Ogni albero robusto che troneggia sugli esseri umani
deve la sua esistenza ad un nocciolo radicato in profondità
(Morihei Ueshiba, "l'Arte della Pace")

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QUALITY CONTROL OF MICROBIALS

PhD student: Dr.ssa Silvia Volpato

Tutor: Prof. Emilio Stefani

PhD School Coordinator: Prof. Alessandro Ulrici

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1 INTRODUCTION

1.1 SYMBIOTIC AGRICULTURE

There are many doors that have opened in agriculture over the last few centuries: from the conventional to the sustainable, from the integrated to the biological up to the biodynamic one, focused on the anthroposophical spiritual vision of the world developed by Rudolf Steiner for the production of food, in greater balance with the terrestrial ecosystem **(Chalker-Scott, 2013).**

Today symbiotic agriculture comes: an improvement, or an evolution intending to make agricultural practices even more sustainable from an economic point of view **(www.agricolturasimbiotica.it)**.

In 1878, during a conference of European naturalists and doctors, the German pathologist Anton H. De Bary introduced, for the first time, the term symbiosis (from the Greek: συμ 'together', βίωσ 'vita') to describe the phenomenon in which two or several organisms live together, all having their advantages. Since then the term found widespread use in the psychoanalytic world and in everyday life, where it is used to indicate a close relationship created between individuals living together and the consequent mutual dependence **(Oulhen et al., 2016)**.

In the biological sector, and in accordance with the original definition of De Bary **(1878)**, symbiosis is described as an association between individuals of different species, which persists over time and in which the trophic relationships established between the partners of the association are crucial. In fact, symbiosis does not imply an exclusive concept of mutual benefit: it includes relationships that are established with mutual benefit (mutualistic symbioses), antagonistic relationships (pathological symbioses) and relationships from which a single organism benefits (commensalism).

These relationships are not unchangeable properties of the association, but often depend on the environment in which the association is found or, additionally, on the developmental stage of one or both symbiotic partners **(Douglas, 1994).**

However, from the years 1915-1920 onwards, a univocal positive meaning was given to the term: Symbiosis is a close relationship between two or more organisms from which everyone benefits. It is precisely from this bond that exists in nature that symbiotic agriculture was born. It is a new method of cultivation and breeding based on the respect and enhancement of the symbiosis between the actors of the food chain: the soil, animals, microbial communities and humans. Among these actors, it is precisely in soil care and management that symbiotic agriculture achieves its maximum productivity. This kind of cultivation, in fact, focuses on all the good and functional microorganisms present and living in soil - such as fungi, bacteria and yeasts - because they work in symbiosis with the plant, supporting and promoting its growth, keeping it healthy and guaranteeing the production of very high quality crops. An approach, therefore, that takes into account and sustain the enormous microbiological variety present in the soil.

Soils are highly diverse. It has been estimated that 1 g of soil contains up to 1 billion bacterial cells, consisting of tens of thousands of taxa, up to 200 m fungal hyphae, and a wide range of mites, nematodes, earthworms, and arthropods **(Roesch et al., 2007; Bardgett, 2005).** This vast and hidden biodiversity contributes to the total terrestrial biomass and is intimately linked to above-ground biodiversity **(Fierer et al., 2009; Wardle et al., 2004).**

A major effort was made to increase the positive impact of organic farming practices. One possibility could be the synergic effect of combiningthe organic farmingwith the use of selected microorganisms able to promote both the plant growth and the bioremediation of contaminated soils derived from intensive farming **(Baez-Rogelio et al., 2016).**All plant-associated microenvironments, especially the rhizosphere, are colonized in high abundances by microbes **(Berg et al., 2005)**. Among these microorganisms we can find several symbiotic fungi, therefore good fungi that colonize plants and have a key role in extending (dimensionally and functionally) the plant root system and, possibly, putting in

communication all the plants. The link between a symbioticfungus and its host plant is called **mycorrhiza (Balestrini and Lanfranco, 2006).**

Mycorrhizae are mutualistic symbiotic associations established between the roots of plants and some soil fungi. Depending on the particular anatomical structures different types of associations can be distinguished from the type of fungus involved and plant colonized. Traditionally, mycorrhizae are distinguished in ectomycorrhiza, when the fungus develops in the host root in the spaces between the cells through intercellular hyphae, and in endomycorrhiza, when the fungus grows endophytically and develops intracellular structures **(Smith and Read, 1997).** Thanks to this kind of interaction, plants and fungi considerably improve their nutrition, as the fungus has access to the carbohydrates synthesized by the host plant and completes its life cycle **(Hampp et al., 1995)**, while the plant takes great advantage in its vegetative development thanks to improved mineral nutrition through the biorganic processes initiated by the fungus. Between these, the most widespread interactions are arbuscular mycorrhizas (AM). This process of mycorrhization is part of the concept of symbiotic agriculture.

Microbial symbiosis in animals is studied and evaluated in terms of nutrition. Feeding of domesticated livestock in animal husbandry (cattle, swine, sheep and poultry) is done giving to domestic animals two basic types of feed: fodder and forage. From the microbial point of view, they are mostly characterized by the same microorganisms that live in soil, and present in plant matrices as endophytes. The animals, as opposed to humans, who are unable to do it, convert cellulose of fodder into digestible proteins. In this way, functional microorganisms move from the cropping sits to plants and then to animals, thanks to feeding **(www.agricolturasimbiotica.it)**.

Figure 1: trademark of symbiotic agriculture: it is a trademark registered by the consortium composed by CCS Aosta, La Granda, Commerciale Agricola, Tedaldi, Erbalatte and Simbiò and indicates a voluntary privat certification system. Symbiotic agriculture has three targets in balance: improve soil's health and fertility, improve the function of intestine, and create functional food and feed.

Symbiotic agriculture involves three aspects along the food production chain and nutrition:

1. Improve soil fertility and plant health. For this reason, in symbiotic agriculture fertilizers and agrochemical products containing heavy metals are banned. Furthermore, hybrid seeds and genetically modified organisms (GMO) are prohibited. Fertilization is done using natural substances, such as manure. Soil protection is also achieved by using mycorrhizae and functional bacteria in the soil. In this way symbiotic fungi (and beneficial bacteria) are protected, as they are particularly vulnerable to chemical inputs in the agro-ecosystem **(Alori and Babalola, 2018)**.

2. Create functional noutrishment products. Animal nutrition and welfare is, as well as environmental protection, the basis of symbiotic products. Use of hormones or antibiotics is banned for this reason. Animal feeding is based at first on mother's milk and then onagricultural products. The result is food that is different for its nutritional properties **(http://www.agricolturasimbiotica.it).** The same approach is followed when producing fruits and vegetables, following the use of microbial consortia in

agricultural production systems that enhance the microbial biodiversity associated in such products.

3. Improve the function of our second brain: the intestine. The microorganisms represented in the soil microbial communities, later colonizing the agricultural products, soon will reach the intestine of consumers and help to stay in good health, through their role in the absorption of nutrients and their inhibition of noxious bacteria. In this way, the intestinal biota is daily reinforced and maintained. An intestine with many functional bacteria enhances the immune response and protect the body from metabolic diseases. Additionally, there are evidences that food allergies are linked to the consumption of industrial food, often produced in agro-systems with a reduced microbial biodiversity: as an example, tomato production in high throughput glasshouses, grown on soilless matrices.

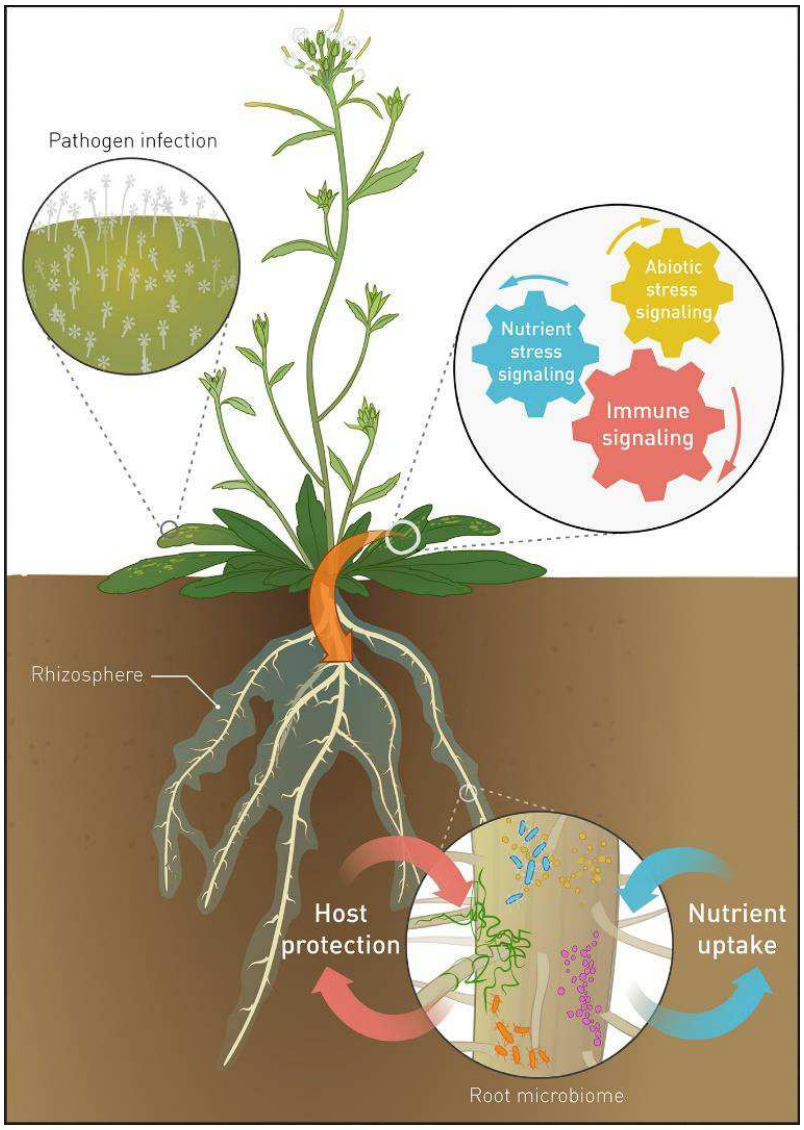


Figure 2: Soil-Borne Legacies: Management of the Root Microbiome.

Plants respond to biotic and abiotic stresses with changes in radical exudates. Radical exudates can act on stresses by improving, for example, the availability of nutrients; they can also influence the radical microbiome. Plants are able to regulate the microbiome in the rhizosphere (the area of soil that surrounds the roots and is influenced by them) by secreting a mixture of stimulating and selective metabolites. Different responses to stress stimulate the functions of the microbiome that the plant requires. It can help the plant absorb nutrients and water or protect it from invasive species, but it can also act as a signal of the adverse conditions experienced by the plant.

Illustration: Jan Karen Campbell **(Bakker P.A.H.M. et al., 2018)**.

The concept of microbial biota

A concept of fundamental importance, linked to symbiotic agriculture, is the **microbial biota**.

The definition of biota is the complex of organisms (plants, animals, etc.) that occupy a certain space in an ecosystem. In this case, we mean the set of symbiotic fungi, bacteria and yeasts, which makes the rhizosphere extremely rich in so-called "good" micro-organisms. These microorganisms constitute the microbial biota, which is necessary for an effective contrast to “harmful” micro-organisms and for growth promotion of agricultural plants **(www.micosat.it)**.

1.2 MICROORGANISMS IN AGRICULTURE

Microorganisms are the smallest forms of life and they play a vital role in any activity within a living organism. Therefore, research based on microbial ecology becomes an important frontier in current biological science. Bacteria, fungi, algae, protozoa, actinomycetes and infectious agents such as viruses are the elements that promote microbial diversity **(Andreote et al., 2014)**. Microbial populations interact and associate with different means. A series of beneficial microorganisms colonizes plants creating a variety of plant-microbe interactions. Some of these interactions are beneficial, while others are harmful to the plant **(Vurukonda et al., 2018)**.

The microorganisms play akey role in crop protection through enhancing the diseaseresistance capacity of plants against pathogens, exhibitingantagonistic activities or actingas biotic elicitors against different biotic and environmental factors. The term "effective microorganism" is usually indicated with a group of known microorganisms with beneficial activity used as microbial inoculants that can be applied to increase the natural microbial diversity of soils and the rhizosphere **(Higaand Wididana, 1991)**.

Microorganisms are valuable in agriculture and forestry and are used in the management of plant diseases, weeds and harmful pests. Fungi, for example, can be useful in case of drought by colonizing the upper parts of plants, increasing tolerance to heat, resistance to insects and various plant diseases **(Singh et al., 2011)**. Thus, the uniqueness of microorganisms and their unpredictable nature andbiosynthetic capabilities make them quite adaptable inspecific environmental and cultural conditions to solvevariousproblems related with crop improvement and disease suppression **(Bhattacharyya et al., 2016)**.

