides.

The microorganisms selected according to the criteria described in Examples 1-3. were plated to Tag media (the composition of this medium is defined above), containing different quantities of the plant protection agents. Colonies were selected which still grew in the presence of 2% (20000 µg/ml) compound, and isolated. If the microorganism selected did not grow in the presence of the smallest amount of plant protection agent, the degree of resistance was improved by the known reinoculation method.

The microorganisms were resistant at least to 2% concentration of plant protection agent, some isolates were able to multiply in the presence of even higher concentrations of plant protection agent.

Example 5.

Cultivation of microorganisms

5A. Cultivation on complete medium:

From the TAg medium (the composition is given above) slant cultures were prepared, and incubated at 30 °C for 48 hours. From the Azospirillum, Azotobacter, Bacillus, Rhizobium, Pseudomonas, Alcaligenes or Micrococcus cultures the following medium (named Ta1i) was inoculated:

| | |
|---|---|
| Glucose (sterilized separately in 50% aqueous solution) | 0,5% |
| Molasses | 1,5% |
| Corn steep liquor (50% dry weight) | 1,5% |
| Gistex Yeast extract | 0,2% |

| | |
|---|---|
| Acid casein | 0,1% |
| Ammonium-sulfate | 0,1% |
| Ammonium-nitrate | 0,1% |
| Calcium-carbonate | 0,3% |
| Potassium-dihydrogene-phosphate | 0,1% |
| NaCl | 0,1% |
| $MgSO_4 \times 7\ H_2O$ | 0,1% |
| Palm oil | 0,2% |

Each 100 ml of medium were put into Erlenmeyer flasks, and sterilized at 121 °C for 30 minutes. The sterile media were inoculated with the microorganisms grown on slants, and in case of the Azospirilum and Bacillus strains at 37 °C, in case of the other microorganisms the cultures are propagated at 30 °C, on a rotary shaker with 260 rpm, for 36 hours. The growth was controlled by microscopic observation, and 5% Talf main fermentation medium was seeded with 5% inoculum. The composition of Talf is the following:

| | |
|---|---|
| Glucose (sterilized separately in 50% aqueous solution) | 1,5% |
| Molasses | 2,5% |
| Corn steep liquor (50% dry weight) | 1,5% |
| Gistex Yeast extract | 0,4% |
| Acid casein | 0,4% |
| Ammonium-sulfate | 0,2% |
| Ammonium-nitrate | 0,2% |
| Calcium-carbonate | 0,3% |
| Potassium-dihydrogene-phosphate | 0,2% |
| NaCl | 0,1% |
| $MgSO_4 \times 7\ H_2O$ | 0,2% |
| Trace element solution* | 0,45% |
| Palm oil | 0,2% |

The composition of the trace element solution is given in Example 1.

The cultures in the flasks were cultivated on rotary shaker, while the microorganisms in the fermenters were grown in the usual way, using v/v aeration, dual influx turbo agitator for 24 hours when cell count per milliliter reached, depending on the bacterium just cultivated, the value of $4 \times 10^8$ - $1,3 \times 10^9$.

For obtaining higher amount of culture on the Tal1 medium as defined above, using the fermentation conditions described above, 10 cultures were prepared, and 5-5 liters were used to inoculate of each 100 l of sterilized Tal1 and Tal2 medium. Cultivation was carried out for 24 hours under the fermentation conditions as defined above, and the culture grown on Tal2 medium, after controlling, was applied on the locus while 50 l of the culture grown on the Tal1 medium was used to inoculate 1000 l of sterilized Tal2 medium. The cultivation was conducted for 24 hours under the fermentation conditions as given above, and after controlling, the culture was utilized. In case of extraordinarily strong foaming then 0.01% polypropylene-glycol was added as anti-foaming agent.

5B. Cultivation on semi-minimal medium

The process described in Example 5A. was repeated, with the difference that instead of the Tal2 medium Ta2f medium was used, with the following composition:

| | |
|---|---|
| Glucose (sterilized separately in 50% aqueous solution) | 1,5% |
| Molasses | 0,5% |
| Acid casein | 0,1% |
| Ammonium-sulfate | 0,7% |
| Ammonium-nitrate | 0,5% |
| Calcium-carbonate | 0,3% |
| Potassium-dihydrogene-phosphate | 0,3% |
| NaCl | 0,1% |
| $MgSO_4 \times 7\ H_2O$ | 0,1% |
| Trace-element solution* | 0,35% |

Palm oil 0,2%

*The composition of the trace-element solution is given in Example 1.