Today many single microorganisms are marketedasmicrobial inoculants. Several fungi pathogenic toinsects (entomopathogenic fungi) are also being used as control agents, including *Beauveria*, *Metarhizium*, *Verticillium* and *Paecilomyces*. These are most frequently used against leaf caterpillars in greenhouses or other places where

the humidity is relatively high. *Beauveria bassiana* can affect a wide variety of arthropods. Environmental conditions, particularly temperature and humidity are important factors affecting the success of fungal microbial treatments, particularly when using preparations of fungal spores. However, establishment of the pathogen may result in death of insects well beyond the period attained by chemical controls. Indeed, the risks of resistance are less and the effects on non-target organisms hugely reduced. In general, when a spore interacts with insect cuticle and attaches, germinates, penetrates the cuticle. Naturally, various entomopathogenic fungi release a wide range of toxins and molecules that induce change in or modify behaviour (*Cordycipitales*, *Trichocomaceae* etc). Since then the fungus has been used successfully to control the larvae of many Lepidoptera **(Sarwar, 2015; Roberts**, **1874)**. The term "biological control", as a feasible strategy for plant disease management was usedfor the first time by C. F. Von in 1914. Since then, various biocontrol products were found to be very effective in controlling the plant diseases. **Sanford (1926)** observed that the potato scab was suppressed by green manuring antagonistic activities. **Weindling (1932)** reported the parasitic nature of *Trichoderma lignorum*on several plant pathogens. **Grossbard (1948)**, **Wright (1954)** and others demonstrated that antibiotics were produced in soil by *Pencilium*, *Aspergillus*, *Trichoderma*, *Streptomyces* spp. After, **Kloepper (1980)** demonstrated the importance of siderophores produced by *Erwinia carotovora*: plant pathogens contain molecules called siderophores, which carry the iron necessary to ensure their survival. It was observed that *Pseudomonas fluorescens* B10 strain effectively complexes iron (III) in soils and makes it unavailable to pathogens. **Howell (1988)** reported P and Q strains of *Trichoderma* sp. **(Junaid at al., 2016**): These two strains of the fungal antagonist *Trichoderma harzianum* were divided into groups "P" and group "Q", based on their production of secondary metabolites. Both strains produced antibiotic substances that make the fungus an effective biocontrol agent. The first commercially registered mycoherbicide consisted of a suspension of chlamydospores of *Phytophthora palmivora* to control *Morrenia odorata* **(McRae, 1988)**.

Several microorganisms such as *Trichoderma harzianum* **(Mohiddin et al., 2010**), *Pseudomonas fluorescens* **(Peighami-Ashnaei et al., 2009)** and *Bacillus subtilis* **(Dawar et al., 2010)** are able to control many foliar and soil-borne fungi, *e.g. Fusarium* spp., *Rhizoctonia solani*, *Pythium* spp., *Sclerotium rolfsii* in vegetables, fruit and industrial crops **(Ngo et al., 2006)**. *Trichoderma* is known to have species containing effective mycoparasitic strains active as biocontrol agents against plant fungal pathogens under a wide range of adverse environmental conditions **(Manczinger et al., 2002)**. *Bacillus* species were found to colonize the root surface, increase the plant growth and cause the lysis of fungal mycelia **(Turner and Backman, 1991; Podile andPrakash, 1996; Takayanagi et al., 1991)**. *B. subtilis* cells can form dormant spores that are resistant to extreme conditions and thus can be easily formulated as biopesticied (or microbials) and stored **(Piggotand Hilbert, 2004)**. *B. subtilis* also produces a variety of biologically active compounds with a broad spectrum of activities toward phytopathogens and that can induce host systemic resistance **(Bais et al., 2004; Stein, 2005; Butcher et al., 2007; Nagorska et al., 2007; Ongena et al., 2007; Ongena and Jacques, 2008)**. Various strains of *B. subtilis* have also been shown to be capable of forming multicellular structures or biofilms **(Branda et al., 2001; Hamon and Lazazzera, 2001; Bais et al., 2004)**. Due to these beneficial traits, *B. subtilis* is potentially useful as a biological control agent. Indeed, it has been reported that some *B. subtilis* strains can effectively suppress the wilt disease in several plant hosts caused by *Ralstonia* spp. **(Lemessa and Zeller, 2007; Aliye et al., 2008; Ji et al., 2008)**. Bacteria, mycorrhiza and other fungi can all contribute to growth promotion and biocontrol in agricultural ecosystems. These organisms may help the plant to acquire nutrients such as iron, phosphorus, nitrogen or water; they may act as biocontrol agents, thereby reducing damage to the plants from pathogenic agents; or they may modulate the levels of important plant hormones such as auxin and ethylene **(Glick, 1995).** The most efficient N-fixing strains belong to the genera *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium* and *Allorhizobium* **(Bloemberg and Lugtenberg, 2001)**. *Pochonia chlamydosporia*

(Kerry, 2000; Khan, 2008), and *P. fluorescens* **(Pal, 2000)** can effectively control the diseases caused by nematodes **(Trabelsi and Mhamdi, 2013)**.

Many progresshas been achieved in recent years in the development and commercialization of bionematicides **(Wilson and Jackson, 2013)**. Examples of this are products containingthe actinobacterium *Streptomyces avermitilis*, which producessecondary metabolites known as avermectins: these compounds are model pesticides, as they are non-toxic to mammals and active against nematodes, even at very low doses. Finally, filtrates of *B. firmus* induce paralysis and mortality of adult nematodes and larvae, including *Radopholus similis*, *Meloidogyne incognita*, and *Ditylenchus dipsaci*, which suggests that the synthesis of toxic metabolites is involved in the control of these pests **(Mendoza et al., 2008)**.

Changes in the soil bacterial community structure caused by the inoculation of microbial-based products could be buffered by ecosystem resilience, which is driven by the level of diversity and interactions of the plant-soil-biota **(Kennedy, 1999)**. The loss of certain bacterial species may, however, not change the functioning of the system because of the bacterial redundancy, since different bacterial species may carry out the same functions **(Nannipieri et al., 2003)**. Microbial inoculants are environmentally friendly termed as eco-friendly: for this reason, microbial formulations are potential alternative to chemical inputs. They could be phyto-stimulants, biofertilizers or microbial biocontrol agents. Naturally occurring microbes, isolated from the natural environments and applied to plants can also be used as bioherbicides. To use microbial inoculants, relatively high numbers of microbial cells are introduced into the competitive soil environment **(Babalola and Glick, 2012)**. Many microorganisms have been studied and are used routinely as microbial inoculants. A contemporary improvement in Classical Microbiology has been observed through the discovery of new species, selection and improvement of known strains until the introduction of non-native genes for the acquisition of expressed products or new functional traits **(Vitorino and Bessa, 2017)**.

Globally, there are several companies in that deal with the research, development and production of microbial consortia containing microorganisms. Therefore, it is particularly important to evaluate the strategy, procedure and methods used by these innovative companies to during the production chains of such microbials, in order to guarantee that effective microorganisms are present in their commercial products.

1.3 MYCORRHIZATION IN AGRICULTURE

Mycorrhization is a very important element to consider in the context of symbiotic agriculture and in the use of microorganisms in agriculture.

Symbiotic mycorrhizal fungi, such as arbuscular mycorrhizal (AM)fungi or ectomycorrhizal (EM) fungi, are ubiquitous component of most ecosystems throughout the world and form a key component of soil microbiota influencing plant growth and uptake of nutrients **(Bethlenfalvay and Linderman, 1992; Van der Heijden et al., 1998)**.

Soil microbial communities surrounding mycorrhizal rootsand extrametrical mycelium are different from those of the rhizosphere ofnon-mycorrhizal plants and the bulk soil **(Katznelson et al., 1962; Garbaye and Bowen, 1987, 1989; Garbaye, 1991)**. Hence, the rhizosphere concepthas been widened to associate this fungal effect, resulting in the introductionof terms "mycorrhizosphere" and "hyphosphere" **(Rambelli, 1973; Linderman,1988)**. The mycorrhizosphere named the zone influenced by both the rootand themycorrhizal fungus, whereas the hyphosphere is the zone surrounding individual fungal hyphae **(Linderman, 1988)**. Specific relationships occurbetween mycorrhizal fungi and mycorrhizosphere microbiota and there isabundant literature attesting that mycorrhizal symbiosis is largely influencedby soil microorganisms **(Rambelli, 1973; Bowen, 1980; De Oliveira, 1988; De Oliveira and Garbaye, 1989)**. However, these interactions have beenmainly focused on the effects of mycorrhizosphere microbial communities on the mycorrhizal formation (extent of mycorrhizal colonization) and on themycorrhizal efficiency on the host plant growth. Recently, it has been proposed that mycorrhizal symbiosis is a component of a microbial complex regulated by multitrophic interactions **(Frey-Klettet al., 2005)**. In addition to their known direct effect on plant growth, mycorrhizal symbionts couldpositively act on host plant development through a selective effect on bacterial communities involved in soil functioning and soil fertility. This new concept of the mycorrhizal symbiosis is

particularly important in tropical and Mediterranean areas, currently subjected to desertification. A desertification process usually results from degradation of natural plant communities (population structure, succession pattern and species diversity) and of physico-chemical and biological soil properties (nutrient availability, microbial activity, soil structure, etc.) **(Garcia et al., 1997; Requena et al., 2001)**. In addition, the loss or reduction of mycorrhizal fungi activity was often detected **(Bethlenfalvay and Schüepp, 1994).** Hence, the management of soil mycorrhizal potential in tropical and Mediterranean environments is of great importance, since mycorrhizal symbiosis determines plant biodiversity, ecosystem variability and productivity directly from its influence on plant mineral nutrition but, also indirectly from its impact on soil microbial functioning. The natural role of mycorrhizosphere microorganisms has been marginalized in intensive agriculture and forest management but, due to the increased environmental awareness, particular interest has been raised on low-input cropping systems. In low-input, sustainable agrosystem production, natural activities of microbes contribute to the biocontrol of pathogens and improve supply of nutrients **(Siddiqui et al., 2008).**

1.4 DISCUSSION AND PURPOSE OF THE THESIS

Experiments carried out in recent years have highlighted the importance of the qualitative and nutraceutical aspects of the products destined to the agri-food chain.

A plant in harmony with beneficial microorganisms (with a "good biota") has a low environmental impact, consumes less resources (in terms of nitrogen and water, for example) and requires less care. In fact, agriculture has always been a collaboration between human attention and a vast community of microbes. The microorganisms that live in the crops of food chains in some cases guarantee the safety of food and offer an environmentally friendly alternative to the massive use of fertilizers and pesticides. They are considered fundamental for the maintenance and/or reconstitution of the microbiological biodiversity of agricultural soils, so often damaged and reduced by treatments with chemical products made by man **(www.micosat.it).**

Microorganisms are then fundamental not only in agriculture, but also for man and his health.

Man cohabits with many micro-organisms, some belonging to each individual, others deriving from food. Thanks to food, in fact, microorganisms move into the intestine, our second brain. As a result, the ground biota reaches the intestinal biota. Intestinal biota affects human health. Diabetes and obesity (up to the highest contradiction of shaky obesity) are two modern diseases that could depend on our intestinal biota **(Calvani et al., 2018).**

It is clear from what has been reported that the microbial community is complex and dynamic and that its composition is extremely various. This represents a real challenge in soil ecology: in fact, the use of microbial inoculants for agriculture is an increasingly accepted way and studied by the scientific community.

However, the potential environmental impacts related to inoculation have always been neglected. It consists in providing the host rhizosphere with high density of

viable and efficient microbes for rapid colonization: this inevitably leads to a transient (or not) perturbation of the balance of the microbial communities already present in the soil, which could lose important native species, an undesirable aspect of changes in the microbiome. The beneficial microorganisms therefore affect subsequent crops **(Whipps, 2001).**

While on the one hand the use of microorganisms in agriculture becomes an increasingly studied and applicable perspective, on the other hand, these factors must also be kept in mind and it is therefore necessary that companies that produce and market microbial formulations provide quality products, which are safe for the environment and for man. **(Nuti and Giovannetti, 2015)**.

Therefore, the main purpose of the research is to set up rules, procedures and protocols to be used during the industrial production of microbials, in order to obtain an efficient, effective and sustainable microbial formulation, whose quality is checked along the production chain. Only by performing quality control checks, microbials can be a valid alternative to chemical pesticides.

Research started from a commercial biostimulant, named Micosat F UNO, consisting of a microbiological consortium. One of the aims of the research was the development of a "quality protocol" considering both the individual microorganisms present in the product and the consortium as a whole.

2 ELEMENTS THAT DETERMINE THE QUALITY OF A MICROORGANISM

For innovative companies producing biocontrol microorganisms it is important to ensure good quality of the individual microorganisms that are inserted into the final commercial product. To make this possible there are some elements and steps that these companies should consider in order to produce and commercialize high quality microbials to be efficiently used in symbiotic agriculture. These elements should be following: i) origin of the single microbes; ii) their precise taxonomic identification; iii) definition of their antagonistic activity; iv) definition of their plant growth promoting features; v) biosafety; vi) population structure inside the consortium or consortium composition; vii) microbial viability of the consortium; viii) efficacy of single microbes in the final commercial product(s); ix) dynamics of microbes in agricultural environments (soil, surface and irrigation water, rhizosferecolonisation, internalisation into host plants).

2.1 MATERIAL

One of the aims of the thesis is the detailed evaluation of a characteristic product composed by microorganisms: **Micosat F UNO** was selected and used along the research. It is a microbial consortium produced and marketed by the The CCS (Centro Colture Sperimentali) of Aosta (AO). It is a company located in the Aosta Valley and that has chosen to specialize exclusively in biotechnology; its activity is aimed to the research, selection and marketing of microorganisms useful for use in agriculture.

Micosat F UNO is a microgranular product that contains a set of selected microorganisms from the CCS Aosta colllection, including mycorrhizal fungi, streptomycetes, rhizosphere bacteria and saprophytic fungi. This biological community constitutes a set of populations of different species that live in the same natural environment and among which relationships are created, completing an ecosystem with a high degree of adaptation and stability in the natural environment that hosts it.

The product contains:

• **Crude inoculum**: mycorrhizal and crushed roots, spores and symbiotic fungi on green composted soil conditioner and / or acid, neutral or humid peat containing the species belonging to the genera:

Glomus (*Glomus spp*. GB67)

Funelliformis (*Funelliformos mosseae* GP11)

Septoglomus (*Septoglomu sviscosum* GC41)

These microrganisms are capable to enter into the host roots (arbuscles) in a minimum percentage of 30%.

• **Rhizosphere bacteria:**

Agrobacterium radiobacter AR39

Bacillus amyloliquefaciens BA41

- **Saprophytic fungi:**

Pochonia chlamydosporia PC50

Trichoderma harzianum TH01

- **Actinomycetes:**

Streptomyces spp. SB14

- **Yeasts:**

Pichia pastoris PP59

The research also analyzed and studied other microorganisms collected and produced by CCS Aosta, that are not present in the commercial product Micosat F UNO, but that are part of other microbial formulations, such as some Pseudomonas including *Pseudomonas granadensis* PT65 and strains PA29, PM46 and PN53 of *Pseudomonas spp*; some Actinomyces as the strain SA51 of *Streptomyces*, some Bacillus as BF90, an *Ulocladium oudemansii* UO18.

2.2 ORIGIN

The first step to insert a new microorganism in the production is the discovery of the origin of the microorganism (natural niche).

There are two possible routes:

- **To buy** the strains of interest from one of the official microbial collections.

There are many international microbial collections, among which it is possible to quote Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures **(https://www.dsmz.de/)**. It is the most comprehensive biological resource centreworldwide. One of the world largest collections, the DSMZ currently comprises more than 70.000 items, including about 31.000 different bacterial and 6.500 fungal strains, 830 human and animal cell lines, 1.500 plant viruses and antisera, 2.000 plasmids and bacteriophages and 13.000 different types of bacterial genomic DNA. All biological materials accepted in the DSMZ collection are subject to extensive quality control and physiological and molecular characterization by its central services.

Another example of a collection, from which to buy microorganisms, is the American Type Culture Collection (ATCC) **(http://www.lgcstandards-atcc.org/)**. The headquarters and bioproduction facilities are located in Manassas, Virginia, U.S. The collection was established in 1925, when a committee of scientists recognized the need for a central collection of microorganisms that would serve scientists all over the world. The early years were spent at the McCormick Institute in Chicago until the organization moved to Georgetown University in Washington, DC in 1937. As research in the biosciences expanded ATCC began to diversify its holdings, and as the collections grew, ATCC occupied a series of sites, each providing more storage space.

This is the premier global biological materials resource and standards organization, whose mission focuses on the acquisition, authentication, production, preservation,

development, and distribution of standard reference microorganisms, cell lines, and other materials. While maintaining traditional collection materials, ATCC develops high quality products, standards, and services to support scientific research and breakthroughs that improve the health of global populations.

- **To isolate** micro-organisms directly from the soil, rhizosoil or the rhizosfere.

Another possibility for companies is to independently select the microorganisms they intend to produce. To do this it is necessary to understand which microorganism to select, and then proceed to the isolation and characterization. CCS Aosta boasts a rich collection of strains isolated from soil. One of the possible strategy used for the search of efficient microbial biocontrol agents is to search healthy plant within a highly compromised cultivation due to a pathogen. Subsequently the plant is taken to the laboratory and through specific culture medium.

- Plate Count Agar (PCA) or Tryptone Soy Agar (TSA) medium for *Bacteriaspp*.
- Potato Dextrose Agar (PDA) or Malt Agar (MA) for fungi and yeasts,
- A medium composed by malt extract, yeast extract, glucose and agar (called TAM) + *cycloexemide* for *Streptomyces* spp.
- King's Medium B (KING B) for *Pseudomonas* spp.

Both the microorganisms present on the root surface of the plant and any endophytes are selected on the plate. The choice of potentially interesting microorganisms is made on the basis of the disease present in the field, in which the plant was collected, and the bibliographic knowledge that allows knowing which microorganisms are potential antagonists of the pathogen.

2.3 CHARACTERIZATION

Any microorganism that grows forming visible colonies on a nutrient substrate, selective and/or general, before being identified should be obtainedin pure culture. In nature, bacteria rarely exist as pure cultures. By pure culture we mean a population of cells derived from a single cell.

Methods for the characterization and taxonomical identification of microorganisms are based upon an analysis of their morphology and their biochemical, metabolic, serological and genetic fingerprints. Initially, characterization is done based on colony morphology:

- Colour and texture;
- Size, margins, elevation;
- Morphology variations on different media.

It is also possible to set up a microscopic preparation stained with methylene blue on a slide to be observed under a microscope. Since the density of bacteria is slightly higher than that of water, they are almost transparent from observation under a microscope. To see them better, colorants are then used to increase the contrast between the body and the background, in order to better observe and differentiate the different cellular shapes (round, rod, corineform, etc...). To prepare the bacteria for staining (for example bacilli such as *Bacillus subtilis*), a thin cellular film is made on the slide which is fixed to the slide to prevent it from being washed off during the staining procedures. The fixing is carried out by passing the slide on a flame: in this way the bacteria, in addition to being "bonded" to the slide, are killed and this allows an easy entry of the dye into the cell. Once the preparation is fixed, add the dye and wait for about a minute, so that it penetrates into the cells; the excess dye is then washed away with water. The use of a single basic dye to increase the contrast between bacteria and background is called simple staining and provides information on cell shape and size.

Annex 1: Preliminary characterization table of some of the microorganisms present in the collection of CCS Aosta.

Following the preliminary morphological observation, it is desirable to identify a bacterial isolate with biochemical methods. The metabolic (biochemical) characteristics may concern the determination of the sources of energy, carbon and nitrogen, the determination of the products deriving from the use of nitrogen compounds, the sensitivity to antimicrobial substances, the ability to ferment carbohydrates. The biochemical activities of the bacteria can be determined with traditional methods that involve the inoculation of the culture to be tested in substrates that contain compounds on which the enzymes involved in the specific metabolic process act. Alternatively, multi-test identification systems can be used. In general, the result of enzymatic activity is accompanied by chromatic modifications of the substrate.

Figure 3: A plate with *Pseudomonas* sp., strain PN53. grown on King's B medium. Since King's B medium stimulates the production of fluorescein, it is possible to observe a change in the normal pigmentation of the plate, which is yellow-green instead of the usual whitish colour.

Visual and biochemical features can give good results, but a more precise characterization can only be obtained through molecular identification techniques. Technological improvement has made this technology more affordable also from an economic point of view; therefore, it's necessary that companies wishing to insert new micro-organisms into their products and their collections use these types of services. Microbial identification services provide the organism's species. A commonly used method to identify bacterial strains consists in analyzing the sequence of the gene that encodes 16S ribosomal RNA (16S rRNA). The sequencing of this gene is the standard tool for phylogenetic and taxonomic studies thanks to the degree of conservation of the gene itself among the bacterial species.

2.3.1 Isolation and characterization of some CCS Aosta's strains

Introduction:

An example in this regard is the strain PT65 (*Pseudomonas* sp.) marketed by CCS Aosta in its Micosat F products. The strain does not derive from a cultivation attacked by pathogens, but was isolated in the spring of 2007 from the flatbed of a truffle plant, specifically from *T. melanosporum*. It is now known that in ectomycorrhizal symbiotic associations some bacteria that receive nutrients and water from the fungus-plant system play a fundamental role and can produce biostimulant substances that contribute to the aroma of the ascocarp or can act as biological control agents. The sporocarps produced by ectomycorrhizal fungi host cultivable populations of chemo-organotrophic bacteria referable to psudomonads and sporogenic bacilli. Tests were carried out on these bacteria to evaluate production of siderophores. These trials gave positive results, therefore CCS Aosta decided to continue the procedures for inserting the strain within the company microbiological bank.

Another important analysed microorganism belonging to the collectionof CCS Aosta is strain SA51. It was isolated in the rhizosphere of an Olive groove in Arnasco (SV, Italy) in 2004. It is a *Streptomyces* spp. which, as we have seen, belongs to the rhizosphere and the ability to act as a plant growth promoter has also been studied **(Dias et al., 2017)**.

Streptomyces are very common in nature and beyond that in the soil, they can be found as endophytes of some plants.

Streptomyces spp. is a genus of *Actinomycetes*, aerobic gram-positive bacteria. It forms a very branched and often pigmented vegetative mycelium. The SA51 analyzed strain, for example, has a pinkish colour.

They are filamentous and produce spores, an aspect that allows them to survive in adverse environmental conditions and make them able to compete with other microorganisms present in the rhizosphere.

During the metabolic processes *Streptomyces* spp. are able to produce different lytic enzymes capable of degrading polymers such as cellulose and chitin and making them more easily absorbed by ABC transporters **(Bertram et al., 2004; Chater et al., 2010; Thompson et al., 2010; Seipke et al., 2012).** They are also known for the solubilization of phosphates and the production of siderophores. Their ability to transform complex nutrients into simple mineral forms makes them good natural bio-fertilizers. **(Viaene et al., 2016; Massalha et al., 2017; Bais et al., 2006; Jog et al., 2016)**.

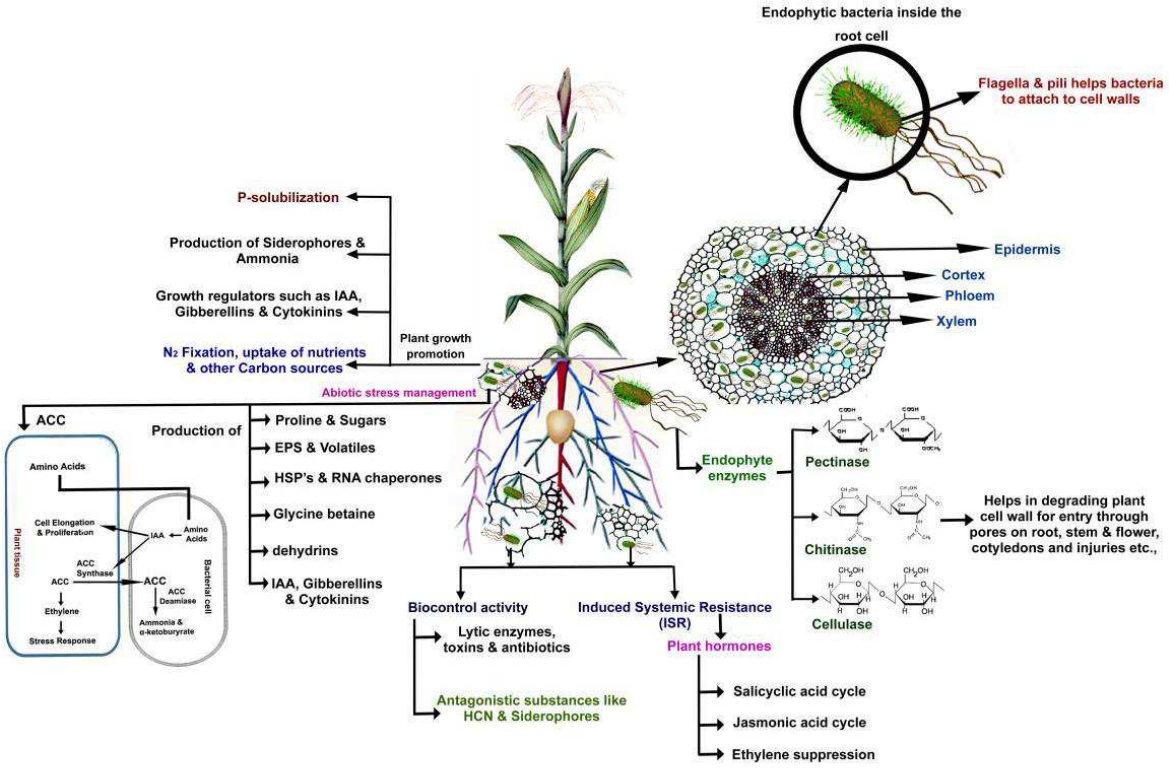


Figure 4: Representation of possible plant–microbe interactions favoring plant growth and/or biocontrol of phytopathogens by streptomycetes as rhizosphere competent microorganisms and/or endophytes **(Vurukonda et al., 2018).**

Materials and Methods:

Strain PT65: we identifiedthe bacterial strain "*Pseudomonas* sp. PT65" analyzing the 16S rRNA sequence, *rpoB* and *rpoD* coding genes, which were retrieved from the assembled genome provided for the strain. The 16S rRNA sequence was then compared from the strain PT65 against a qualified database. Moreover, they concatenated the nucleotide sequence of *rpoB* and *rpoD* genes for the strain PT65, which was aligned and was compared with the homologous sequences from the type strains of the most phylogenetically related species.

Annex 2: Microbionanalisys on strain PT65

Strain SA51: Rizosphere soil samples were collected and suspended in a sterile saline solution. Serial dilutions were made and 50 μL were plated on ISP-2 (International Streptomyces Project) agar medium and incubated for 7 days at 28°C **(Shirling and Gottlieb, 1996)**.

The colonies resembling streptomycetes were purified on the same medium and as a result of other antagonistic tests the SA51 strain was chosen.

Before DNA extraction, the SA51 strain was subjected to three successive sub-cultures on ISP-2. Single strain colonies were grown in Tryptic soy broth (TSB) for 3 days at 28°C. Genomic DNA was extracted and purified using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) and its quantity and quality was checked using the NanoDrop One Microvolume UV-Vis spectrophotometer (Thermo Fischer Scientific, Waltham, USA), followed by gel electrophoresis.

An Illumina HiSeq2000 sequencer for DNA sequencing was used. The high-quality Illumina sequence libraries were prepared using the Nextera DNA Flex preparation kit.

The parameters of the assembler module available in the *Geneious*v 1.0 software **(www.geneious.com)** were used to perform the assembly of the genome from sequential readings in pairs. Through the software, quality control, cutting and assembly phases were also performed using predefined parameters.

Through ClustalW and "Map to a reference" tools, available in the *Geneious*v 1.0 software, the sequence alignment was performed. Based on the alignment to the reference genome of *S. avermitilis* (NC_003155.5) available in GenBank, coverage was determined at 30x and the coverage breath was 95.3%.