At the end of cultivation the cultures contained, depending on the type of bacterium, 1-7 x $10^8$ cells per milliliter.

Example 6.

Improvement of the structure of the soil and plant cultivation experiments

6A. Experiments for improvement of the structure of the soil

Sandy soil collected at the river Danube at Dömös, and clay collected in Esztergom were placed into 90 x 90 cm trays, the height of the soil was 25 cm. The sandy soil was treated with 10 g of ammonium-nitrate and 5 g of calcium-phosphate per tray. The soil was seeded with maize, 81 seeds per tray. At evaluation, the data of the five biggest and the five less developed plants were not considered. The mass of the roots, washed with distilled water, was measured after two days desiccation at 45 °C. No. 1 trays were abundantly watered, No.2 trays were abundantly watered at bed-planting, later they were not watered at all. The trays marked "a" were inoculated with the cultures of Bacillus circulans var. Res.97, Rhizobium meliloti PolRes.7 and Alcaligenes faecalis var. Res36 strains, prepared according to Example 5A. in a rate of $10^8$ cells per square meter. The trays marked "b" were not treated with microorganisms.

In Table 3. the results are given at 33 days after bed-planting. The results are given as average, calculated to one plant.

Table 3.

| Soil | Tray | Treatment | Height of the plant (cm) | Mass of the root (mg) |
|---|---|---|---|---|
| Sandy | No.1 | a | 23 | 956 |
| | | b | 16 | 655 |
| | No.2 | a | 19 | 876 |
| | | b | 11 | 766 |
| Clay | No.1 | a | 28 | 1154 |
| | | b | 23 | 1222 |

| | | | |
|---|---|---|---|
| No.2 | a | 21 | 1015 |
| | b | 18 | 677 |

In Table 4, the friability and fracture of the non-watered (No.2 trays) sandy and clay soils is given. Friability means that the size of most of the soil pieces shot to a paper and slightly joggled, not falling apart, are under 2 mm (-), between 2-5 mm (+), or are over 5 mm (++). The degree of fracture of the surface of the soil is marked according to the followings: no fracture (++), mild, rather capillary fracture (+), and the big fractures, characteristic of the droughty fields are marked by (-).

Table 4.

| Soil | Treatment | Friability | Fracture |
|---|---|---|---|
| Sandy | a | + | + |
| | b | - | - or + |
| Clay | a | ++ | + |
| | b | ++ | ++ |

It concludes from the data of Table 3. and 4., that the presence of the microorganisms producing polysaccharides helps the development of the plants and the formation of the advantageous soil structure.

6B. Greenhouse experiments

The experiments were conducted with soils collected from different fields of Hungary, containing 110-620 mg phosphorous, 40-110 mg nitrogen and 600-3800 mg potassium per kilogram. The desired temperature of the greenhouse was assured by heating and ventilation, the humidity of the air was controlled by vaporization.

The seeds were planted according to the method of Example 6A., the results are always calculated to one plant. Evaluations were done on the 23-92. days, counted from planting.

The results of the experiment performed with maize are on Table 5A. and 5B., those of the wheat in Table 5C., while results with tomato (Lycopersicon lycopersicon,

Kecskeméti zömök) in Tables 5D. and 5E. The maize and the wheat were treated with the aqueous formulation according to Example 7B., the tomato was treated according to the example 7C. 8 tomato seedlings were cultivated in one tray.

In the first column (Treatment) 1 shows the results received with the plants grown on the non-treated trays, 2 shows the results received with the plants grown on the trays treated with the optimal amount of fertilizer, 3 shows the results received with the plants grown on the trays treated with the bacterium, while 4 shows the results received with the plants grown on the trays treated with the optimal amount of fertilizer and bacterium.

Table 5A.

Development of maize on sandy soil

| Treatment | The height of the plant (cm) | The mass of the roots (mg) |
|---|---|---|
| 1 | 16 | 790 |
| 2 | 17 | 780 |
| 3 | 19 | 990 |
| 4 | 22 | 910 |

Table 5B.