The SEED project **(Overbeek et al., 2005)** provided the standard parameter **(Aziz et al., 2008)** of the rapid annotation subsystem (RAST) technology for the analysis of annotations and subsystem coverage. Genome annotation with RAST identified 6,040 coding sequences (CDS), 32 tRNAs, and 13 rRNAs in the SA51 genome. The

amplification of the short subunit (SSU) 16S rRNA was performed using the strepB (5′-ACAAGCCCTGGAAACGGGGT-3') and strepE (5′-CACCAGGAATTCCGATCT-3') primer pair **(Ramazani et al., 2013).**

PCR reactions were performed for amplification in a total volume of 25 µL containing:1x GoTaq Buffer, 0.8 µM of each primer, 2µL DNA template, 200 µM dNTP's, 1.250 mM $MgCl_2$, 1 U TaqPolymerase, and remaining volume added with nuclease-free water.

PCR conditions:

- pre-denaturation at the temperature of 94°C for a time of 5 minutes,
- denaturation at the temperature of 94°C for a time of 60 seconds,
- annealing at the temperature of 55 °C for a time of 60 seconds,
- elongation at the temperature of 72 °C for a time of 30 seconds,
- post-elongation at the temperature of 72 °C for a time of 5 minutes.

Sanger sequencing of the 16s rRNA gene PCR, followed by nucleic sequence blast **(https://blast.ncbi.nlm.nih.gov/Blast.cgi)** analysis, revealed that strain SA51 was identified as *Streptomyces avermitilis* with identity of 97% with the e value of 0- 3e-77 with the strains present in NCBI GenBank.

Furthermore, using the Kyoto Encyclopedia of Genomes and Genomes (KEGG) **(Kanehisa and Goto, 2000)**, a construction of the metabolic profile of SA51 was carried out to identify the genes involved in promoting growth.

Results:

According the analysis of the 16S rRNA sequence, the strain PT65 resulted related to *Pseudomonas fluorescens* group within the *Pseudomonas fluorescens* lineage (Annex C), which includes also *Pseudomonas gessardi*, *Pseudomonas fragi*, *Pseudomonas mandelii*, *Pseudomonas jessenii*, *Pseudomonanskoreensis*, *Pseudomonas corrugata*,

Pseudomonas chlororaphis and *Pseudomonas asplenii* subgroups **(Gomilaet al., 2015; Mulet et al., 2012).**

The analysis based on the concatenated sequence of *rpoB* and *rpoD* genes revealed the correlation of the strain PT65 with the type strain of the species *Pseudomonas granadensis* F-278,770.

Above all considered, the bacterial strain PT65 from CCS Aosta belongs to the species *P. granadensis*.

The study of strain SA51 total size genome showed that it is 5,465,072 bp (including 792 assembled contigs and 2959 unassembled reads) with a GC content of 70.1%. The average length of the contig was 2,832 bp, while the shortest and longest contigs were respectively 707 bp and 23.079 bp (length N50: 3.517 bp) (*Table 1)*.

The metabolic profile showed the presence of genes involved in the pathway for the alkaloid indole biosynthesis and in the transport and metabolism of iron, together with genes that code for proteins that act in the regulation of iron homeostasis.

Thanks to the RAST annotations, proof of the presence of genes and operons related to metal transporters and antibiotic biosynthesis was also provided, a result that suggests the possible involvement of SA51 in the biological control of plant pathogens and / or soil remodeling microbiota.

Overall, these preliminary studies suggest that the strain SA51 strain deserves further study into its ability to act as a PGPR in agricultural systems, along with its possible role in helping plants in the resistance system.

Statistics	Unassembled Reads	All Contigs	Contigs>=100 bp	Contigs <=1000 bp
Number of	2,959	792	792	74
Min Length (bp)	398	707	707	1,001
Median Length (bp)		2,179	2,325	2,179
Mean Length (bp)	1,088	2,832	2,832	2,969
Max Length (bp)	14,732	23,079	23,079	23,079
N50 Length (bp)		3,517	3,517	3,565
Number of Contigs >= N50		199	199	193
Length Sum (bp)	3,221,808	2,243,264	2,243,264	2,197,473

Table 1: Length of genome contigs assembly report of strain SA51.

Data availability: The draft genome sequences were deposited at DDBJ/ENA/GenBank under BioProject number PRJNA545025 with accession numberVEXM00000000.1. The version described in this paper is the first version VEXM01000000.

2.4 PRODUCTION

2.4.1 Cultivation medium

The first aspect to be taken into consideration for the production of microorganisms in the laboratory and, subsequently, on a large scale is the choice of the culture medium.

The cultivation of microorganisms in the laboratory requires the use of so-called "media" or "culture media" to try artificially to reproduce an environment able to satisfy the metabolic needs of the micro-organism that we want to cultivate.

The medium, before plating, shouldbe sterile and contained in sterile containers equipped with a closing system that guarantees the sterility of the contents.

All operations relating to platingshouldbe carried out observing the necessary precautions to avoid contamination of the soil by bacteria present in the environment.

The uses of culture media are numerous:

- counting of microorganisms that contaminate food and surfaces;
- isolation of microorganisms;
- maintenance in culture;
- identification and study of biochemical characteristics;
- cultivation for the production of antibiotics, enzymes, toxins, antisera, vaccines, starter cultures;
- evaluation of the activity of pharmacologically active preparations (for example: antibiotics; research inhibitory substances).

The components of the culture media shouldmeet the nutritional requirements of microorganisms. To multiply, they need sources of hydrogen, carbon, nitrogen, oxygen, sodium, magnesium, phosphorus, sulfur, potassium, manganese, iron.

The components of the culture media can be divided into the following classes: peptones, carbohydrates, indicators, mineral salts, selective agents, solidifying agents, and other additional components.

The chemical composition of the culture media is different in relation to the nutritional needs of the species that you want to cultivate. For most bacteria it is possible to set up synthetic culture media, of defined composition, in which the individual substances that the microorganism in question needs are present in well-defined quantities.

The sources of nitrogen can derive from food such as milk, eggs, infusions of meat, potatoes, tomatoes. Currently, several highly reliable industrial products are available that guarantee a high reproducibility of their fundamental characteristics. These are hydrolysates of proteins of biological origin called peptones. The basic materials for their production are meat, casein, soy, yeast cells, jelly. The peptones consist of free and polymerized amino acids in chains of variable length depending on the type of hydrolysis undergone.

Sugars are nutritional essentials elements. They are a source of carbon and are also used as fermentable substrates for the differentiation of microorganisms. The most used are glucose, lactose, mannitol and sucrose.

Salts have three fundamental functions: to supply the metals necessary for microbial growth (Mg, Mn, Fe, Ca, Zn, Cu), to provide a buffer action to the soil (KH_2PO_4 and K_2HPO_4) and to maintain an adequate osmolarity to the culture medium (with NaCl).

The preparation phases of the culture media include the choice of the components, their solubilization and sterilization. Generally, dehydrated culture media are used thatalready contain the individual ingredients in the desired proportions, other times the individual ingredients are weighed. To prepare a mediumproceed carefully weighing the desired quantity of powders and dissolving them in the

amount of water required by stirring until the ingredients are completely dissolved. Sometimes pH correction is required through buffer solutions. The solution is then transferred to bottles with screw caps (half-turn loose) and with autoclave sterilization (120 ° C for 25 min).

Quality control in media preparation is ensured when all equipments and tools are regularly checked for their quality and functions: scales, distilled water (*e.g.*: conductivity) and distiller, pH-meters, autoclave, sterile hood. Availability of checking reports for inspections and audits should be ensured.

2.4.2 Cultivation conditions

In addition to nutritional needs, a growing medium shouldalso respond to the physiochemical requirements of the metabolism of the microorganism that must be grown. In order for the cultivation to lead to the growth of the bacterium, it is necessary that the environment in which the soil is maintained is suitable for the particular needs of the micro-organism in question.

Temperature

Temperature is one of the most important environmental factors that can influence the growth and survival of microorganisms. The absolute values of minimum and maximum temperatures vary widely from one microorganism to another and generally reflect temperature fluctuations and the average temperature of their habitats.

As the temperature increases, the chemical and enzymatic reactions of the cell proceed at a greater speed and the growth becomes faster. However, some proteins can be irreversibly damaged beyond certain values. There is both a

temperaturerange whose metabolic functions and growth increase, and a point where inactivation reactions begin, above this point the cellular functions are cleared **(Madigan et al., 1970)**. The growth medium must then be incubated at the optimum temperature for the metabolism of each microorganism. Most bacteria are mesophilic and grow better between 30 and 37°C, with optimum temperatures around 36-37°C. The psychrophilic forms grow well at low temperatures, between 15 and 20°C, the thermophiles between 50 and 60°C. Quality control when growing or storing microorganisms is verified when incubators and refrigerators are regularly checked and checking reports are always available. In general, temperature variations should not exceed ±1°C in case of incubators and ±2°C in case of refrigerators.

pH conditions

Each microorganism grows in a certain pH range within which it is possible to identify an optimal pH. Most bacteria develop best at pH values between 6.0 and 8.0 (neutrophils), but some exhibit an optimal lower pH of between 2.0 and 4.0 (acidophilic) and other optimal pH between 8.0 and 11.0 (basophils). Buffers are often added to the soils to maintain the optimum pH for as long as possible **(Madigan et al., 1970)**.

Osmotic pressure

Water availability is one of the main factors affecting microbial growth in nature. The availability of water does not depend exclusively on the content of water in the environment but is also a function of the concentration of solutes such as salts, sugars or other substances that are dissolved within it. Generally, the availability of water is expressed in physical parameters such as water activity, which represents

the ratio between the vapor pressure of the air in equilibrium with a substance or with a solution and the vapor pressure of pure water.

Often it can be important to check the osmotic pressure or the salt concentration of the environment in which a microorganism is cultivated.

Common growth media have satisfactory characteristics for most micro-organisms and generally they multiply better in soils with lower osmotic concentration than their own. For example, for bacteria that grow in solutions with a high concentration of sugar, osmotic pressure and ionic strength should not be neglected. The microorganisms that live well at high salt concentrations are defined as halophiles; osmophiles require high osmotic pressure. Most bacteria tolerate large shifts in osmotic pressure and ionic strength because they are able to regulate their internal salt concentration **(Madigan et al., 1970)**.

Oxygen request

Bacteria present a wide variety in their demand for atmospheric gaseous oxygen. Some don't grow in its absence, others do not develop in its presence and some can adapt both to the presence and absence.

For aerobic bacteria the presence of oxygen must be ensured (it will be necessary to proceed with forced aeration, through the introduction of sterile air, of the cultures of the aerobic bacteria obliged in liquid soils where, otherwise, there would be development only in the surface layers of the ground). The supply of air to cultures of aerobic microorganisms is a vital technical problem; to introduce oxygen into liquid culture media, they shake using shakers where they place the flasks or inject air into the ground through aeration or suction systems. When microorganisms reach high concentrations ($14X10^9$ C.F.U./ml) (Colony Forming Unit), the oxygen presence can become a limiting factor for aerobic growth. On the contrary, for the cultivation of the obligate anaerobes, it is necessary to proceed with the elimination

of oxygen, something that can be done in various ways: by adding to the liquid cultures the reducing substances such as sodium thioglycolate, sealing the tubes containing agar with a layer of paraffin, or placing the culture vessel in another vessel from which oxygen is removed by vacuum or chemical processes.

2.4.3 Cultivation course and bioprocess kinetics

Introduction:

Growth is defined as the increase in the number of bacterial cells in a population; this increase can also be measured as an increase in microbial mass. The term growth rate refers to the change in the number of cells or mass per unit of time. The generation time is the time required for a population to duplicate, therefore it is also called duplication time.

The growth cycle of a bacterial population can be divided into different distinct phases.

Growth of a microbe inoculated in fresh medium usually does not begin immediately, but requires a defined period of latency and can be more or less long depending on the crop. An exponential phase follows, in which the number of cells doubles and, eventually, a stationary phase, when the number of living cells is do not increase so fast, due to lack of nutrients and catabolites released in medium. If the incubation proceeds beyond the stationary phase the cells can enter the phase of death.

Relative estimate of the cell mass is done through the measurement of the turbidity witha spectrophotometer, the measurement of the pH and the quantification of the sugars through the Brix degrees.

The test was aimed to the study of a liquid medium suitable for the growth of the BF90 strain.

BF90 is a strain not yet included in the Micosat F production. It was purchased by CCS Aosta on DSMZ and his original name is *Bacillus firmus* NRS613. The name was assigned by **Werner (1933)**, who also described it for the first time. *B. firmus* is the name of the most ancient species represented, it is a Gram+ bacterium, ellipsoidal and an alkaliphilic *Bacillus* **(Fritze G. et al., 1990)** which was grouped in the complex *Bacillus firmus-Bacillus lentus* **(Gordon and Hyde, 1982).** The origin country is unknown.

Materials and methods:

Three different liquid media (T1, T2 and T3) have been tested with strain BF90 to understand which of the three substrates was the most suitable and performing for the growth of the Bacillus.

The three media differed in their composition. T1 and T2 are two nutrient media found in literature **(www.dsmz.de)** for the growth of *Bacillus firmus,* which differ only in the addition of soil extractcomponent, the third was a medium already used by CCS Aosta for the growth of Bacilli.

The nutrient medium composition used was: 5g of peptone and 3g of meat extract diluted in 1 liter of distilled water. pH adjusted to 7.0 and sterilized for 20 min at 120°C.

The soil extract is a natural mineral soil ideal for the growth of soil bacteria obtained by mixing soil garden with neutral pH and water. It was produced sterilizing 400 g of air-dried garden soil (with high content of organic matter) in 1 liter of tap water for one hour at 121°C. Allowed it to sediment for overnight at roomtemperature (20°C ±2). Centrifuged the supernatant for 10 min at 3500 revolutions per minute

(rpm) and collected the clearsupernatant solution thus obtained. Adjusted pH to 6.8 - 7.0 and sterilized.

The soil extract thus obtained was used in T1 in addition to 50% of the growth medium for *Bacillus firmus* as recommended by DSMZ.

The T3 medium composition was: 20 g of glucose, 10 g of malt extract, 5 g of yeast extract, 10 g of corn steep atomized (CSA) and 1 g of casein peptone diluted in 1 liter of distilled water. pH adjusted to 7.0 and sterilized for 20 min at 120°C.

Each flask containing a different growth medium (three repetitions for each) was inoculated with BF90 and placed in a shaker at 30°C and 100 rpm.

Checks were therefore carried out on each type of medium at regular intervals of 17, 24, 41 and 48 hours.

The pH was measured using a bench-top pH meter, the Brix grades for the sugars were measured with a professional refractometer, the Optical Density (O.D.) 600 nm were calculated with a spectrophotometer.

A slide was prepared and observed with an optical microscope.

Results and discussion:

The experiment has shown that, both from the slides and from the analysis data, the T1 growth medium is the one that determines a greater bacterial growth of the strain.

Results are superior both as regards pH and O.D. 660 nm.

Brix degree are the same in T1 and T2. The growth of the T3 strain has instead given negative results throughout all the observation period.

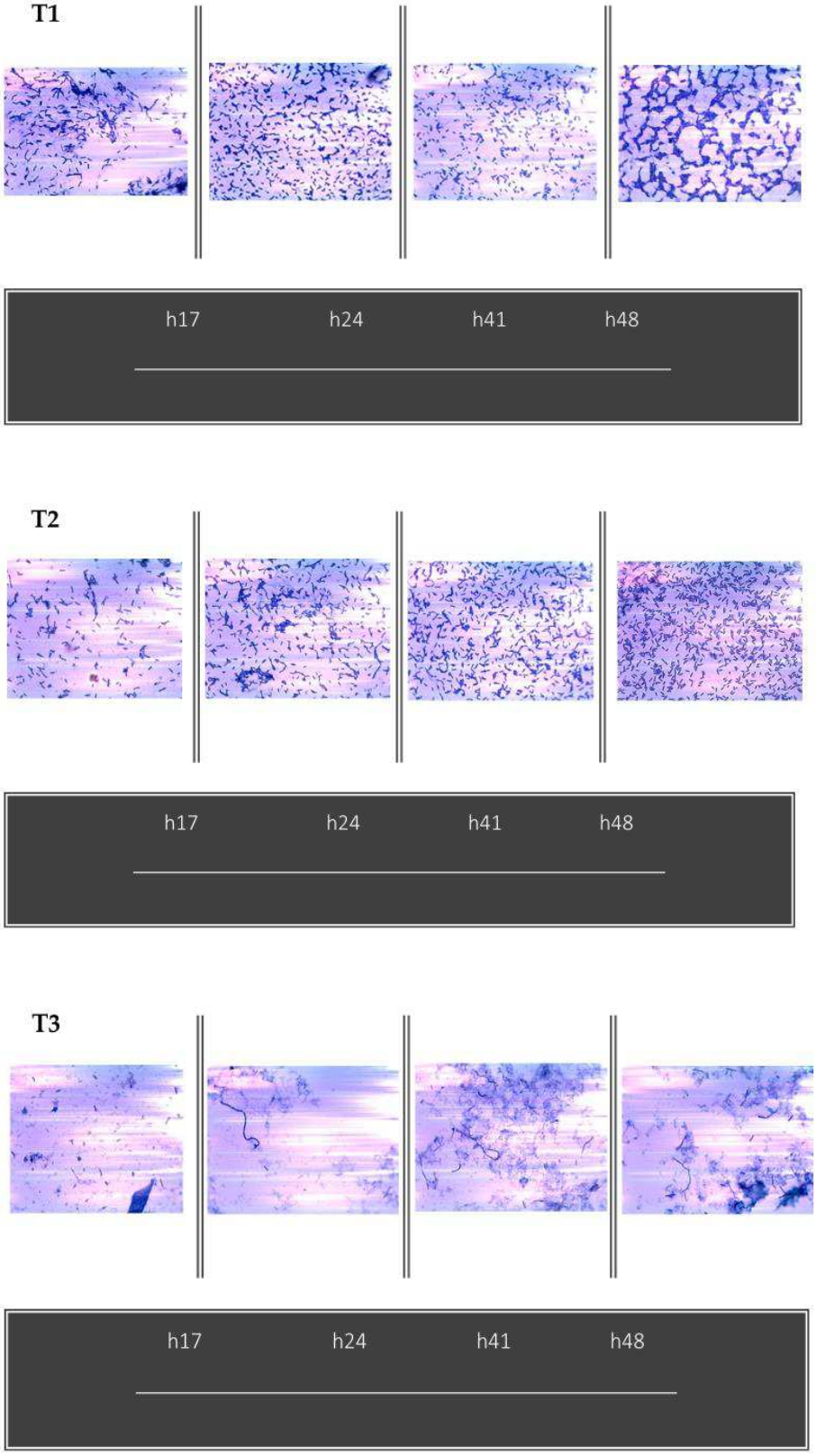


Figure 5: Images of strain BF90 growth slides at regular intervals, on growth media T1, T2 and T3. Pictures show that BF90 strain grows better in the T1 medium.

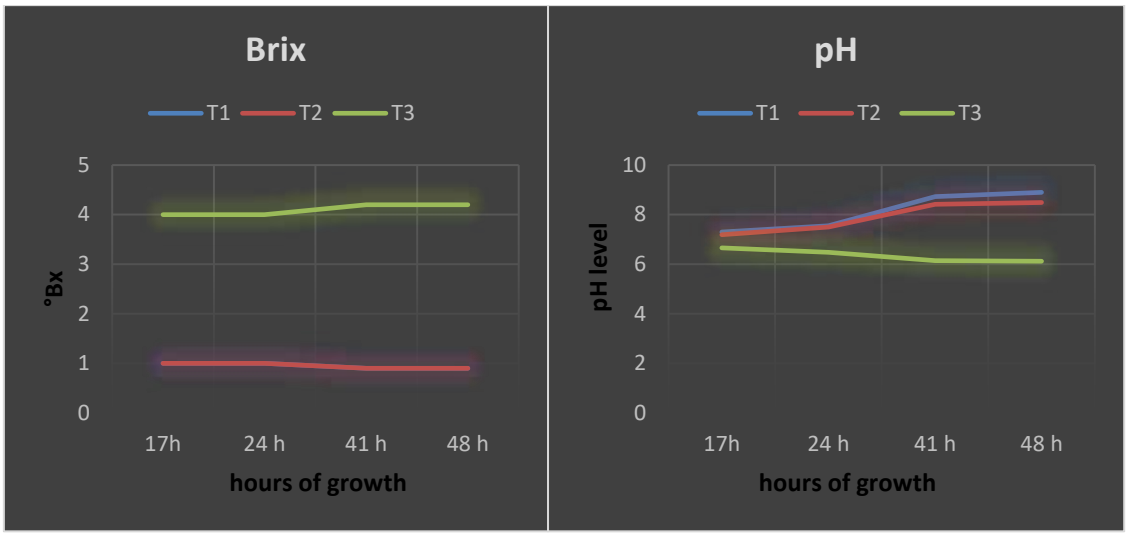


Figure 6: The graph shows the Brix and pH levels in the three substrates T1, T2 and T3 in regular time intervals at 17, 24, 41 and 48 hours after BF90 inoculation. In the graphic representing the Brix degree, the representative lines T1 and T2 are overlapped because the measured values were the same.

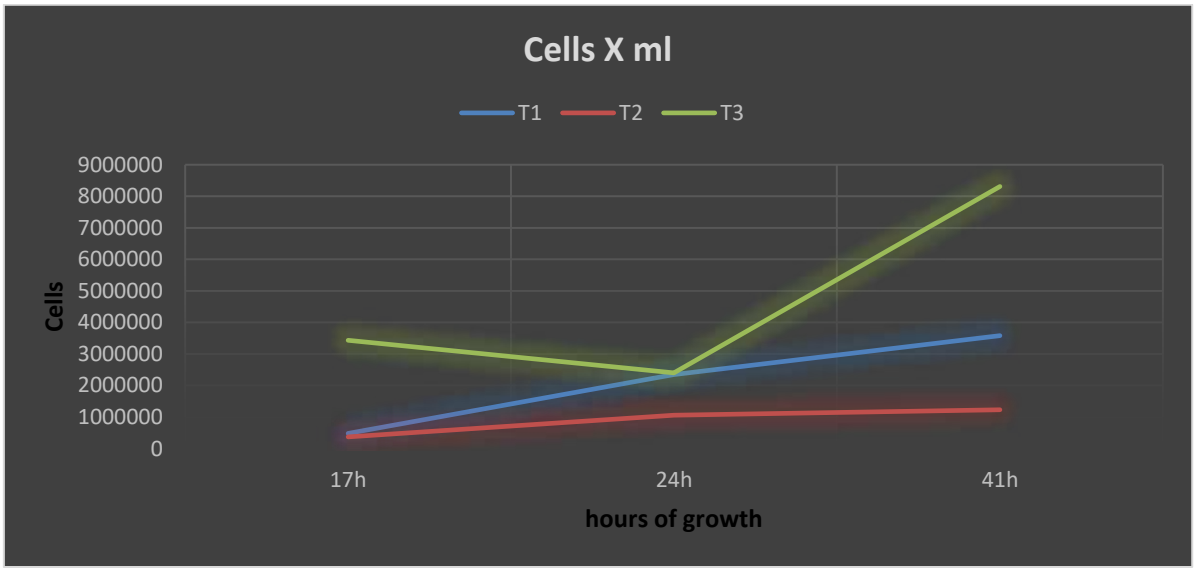


Figure 7: The graph shows the turbidity levels (O.D. 600 nm, 0.1) of the three-liquid media after 17, 21 and 24 hours from the BF90 inoculation. Since the growth of T3 is not linear, the most significant is the T1 medium.

The cultivation of microorganisms can take place both on a small scale in the laboratory (in a flask) and on an industrial scale.

Working on an industrial scale, the multiplication of microorganisms takes place in bioreactors.

2.5 BIOCONTROL ACTIVITY

Many microorganisms act as biocontrol agentsto protect plants against various phytopathogens. The organisms potentially more active in this are the bacteria *Bacillus* spp., *Pseudomonas* spp. and *Streptomyces* spp. and the fungi *Trichoderma* spp., *Gliocladium*spp. The mechanisms used by these biocontrol agents include following:(i) Niche exclusion, (ii) Competition for nutrients, (iii) Production of chitinase by *Serratia marcescens* **(Ordentlich et al., 1987)** and by *Trichoderma* **(Sivan and Chet, 1989),** (iv) Production of AFMs (antifungal metabolites), (v) ISR (Induced Systemic Resistance), which is triggered by certain non-pathogenic rhizobacteria as *Pseudomonas* spp. andmake the host plant extremely reactive towards a range of pathogens **(Van Peer et al., 1999; M'piga et al., 1997)**.

Different stains of *Bacillus* spp. and of actinomycetes, particularly *Streptomyces*, have been used against numerous plant pathogenic fungi of economically significant importance. *Bacillus spp*. have special features that make them good candidates as BCAs (Biological Control Antagonists). These strains are ubiquitous. They have resistance spores, different types of antifungal compounds and have shown significant inhibitory activity against pathogens such as *Ceratocystis ulmi* **(Gregory et al., 1986)**, *Puccinia pelargonii-zonalis* **(Rytter et al., 1989)**, *Euthypalata* **(Ferreira et al., 1991)**, *Fusarium moniliforme* **(Agarry et al., 2005)**, *Colletotrichum musae* **(Mahadtanapuk et al., 2007; Alvindia and Natsuaki, 2009)**, and *C. gloeosporioides* **(Havenga, 1999; Demoz and Korsten, 2006)**. The antagonistic activity of Streptomyces is usually related to the production of extracellular hydrolytic enzymes and secondary antifungal metabolites. The *Streptomyces* species have the potential for biological control of fungal diseases caused by *Phytophthora capasici* **(Jo, 2005)**, *Phytophthora cinnamomi* **(Aryanta and Guest, 2006)**, *F. oxysporum, Botrytis cinerea*, and *Monilinialaxa* **(Lu et al., 2008)**, *Sclerotium rolfsii* and *C. gloeosporioides* **(Prapagdee et al., 2008).**

The filamentous fungi, *Trichoderma* and *Gliocladium*, are well studied and have shown efficiency in the biocontrol of different phytopathogens such as *Alternaria*, *Botrytis*, *Colletotrichum, Diaporthe, Fusarium, Monilinia, Phytophthora, Phythium, Rhizoctonia, Sclerotinia, and Verticillium* **(Bell et al., 1982; Yu and Sutton, 1997; Balaž et al., 2000; Begum et al., 2008; Hajieghrari et al., 2008; Imtiaj and Lee, 2008)**. Many strains of *Trichoderma* are strong opportunistic invaders, fast growing, prolific producers of spores and powerful antibiotic producers **(Woo et al., 2006)**. The *Gliocladium* species are effective and versatile antagonists. These fungi have the advantage of abundant production and the long-term viability of inoculum attributes of key importance for commercial use **(Sutton et al., 1997).**

2.5.1 *In vitro* antagonism and production of volatile organic compounds (VOCs)

Introduction:

Beneficial microorganisms frequently show antagonism towards pathogenic microbes. It's therefore necessary to evaluate *in vitro* any antagonistic activity of astrain prior to its production. To do this it is important to have some pathogenic strains on which to evaluate antimicrobial activity.

In following experiments, the aim was to observe and evaluate the antagonistic activity *in vitro* of a set of beneficial microorganisms by measuring the growth inhibition of a set of pathogens possibly induced by the presumptive antagonists. Such experiments were done with pure cultures grown in the same plate, in order to have a strong evidence that some kind of molecule(s) is(are) released into the substrate, thus contributing to affect pathogen growth and viability.