Development of maize on clay soil

| Treatment | The height of the plant (cm) | The mass of the roots (mg) |
|---|---|---|
| 1 | 21 | 790 |
| 2 | 21 | 810 |
| 3 | 26 | 1120 |
| 4 | 25 | 940 |

Table 5A.

Development of wheat on sandy soil

| Treatment | The height of the plant (cm) | The mass of the roots (mg) |
|---|---|---|
| 1 | 15 | 350 |
| 2 | 24 | 475 |
| 3 | 21 | 870* |
| 4 | 24 | 840* |

*The roots are more branching than usual, the root-hairs are extremely developed.

Table 5D.

Development of tomato on sandy soil, with weak watering, on the 65th day of cultivation

| Treatment | The mass of the green parts of the plant (g) | The number of flowers |
|---|---|---|
| 1 | 1250 | 8 |
| 2 | 1740 | 9 |
| 3 | 1910 | 12 |
| 4 | 2110 | 14 |

Table 5E.

Development of tomato on rich, humic soil, with ample watering, on the 65th day of cultivation

| Treatment | The mass of the green parts of the plant (g) | The number of flowers |
|---|---|---|
| 1 | 2300 | 17* |
| 2 | 2210 | 15* |
| 3 | 2540 | 19 |
| 4 | 2370 | 15 |

*On the leaves spots, indicative of fungal (or bacterial) infection can be seen, later these turn to strong blight.

Example 7.

Preparation of compositions containing pesticide resistant microorganisms according to the present invention

7A. Preparation of microorganism mixture:

The following cultures: Azospirillum lipoferum ssp. lip7R 885 (NCAIM P(B) 001253), Azospirillum amazonense ssp.: K21R 887 (NCAIM P (B) 001256), Azospirillum irakense ssp. 5041R 889 (NCAIM P (B) 0011255), Azospirillum brasilense ssp. A41R 879 (NCAIM P (B)001254), Azotobacter vinelandii ssp. ESZ 2132 (NCAIM P (B) 001257), Pseudomonas sp. Szeged-344 O.P. 14 (NCAIM P (B) 001251), Pseudomonas fluorescens var. MOB24, Res24 (NCAIM P (B) 001252), Bacillus circulans var. Res. 97 (NCAIM P (B) 001261), Bacillus megaterium var. Res. 54 (NCAIM P (B) 001250), Rhizobium meliloti var. PolRes. 7 (NCAIM P (B) 001259), Alcaligenes faecalis var. Res36 (NCAIM P (B) 001260) and Phyl106-R+324 (NCAIM P (B) 001258), prepared according to Example 5, were mixed in equal amounts, between 5-50, advantageously 25 liters/hectare amount, and they were applied on the fields to be treated in any frost-free period of the year, advantageously between March and October.

7B. Composition for the treatment of monocotyledonous plants

Composition was prepared according to the procedure of 7A., with the difference, that the following microorganisms were used: Azospirillum lipoferum ssp. lip7R 885 (NCAIM P(B) 001253), Azospirillum amazonense ssp.: K21R 887 (NCAIM P (B) 001256), Azospirillum irakense ssp. 5041R 889 (NCAIM P (B) 0011255), Azospirillum brasilense ssp. A41R 879 (NCAIM P (B)001254), Azotobacter vinelandii ssp. ESZ 2132 (NCAIM P (B) 001257), Pseudomonas fluorescens var. MOB24, Res24 (NCAIM P (B) 001252), Bacillus circulans var. Res. 97 (NCAIM P (B) 001261), Bacillus megaterium var. Res. 54 (NCAIM P (B) 001250) and Phyl106-R+324 (NCAIM P (B) 001258).