Organisms can also produce a wide range of secondary enzymes and metabolites. Many fungal species issue low concentrations of volatile organic compounds (VOCs), some of them have antimicrobial activity.

A VOC is any carbon compound, excluding carbon monoxide, carbon dioxide, carbonic acid, metal carbides or carbonates and ammonium carbonate, which participates in photochemical atmospheric reactions, with the exception of those designated by the Agency for Environmental Protection (EPA) that have negligible photochemical reactivity.

VOCs are organic chemical compounds, whose composition makes it possible for them to evaporate under normal indoor atmospheric conditions of temperature and pressure. This is the general definition of VOCs that is used in the scientific literature **(https://www.epa.gov/indoor-air-quality-iaq/technical-overview-volatile-organic-compounds).**

Material and methods:

Many different isolates belonging to the CCS Aosta collection were analyzed for their antagonistic activity against six major phytopathogenic microorganisms. They were three pathogenic fungi: *Sclerotium* sp. (SP75), *Fusarium* sp. (FP76), *Rhizoctonia* sp. (RP77) and three pathogenic bacteria: *Xantomonas arboricola pv. Pruni* (XP89), *Xantomonas arboricola pv. juglandis* (XJ88) and *Pseudomonas syringae* sp. (PS87).

For the evaluation of antifungal activity PDA medium plates were prepared and microorganisms were inoculated on through a 4 mm diameter rod. They were incubated for 48 hours at 27(± 2)° C. After that, the Petri dishes were taken out of the incubator, opened inside a sterile hood and inoculated with a 4 mm diameter rod of the 3 the different pathogenic fungi and incubated at 27(± 2)° C.

Following an incubation period suitable for the growth of microorganisms, the percentage of inhibition of the various microorganisms in relation to pathogenic

fungi was calculatedcomparing the diameter of the pathogen in the presence and absence of the fungus.

For each putative bacterial antagonist, a 20 μl droplet of bacterial suspension at a concentration of 10^8 C.F.U./ml (0.157 O.D. at 600nm) for strain SA51 and 10^8 C.F.U./ml (0.140 O.D. at 600nm) for strain PA29 spectrophotometrically measured, was spot inoculated on NSA (Nutrient Sucrose agar) Petridish and incubated respectively for 72 and 48 hours at the temperature of 27(±2)° C to allow the growth of macro colony of the putative antagonist in the centre of the inoculated petridish.

Once the macro colonies were grown, the Petri dishes were taken out of the incubator, opened inside a sterile hood and sprayed with a suspension of pathogenic bacteria at a concentration of $1{,}45*10^6$ C.F.U./ml for PS87, $1{,}77*10^6$ C.F.U./ml for XJ88 and $1{,}78*10^6$ C.F.U./ml for XP89. The Petri dishes were then closed and placed again in the incubator at the same temperature as above for additional 2 days, for to allow the growth of the pathogen.

The inhibition activity of the putative bacterial antagonists against pathogen was measured by calculating the Average Inhibition Area around the macro colony as $AIA = (R^2 * 3.14) – (r^2 * 3.14)$.

To evaluate the possible production of VOCs with antimicrobial activityby antagonistic microorganisms, plastic boxes with hermetic closure were used.

Petri dishes were prepared with PDA medium. Some of them were inoculated with the pathogenic fungus, others with four different *Pseudomonas* spp. belonging to the CCS Aosta collection: PT65, PM46, PA29, PN53.

The inoculated plates were inserted open (without the lid) inside the plastic boxes, specifically they were set up:

- One box with a plate with the pathogen and a plate with the presumed antagonist organism together;
- One box with only the pathogenic microorganism;

- One box with only the antagonist organism.

The sealed boxes were stored at a temperature of 27(±2)° C to allow the growth of microorganisms.

Subsequently the growth rays of the microorganisms alone and those grown in the same box were measured and the relative areas were calculatedwith the circle area formula: πr^2.

Results:

The results show that some of the microorganisms tested as antagonists of three pathogenic fungi *Sclerotium* sp., *Fusarium* sp. and *Rhizoctonia* sp. have shown good antagonistic activity. The most prospective antagonistic strainsseem to be the TH01 and UO18, in particular against SP75 strain.

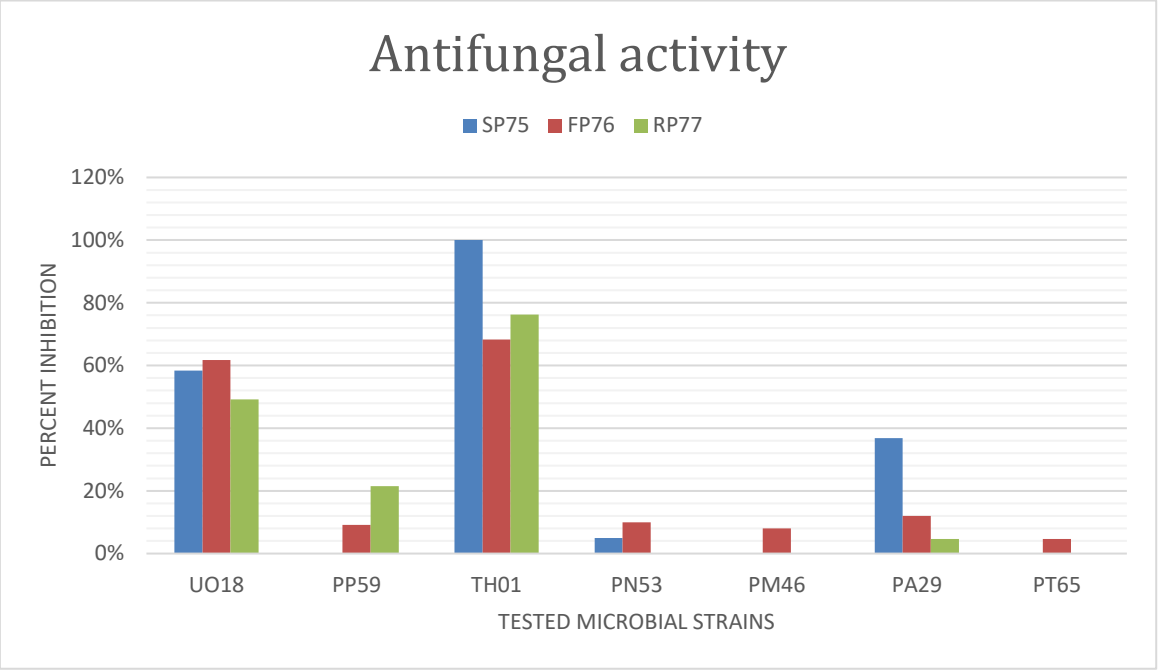


Figure 8: Antifungal activity. In the table, the inhibition percentage of the growth in plate of three pathogens is represented, in relation with some strains of CCS Aosta collection. Data show that TH01 has the best inhibiting activity as it inhibits the growth of SP75 at 100%, the growth of FP76 at 68% and the growth of RP77 at 76%. UO18 strain has a good inhibition activity, respectively 58%, 62% and 49% against SP75, FP76 and RP77. The others tested microorganism have a lower inhibiting capacity.

In the two picture is represented the inhibition of SP75 with TH01 and FP76 with TH01.

The results obtained from antagonism tests against pathogenic bacteria show an area of inhibition both around strain SA51 and strain PA29 tested, this indicates and confirms the antagonistic activity of the two strains against the tested pathogenic microorganisms. The best effects were obtained for the PA29 strain.

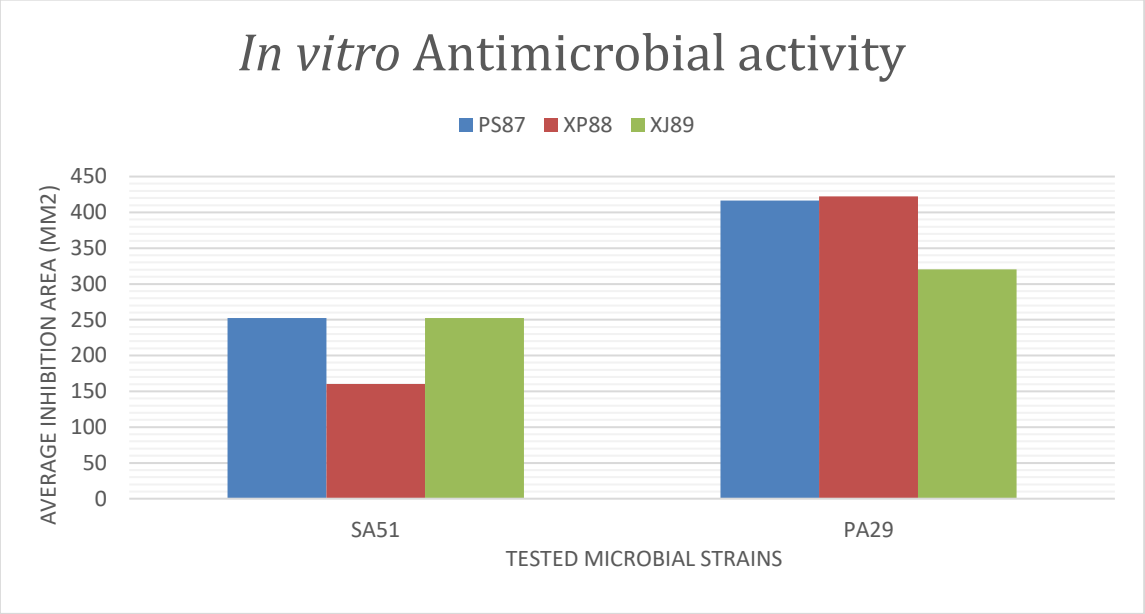
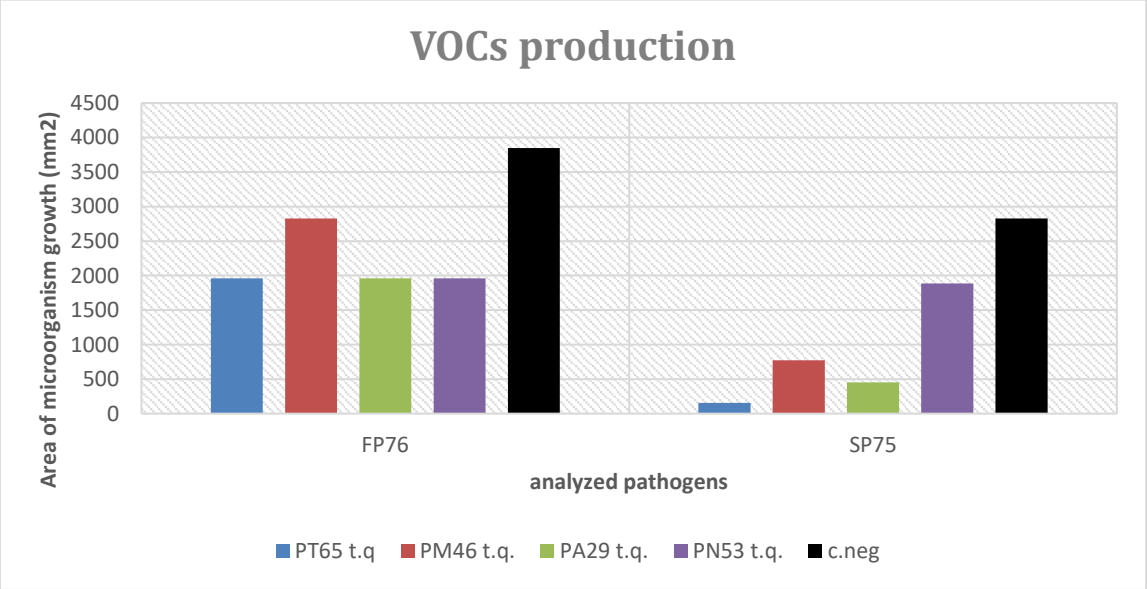


Figure 9: Average inhibition area *in vitro* that shows the antimicrobic activity of two strains of CCS Aosta collection in relation withthree pathogenic bacteria. The SA51 AIA measured with the pathogens PS87, XP88 and XJ89 is 252, 160, 252 mm^2 respectively. The PA29 AIA measured with the pathogens PS87, XP88 and XJ89 is 416, 422, 320 mm^2 respectively.

In the VOCs production tests, the areas of growth of pathogenic fungi, calculated in the presence and in the absence of the studied strains, have shown a reduction in the growth of the pathogen in the presence of *Pseudomonas* therefore it can be hypothesized that they produce VOCs with antimicrobial power.



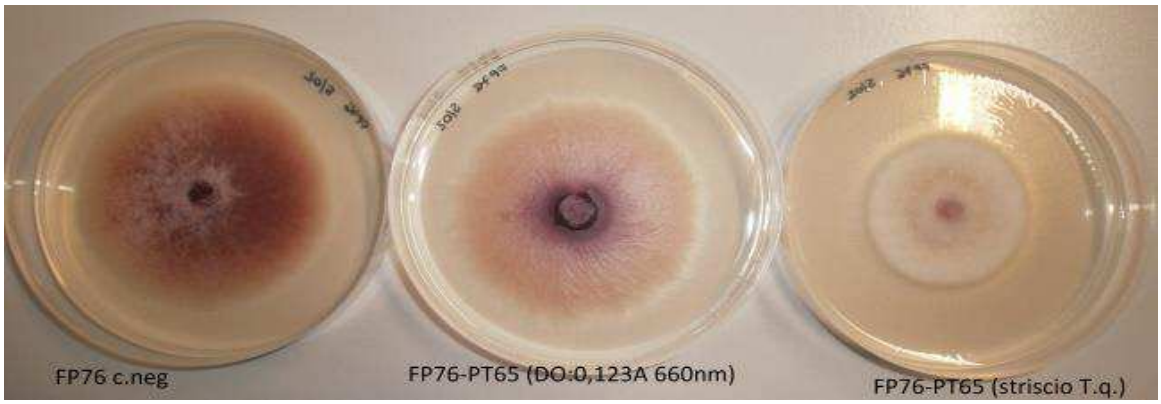


Figure 10: Above: VOCs activity of strains FP75 and FP76 against four different pathogens. The black column is the area of the pathogen grown alone and it's 3846 mm² for FP76 and 2826 mm² for SP75; in both cases it can be observed that the area is greater than the growth area of the pathogen in the presence of the antagonist. Pictures represent *Fusarium* sp. growth alone and with different concentrations of strain PT65.