7C. Composition for the treatment of dicotyledonous plants

Composition was prepared according to the procedure of 7A., with the difference, that the following microorganisms are used: Azospirillum lipoferum ssp. lip7R 885 (NCAIM P(B) 001253), Azospirillum amazonense ssp.: K21R 887 (NCAIM P (B) 001256), Azospirillum irakense ssp. 5041R 889 (NCAIM P (B) 0011255), Azospirillum brasilense ssp. A41R 879 (NCAIM P (B)001254), Azotobacter vinelandii ssp. ESZ 2132 (NCAIM P (B) 001257), Pseudomonas sp. Szeged-344 O.P. 14 (NCAIM P (B) 001251), Pseudomonas fluorescens var. MOB24, Res24 (NCAIM P (B) 001252), Bacillus circulans var. Res. 97 (NCAIM P (B) 001261), Bacillus megaterium var. Res. 54 (NCAIM P (B) 001250), Rhizobium meliloti var. PolRes. 7 (NCAIM P (B) 001259), Alcaligenes faecalis var. Res36 (NCAIM P (B) 001260) and Phyl106-R+324 (NCAIM P (B) 001258) and applied on the fields to be treated, in any frost-free period of the year, advantageously between March and October.

7D. Preparation of lyophilized composition

2 liters of the microorganism suspension prepared according to Example 7A. were lyophilized in Gelman SP54 lyophilizer, according to the instructions appended to the instrument. The dry microorganism powder was used, depending of the intended use, per se, or mixed with calcium-carbonate, starch, glucose or cellulose in a ratio of 1:1 - 1:1000 until use, the preparation was stored at 4 °C to 10 °C. Before use an aqueous suspension was prepared therefrom and introduced into the arable soil.

7E. Preparation of a composition containing vehicle

The cultures prepared on the medium according to Example 5B. were mixed preferably in equal ratio, and the mixture was blended with manure, soy-flour (average size 4 mesh), methyl cellulose or potato starch, so that the preparation contained 5 x $10^8$ to $10^{10}$ microorganisms per gramm, preferably 5 x $10^9$ microorganism cells per gramm, and the preparation, wet or dried at about 40 °C was applied on the arable soil to be treated with an amount of 2-20, preferably 5 kg/ha. At least $10^{13}$ microorganism cells were introduced into the one hectare of arable land.

7F. Treatment of the seeds with the preparation according to the invention

To ten litre of the mixture of equal amounts of cultures prepared according to Examples 5A. or 5B. diluted with water, corn was added (advantageously 300 kg), so that the suspension remained wet. Then the seeds were dried at 40 °C by spreading, and planted. Altogether about $10^{14}$ microorganism cells adhered to the seeds. In this way, about $10^{8}$ microorganism cells were taken up by a seed.

CLAIMS

1. Preparations containing a living microorganism or microorganisms useful for the treatment of the soil and plant seeds, which are able to multiply in certain type of soil and in the environment of a plant and are pesticide resistant, wherein as microorganism the said preparation contains the culture of at least one of the following microorganisms deposited at the National Collection of Agricultural and Industrial Microorganisms: Azospirillum lipoferum ssp. lip7R 885 (NCAIM P(B) 001253), Azospirillum amazonense ssp.: K21R 887 (NCAIM P (B) 001256), Azospirillum irakense ssp. 5041R 889 (NCAIM P (B) 0011255), Azospirillum brasilense ssp. A41R 879 (NCAIM P (B)001254), Azotobacter vinelandii ssp. ESZ 2132 (NCAIM P (B) 001257), Pseudomonas sp. Szeged-344 O.P. 14 (NCAIM P (B) 001251), Pseudomonas fluorescens var. MOB24, Res24 (NCAIM P (B) 001252), Bacillus circulans var. Res. 97 (NCAIM P (B) 001261), Bacillus megaterium var. Res. 54 (NCAIM P (B) 001250), Rhizobium meliloti var. PolRes. 7 (NCAIM P (B) 001259), Alcaligenes faecalis var. Res36 (NCAIM P (B) 001260) and Phyl106-R+324 (NCAIM P (B) 001258)

2. The preparation according to claim 1 wherein the cell(s9 are present in an amount of 5 x $10^8$-$10^{10}$ cells per gramm and a wet or dry vehicle which is not toxic to the microorganisms and is agriculturally acceptable is also present.

3. Preparations of claim 2 wherein said preparation contains water and/or soy-flour and/or starch and/or cellulose and/or glucose or their derivatives as vehicle.

4. Method for preparing compositions useful in the treatment of soil and plant seeds and/or preparations containing living microorganisms, which are able to multiply in certain type of soil and in the environment of a plant and are pesticide resistant, wherein the following microorganisms, deposited at the Collection of Agricultural and Industrial Microorganisms: Azospirillum lipoferum ssp. lip7R 885 (NCAIM P(B) 001253), Azospirillum amazonense ssp.: K21R 887 (NCAIM P (B) 001256), Azospirillum irakense ssp. 5041R 889 (NCAIM P (B) 0011255), Azospirillum brasilense ssp. A41R 879 (NCAIM P (B)001254), Azotobacter vinelandii ssp. ESZ 2132 (NCAIM P (B) 001257), Pseudomonas sp. Szeged-344 O.P. 14 (NCAIM P (B) 001251), Pseudomonas

fluorescens var. MOB24, Res24 (NCAIM P (B) 001252), Bacillus circulans var. Res. 97 (NCAIM P (B) 001261), Bacillus megaterium var. Res. 54 (NCAIM P (B) 001250), Rhizobium meliloti var. PolRes. 7 (NCAIM P (B) 001259), Alcaligenes faecalis var. Res36 (NCAIM P (B) 001260) and Phyl106-R+324 (NCAIM P (B) 001258), are cultivated separately or together in a medium containing assimilable carbon and nitrogen source and inorganic salts, until the cultures reach a cell count of $5 \times 10^8$ - $5 \times 10^9$, if desired, the culture(s) obtained are blended in a given ratio, and/or applied on and, if desired, freeze-dried or conventionally dried.

5. The method of claim 4. wherein glucose and/or saccharose and/or molasses are used as carbon source, ammonium chloride and/or ammonium nitrate and/or ammonium sulfate and/or corn steep liquor and/or casein hydrolysate are used as nitrogen source, and calcium carbonate and salts dissociating to sodium-, potassium-, magnesium-, calcium-, iron-, nitrate, chloride-, sulfate-, carbonate ions, or salts dissociating to trace elements are used as inorganic salts.

6. Pesticide resistant microorganisms, which are deposited at the Collection of Agricultural and Industrial Microorganisms, under the following deposit numbers: Azospirillum lipoferum ssp. lip7R 885 (NCAIM P(B) 001253), Azospirillum amazonense ssp.: K21R 887 (NCAIM P (B) 001256), Azospirillum irakense ssp. 5041R 889 (NCAIM P (B) 0011255), Azospirillum brasilense ssp. A41R 879 (NCAIM P (B)001254), Azotobacter vinelandii ssp. ESZ 2132 (NCAIM P (B) 001257), Pseudomonas sp. Szeged-344 O.P. 14 (NCAIM P (B) 001251), Pseudomonas fluorescens var. MOB24, Res24 (NCAIM P (B) 001252), Bacillus circulans var. Res. 97 (NCAIM P (B) 001261), Bacillus megaterium var. Res. 54 (NCAIM P (B) 001250), Rhizobium meliloti var. PolRes. 7 (NCAIM P (B) 001259), Alcaligenes faecalis var. Res36 (NCAIM P (B) 001260) and Phyl106-R+324 (NCAIM P (B) 001258).

7. Method for preparing of microorganisms of claim 6, wherein sample is collected from the environment of the given plant, from the upper 40 cm layer of the given soil type, the microorganisms selected are identified, and made them resistant to pesticides generally used for the treatment of the given plant.

8. Method for treating soil with the composition of claim 1, wherein $10^{10}$-$10^{14}$, prepferably $10^{11}$-$10^{12}$ microorganism cells are applied into or onto the soil, in frost-free periods.

9. Method for treating of plant seeds with the compositions of claim 1, wherein the seeds are dressed with liquid composition containing at least one of these microorganisms of claim 1 or their mixture.

10. Method for improving maintenance of the structure of the soil, wherein microorganisms biosynthesizing polysaccharides of microbial origin, preferably succinoglycane and/or curdlane, are introduced into the soil.

11. Method according to claim 9, wherein the compositions containing microorganisms biosynthesizing polysaccharides, preferably Bacillus circulans var Res.97 (NCAIM P(B) 001261) and/or Rhizobium meliloti var. PolRes.7 (NCAIM P (B) 001259) and/or Alcaligenes faecalis var. Res36 (NCAIM P(B) 001260) strains are used.