2.5.2 Induction of systemic resistance in plants

Resistance, according to **Agrios (1988)** is the ability of an organism to exclude or overcome the effect of a pathogen or a harmful factor.

In plants, resistance to disease manifests itself with limited symptoms of the disease, which reflect the pathogen's inability to grow or multiply and spread and often take the form of a hypersensitive reaction (HR), in which the pathogen remains confined to necrotic lesions near the site of infection. Induced resistance is the phenomenon whereby a plant shows a greater resistance if appropriately stimulated with the inoculation of a pathogen **(Ryals et al., 1994; Hammerschmidt and Kuc, 1995)**.

Particularly evident is systemic resistance induced bynon-pathogenic bacteria of the genus *Pseudomonas* that colonize the rhizosphere.

These bacteria are often referredto as rhizobacteria and are known as plant growth-promoting microbes (PGPR), because they are able to suppress deleterious microorganismsin soil and, thereby, improve plant health and productivity.

This induced resistance has been demonstrated in some tests in which the inducing bacterium and the stimulating pathogen have been kept spatially separated for the duration of the experiments and any direct interference by the bacteria with the activity of the pathogens has been excluded **(Van Loon, 1997).**

Many actinomycetes, and particularly *Streptomyces* species, are well known for their production of a wide spectrum of antibiotics. These are often species specific and allow them to develop symbiotic interactions with plants by protecting them from various pathogens; at the same time, plant exudates promote *Streptomyces* growth **(Procópio et al., 2012).**

Quality of microorganismsto be used as biocontrol agents should undergo a thorough testing to verify and confirm their efficacy as a biocontrol agent *in planta*. The activity shouldbe evaluated on host plants for the selected pathogens. Induced systemic resistance should be evaluated in different, parallel experiments: i)

through the phytopathometric evaluation of the disease (*e.g.*: calculation of the area under the disease progress curve: AUDPC); ii) through the analysis of the plant transcriptome; iii) through the analysis of the physiological changes in plants inoculated by the BCAs (*e.g.* measurement of the oxidative stress in the plant tissue after BCA inoculation); iv) through the analysis of expression of selected genes.

2.5.3 Plant biostimulationactivity

Plant biostimulants contain substance(s) and/or micro-organisms whose function, when applied to plants or the rhizosphere, is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality.

Biostimulants have no direct effectagainst pests, and therefore do not fall within the regulatory framework of pesticides **(http://www.biostimulants.eu/).**

To produce a quality microorganism it is therefore important to go and evaluate its biostimulationactivity as well.

It is possible to do this by growing treated and untreated seeds of a plantmodel in cotton / absorbent paper soaked in water and visually evaluating the differences in the growth of the treaty compared to the control. Also in this case it is important to evaluate the activity both on a reference plant and on other easy-to-use models.

2.6 DISCUSSION

There are many elements that allow to assess the quality of a microorganism, which are necessary to obtain a qualitative and valid final product.

Starting from a microbial consortium containing many different microorganisms, it was possible to analyze some of these individually, evaluating intrinsic characteristics or stimulating them through interaction with pathogenic microorganisms.

This allowed to consider how is essential to start from well-known strains for the formulation of a microbial consortium, because a depth study and characterization allow us to know and evaluate the potential and capabilities of the individual microorganisms in question a priori, an aspect that allows to hypothesize formulations, associations with other microorganisms and applications towards potential pathogens even before having carried out in vitro tests and subsequently in the field.

Also the knowledge of the specific growth substrates for each strain, as it may seem superfluous, is very important above all from the business point of view, which has interests in the multiplication of microorganisms in large scale minimizing waste and optimizing the production of the strain to insert in the commercial product.

All the potentials derived from the knowledge of the strain must then be validated in vitro. The evaluation of the antagonist activity in plate allows having clearer and more detailed ideas of the potentialities that the microorganism will have in the field and towards which potential pathogens it will act better or worse.

However, we mightconsider the fact that the conditions in the laboratory are extremely controlled, which is less applicable in the field, even if experimental.

Furthermore, for safety reasons it was not possible to test the antimicrobial activity on more dangerous strains.

3 MYCORRHIZAL INOCULA AND QUALITY STANDARDS DURING THEIR PRODUCTION

Owing to their filamentous organization, fungi exploit very diverse substrates on the basis oftheir nutritional strategy. Saprobes thrive in soil, water and on decaying animal and plant tissues.

A smaller group of fungi, the parasitic and mutualistic symbionts, feed on living organisms **(Carlile et al., 2001)**. Such a classification cannot easily be applied to mycorrhizal fungi, a heterogeneous group ofspecies spread over diverse fungal taxa.

Mycorrhizal fungi associated with higher plant roots. In fact, over 90% of plant species including forest trees, wild herbs and many crops create symbiotic associations with these fungi, which are however able to spend part of their life cycle free.

Both partners benefit from the relationship: the plant is in fact necessary for the growth and reproduction of fungi, which in turn positively influence the host plant helping it in mineral nutrition, water absorption, growth and resistance to diseases **(Smith and Read, 2008)**.

Mycorrhizal fungi colonize various environments ranging from alpine and boreal areas to tropical forests to grasslands and cultivated fields. Through the specific mycelial activity, they play an important role in the nutrient cycle as they absorb them from the soil and supply them to the plant. Their role in the flow of carbon is still rather undefined **(SelosseandRoy, 2009)**.

The term mycorrhiza is derived from the Greek words for 'fungus' and 'root'. Mycorrhizal fungidevelop an extensive hyphal network in the soil, the aptly named wood-wide web **(Helgason et al., 1998)**, which allows the connection of entire communities through a very efficient horizontal transfer of nutrients. Mycorrhizae develop the symbiotic interface, a specialized-areas to interact with the host plant **(Bonfante, 2001; Harrison, 2005; Parniske, 2008)**. Mycorrhizal fungi canbe divided

into two major groups: aseptate endophytes such as *Glomeromycota*, or septate *Asco-* and *Basidiomycota* **(Smith and Read, 2008)**. More commonly, mycorrhiza classifications reflect anatomicalaspects and identify two broad categories, referred to as ectomycorrhizas (EMs) and endomycorrhizas, depending on whether the fungus colonizes the root intercellular spaces or develops inside cells. Endomycorrhizas are further divided into or chid, ericoid and arbuscular mycorrhizas (AMs) **(Bonfante and Genre, 2010)**.

3.1 TYPES OF MYCORRHIZA

3.1.1 Ectomycorrhizae

Hundreds of EM species of *Basidiomycetes* and *Ascomycetes* interact with trees of the families *Pinaceae, Fagaceae, Dipterocarpaceae* and *Caesalpinoidaceae* present in many forests, so much that we can say that the current forests have been shaped by these mushrooms **(Smith andRead, 2008)**. EM fungi colonize the lateral roots of these trees with sheathing mycorrhizas, in which a fungal mantle covers the root tip, and a so-called Hartig net of intercellular hyphae surround epidermal and outercortical cells.

EM fungi may also live independently of plant roots, as demonstrated by their growth capabilities in Petridishes **(Smith andRead, 2008)**.

Molecular techniques allowed the identification of EM fungi in the field **(Gardesand Bruns1993)** and, thanks to metagenomics, the sequencing of organisms that live in an environment **(Handelsman, 2004)**, many sequences have been catalogedfrom the ground **(Martin and Martin, 2010)**, highlighting the rhizosphere biome around EMs.

Use of the Roche 454 Titanium genome sequencer has revealed unexpected levels of fungal biodiversity when applied to the soil of a French forest, in which *Agaricomycetes* were found to be the dominant class including alarge number of EM species **(Buéeet al.,2009)**. These data confirm the dual lifestyle of EM fungi: they can live in the soil as facultative saprotrophs or as symbionts with plant roots **(Martin andNehls, 2009)**.

3.1.2 Endomycorrhizae

Arbuscular mycorrhizae (AM) are the most common symbiotic associations in nature: they affect *Angiosperms* (about 80% of species), *Gymnosperms, Pteridophytes* and some *Bryophytes* and have a very ancient origin. Analysis of fossil and DNA sequences have in fact detected the existence of AM fungi more than 460 million years ago and it has been suggested that these fungi contributed to the colonization of the emerged lands by plants **(Redecker et al., 2000)**. AM fungi are obligate biotrophs and their life cycle depends on their ability to colonize the roots of the host plant. Analysis of specific DNA sequences in 2001 allowed arbuscular fungi to be assigned to a new phylum. This work was performed by analyzing the short subunit of ribosomal DNA (SSU rDNA) and, comparing the sequences, it was necessary to recognize the existence of a *phylum* called *Glomeromycota* **(Schüβler et al., 2001; Fig. 11).**

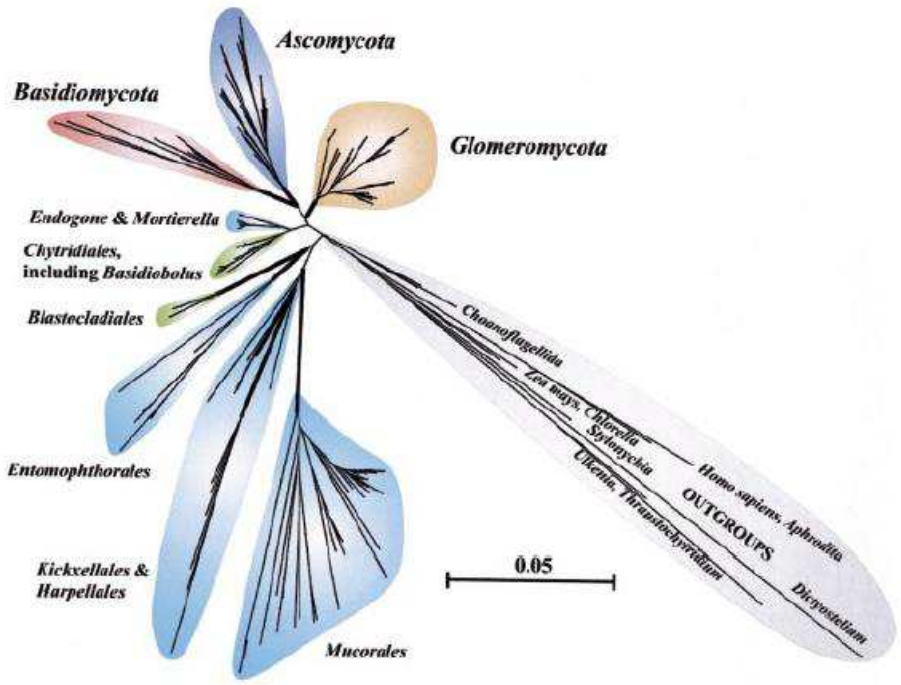


Figure 11: Phylogenetic tree of AM fungi based on the SSU rDNA sequences **(Schüβler et al., 2001)**.

The study shows the existence of a *phylum*, called *Glomeromycota*, to which four orders (*Archaesporales*, *Paraglomerales*, *Diversisporales* and *Glomerales*) belong, which include a total of eight different families. This taxonomy was made possible when approximately 100 ribosomal DNA sequences of *Zygomycetes* were made available in the databases **(O'Donnell et al., 2001; O'Donnell, Cigelnik and Benny, 1998; Tanabe et al., 2000)** and the phylogenetic analyzes have always produced the same results: arbuscular mycorrhizal fungi represent a distinct phylum that shares a common ancestor with the *Ascomycota* and the *Basidiomycota***(Fig. 11)**.

The AM fungi are obligatory symbionts, sincethey manage to complete their life cycle only by establishing a symbiosis with a host plant. During the interaction the fungus receives from the plant carbon compounds originating from the photosynthetic process while it yields mineral nutrients, in particular phosphate. AM symbiosis gives host plants greater tolerance to water scarcity and extreme temperatures **(Smith and Read, 1997)**, protection against radical pathogens **(Pozo et al., 2002)** and pollutants **(Gonzalez-Chavez et al., 2004)**. Furthermore, the extraradical phase of AM fungi extends into the soil through a network of hyphae which contribute to improving the stability of soil aggregates **(Rillig et al., 2004)**. Mycorrhizal fungi, due to their biological characteristics of biofertilizers and bioreproducers, are considered organisms of priority importance for the quality of the environment and for the development of an agriculture based on a decrease in chemical input.

Genetic-molecular studies conducted in the past provided important information that has been the subject of numerous review articles **(Harrison, 2005; Bucher, 2007; Hause and Fester, 2005; Balestrini and Bonfante, 2005; Balestrini and Lanfranco, 2006; Reinhardt, 2007; Paszkowski, 2006, Bonfante and Genre, 2010)**.

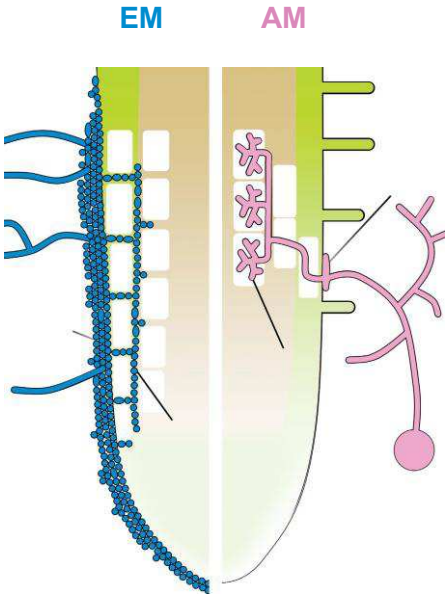


Fig.12: **Illustration of root colonization structures in ectomycorrhizal(blue) and arbuscular mycorrhizal (pink) interactions.** The EM fungus surrounds the root tip with a thick mantle of closely appressed hyphae, forming a *Hartig* net around epidermal cells (green). The AM fungus doesn't colonize the root tip. Hyphae develop from a spore and producing ahyphopodium on the root epidermis.

Intraradical colonization proceeds in and between the cells with the formation of arbuscles: small fungal trees that develop inside the internal cortical cells (brown) and allow the exchange of nutrients **(Bonfante and Genre, 2010).**

3.2 ARBUSCOLAR MYCORRHIZAE

3.2.1 The steps of fungal colonization

The way in which the 150 species of *Glomeromycota*, so far identified, colonize more than 200,000 plant species is decidedly preserved among the different taxa.

Pre-symbiotic phase

The interaction between the plant and the fungus begins before the physical contact between the two symbionts occurs through a molecular dialogue, probably the result of a long coevolution. In nature, AM fungi survive in the form of multinucleated spores which, in the absence of the host plant, pass from a state of quiescence to germination producing hyphae capable of growing only for a few days, given their nature as obligate biotrophs. Near a root the germinative hypha grows more rapidly, forming extensive branches to maximize the possibilities of contact with the host (**Buee et al., 2000; Giovannetti et al., 1993)**. The bioactive molecules present in the radical exudates responsible for hyphal branching have been described as strigolactones **(Akiyama et al., 2005; Besserer et al., 2006)**. There is also experimental evidence of the existence of a diffusible signal produced by the fungus that would be important in the early stages of the AM interaction. In analogy to the *Nod* factor produced by rhyzobia during the nodulation process **(Albrecht et al., 1999; Catoira et al., 2000)**, this signal was called "Myc factor". This factor stimulates, before the contact between the two symbionts, the expression of the *Medicago truncatula* gene MtENOD11 **(Chabaud et al., 2002; Journet et al., 2001; Kosuta et al., 2003)**, a gene that is also induced by the *Nod* factor. Studies showed that a diffusible factor released constitutively from the spores of AM fungi is able to generate a transient increase in cytosolytic calcium in cultured cells **(Navazio et al., 2007)**. Overall these results suggest the existence of fungal signal factors that also

allow the plant host to recognize the partner before penetration and suggest a role of calcium as an intracellular messenger.

Symbiotic phase

The real symbiotic phase begins when a hyphamakes contact with the root of a host plant, where it differs to form appressor-like structures, called hyphopodium **(Genre and Bonfante, 2007)**. The formation of the attachment is accompanied by changes in gene expression, in particular genes associated with calcium-mediated signaling pathways as occurs during pathological interactions **(Breuninger and Requena, 2004)**. Following this event, a specialized hyphais produced to enter and cross the epidermal cell, avoiding direct contact between the fungal wall and the host cytoplasm thanks to a membrane formed by the host cell.

An important role in the colonization process is played by the epidermal cell contacted by the fungus. *In vivo* observations of cellular components labeled with GFP have shown that, before penetration by the hypha, the epidermal cell organizes a transient structure, called pre-penetration apparatus (PPA), with a new cytoskeletal organization. It's hypothesized that PPA plays a fundamental role in the development of a compartment through which the fungus grows and penetrates the cellular lumen **(Genre et al., 2005)**. Once the hyphapenetrates the root, the cortical parenchyma is colonized through the formation of intercellular hyphae and intracellular structures, arbuscules, characterized by the extensive ramifications.

Arbuscule formation, like entry into epidermal cells, is accompanied by profound changes in host cell organization, such as vacuole fragmentation, core migration from the periphery to the center, organelle proliferation and the creation of an interface compartment. Intracellular hyphae, including those that make up the arbuscule, are in fact always surrounded by a thin layer of cell wall and the plasma membrane of the host cell. The construction of this interface by the plant cell is

achieved through the localized deposition of wall material **(Balestrini and Bonfante, 2005)** and the expression of specific genes **(Balestrini and Lanfranco, 2006)**. The arbuscular branches greatly increase the contact surface between the two symbionts. This structure is considered the seat of nutritional exchanges. After a short period of life (a few days) the arbuscule degenerates, but is replaced thanks to the continuous formation of new arbuscules **(Bonfante, 2001)**. Even the extraradical mycelium degenerates after a certain period of time and the new spores produced are released into the soil, where they will start a new development cycle.

The entire process of colonization of the root is asynchronous and the structures of the first stages, like the *appressorium*, coexist with the symbiotic organs, such as the arbuscules.

It is also believed that after the arbuscule collapse the same host cell may undergo a new colonization.

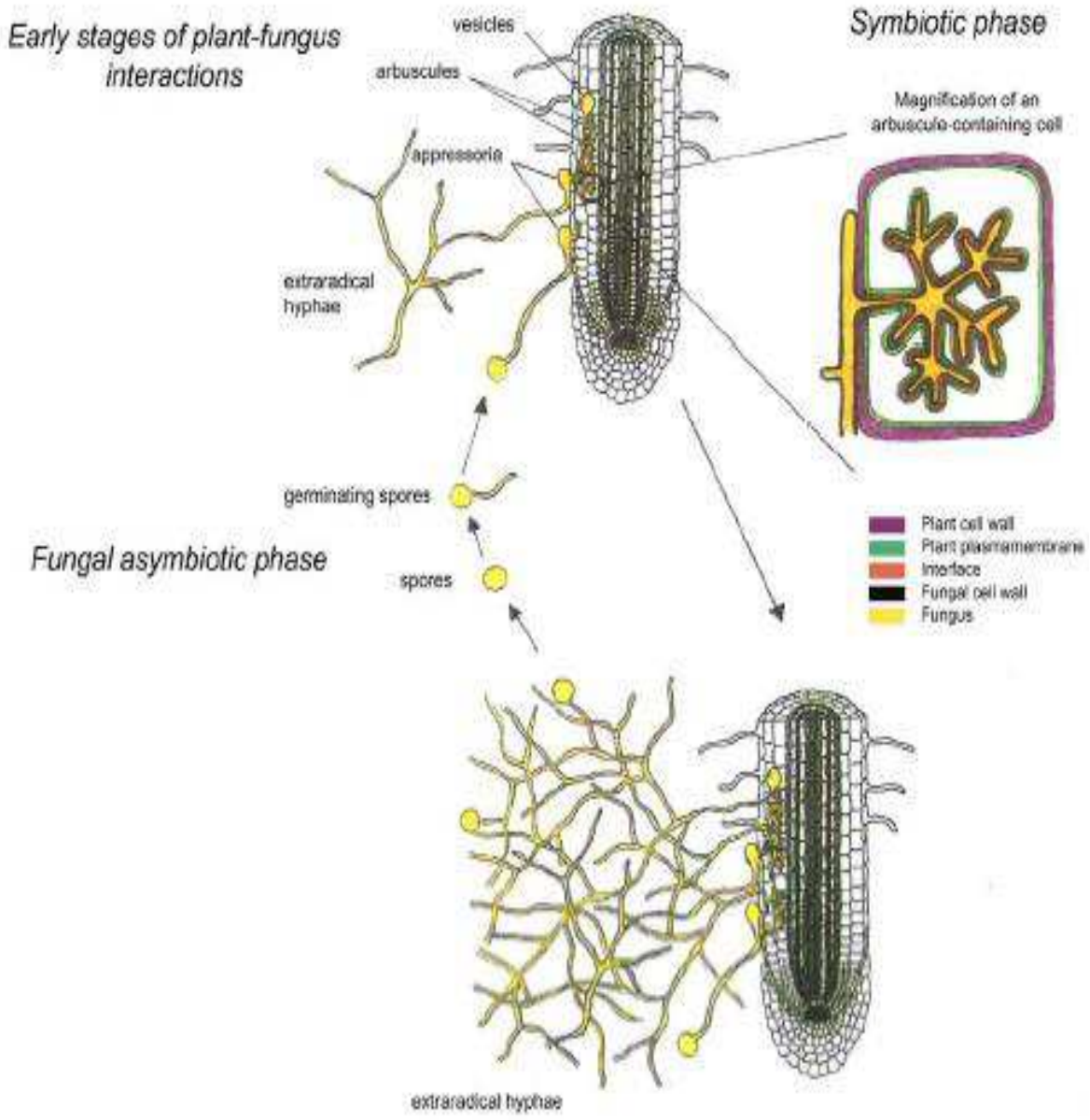


Fig. 13: Scheme of the different stages of root colonization by an arbuscular mycorrhizal fungus **(Balestrini and Lanfranco, 2006).**

3.2.2 The nutrient exchange

The basis for the AM symbiosis is the reciprocal exchange of nutrients. AM fungi depend almost entirely on the host plant for carbon acquisition. From the plant they receive, in a significant fraction (between 4% and 20% depending on the partners involved), compounds originating from the photosynthetic process **(Smith and Read, 1997)**. Some studies suggested AM fungi the presence of "lesions" in the metabolic pathways linked to carbon (C), which seem to be the main cause of the obliged biotrophism of these organisms. In particular, it has been observed that both extraradical hyphae and germinating spores are not able to synthesize fatty acid chains to 16 carbon units; the synthesis of these molecules occurs exclusively in the intraradical phase **(Trépanier et al., 2005)**. Other studies have shown that the fungus fails to accumulate lipids during the asymbiotic phase **(Bago et al., 1999)** and that it takes carbon, probably in the form of hexoses, exclusively inside the root **(Shachar-Hill et al. 1995; Solaiman and Saito, 1997; Bago et al. 2003)**.

Among the many benefits that the plant receives from the AM fungus, first of all is the supply of mineral nutrients. The fungus is able to transfer to the plant mineral elements such as phosphorus **(Pearson and Jacobsen, 1993)**, nitrogen **(Subramanian and Charest, 1998)**, potassium, zinc **(Kothari et al. 1991)** and copper, thanks to its dense and extended mycelial network, which goes beyond the zone of radical depletion (an area that is created due to the high rate of absorption of nutrients by the roots with respect to the speed of diffusion of mineral elements). Most of the studies conducted in the past have focused on the study of phosphate (P) transfer, both through the analysis of the P content in plant tissues, and by radioactive isotopes **(Ratti et al. 2001; Yao et al., 2001; Joner et al. 2000; Ramesh et al. 2000).** Transporter coding genes were isolated in three fungi AM *Glomus versiforme*, *G. intraradices* and *G. mosseae* **(Harrison and van Buuren, 1995; Maldonado-Mendoza et al. 2005; Benedetto et al., 2005)**. As evidence of the involvement of these transporters in the absorption of P from the soil, it has been

shown that the transcripts of these genes are present at extraradical level, even if in *G. mosseae* an expression has also been observed at intraradical level **(Benedetto et al. 2005; Balestrini et al. 2007)**. In parallel, investigations at the level of host plants led to the identification of genes encoding P transporters that are specifically expressed in the cells containing arbuscules and, therefore, should be responsible for the absorption of P released by the fungus **(Javot et al. 2007a)**. For further information on the subject, see excellent reviews **(Bucher 2007; Javot et al. 2007).**

3.3 PRODUCTION AND QUALITY CONTROL OF ARBUSCULAR MYCORRHIZAE

Introduction:

Arbuscular mycorrhizal fungi are obligatorysymbionts, therefore their reproduction and multiplication are possible exclusively *in vivo,* through the reproduction of the fungus in association with a host plant.

In our experience, following tests on various crops, we decided to use sorghum. *Sorghum* spp. is an annual herbaceous plant belonging to the grass family (*Poaceae*). The characteristics of the main types of sorghum are closely related to their final destination. Among food crops, it is one of the most resistant to drought and heat.

For the reproduction of mycorrhizal AM fungi is also important to evaluate the cultivation soil, which must have the characteristics and components suitable for plant growth and promote symbiosis.

Techniques to detect and quantify AM fungi in roots are essential tools. These methods are primarily used to identify mycorrhizal associations and measure the degree ofroot colonization. A range of light microscopy-basedtechniques can be used to detect and quantify AM inroots, including *in vivo* observations of fungal structuresin living roots, non-vital staining methods and vital root staining methods. Alternative means are biochemical methods that measure characteristic biochemical markersfor AM symbiosis (**Bothe et al., 1994; Frey et al., 1992; Schmitz et al., 1991)** and developed quantitative and qualitative molecular tools **(Alkan et al., 2004; Sanders, 2002)**.

Depending on the experimental approach, biochemical and molecular techniques can be reliable tools for the identification and/or quantification of AM in roots and thus a serious alternative to root staining. However, compared withstaining techniques, they are time consuming and costlyand therefore currently not practical for routine use. Thus, the staining of the roots and the counting of the stained fungal

structures in the root by routine light microscopy still remains the standard technique for the quantification of root colonization by AM. Staining and microscopic methods not only provide reliable data on the degree of root colonization but also permit to visualize the presence of key features such as arbuscules, which are the morphological criteria that define AM associations **(Brundrett, 2004).** For the observationof these morphological criteria, it is essential that root material is processed in such a way that these defining anatomical features can be easily detected.

CCS Aosta uses a method for the control of mycorrhizal inoculum that involves the use of the microscope and which has been deposited in the Official Journal.

The method can be used to evaluate the efficacy of the ability to form mycorrhizae, understood as the percentage of mycorrhization of the root system of plants inoculated with *Glomus mosseae, Glomus caledonium, Glomus viscosum, Glomus intraradices, Glomus coronatum*.

The technique is based on the microscopic analysis of colonized roots and on a subsequent one mathematical processing of the values obtained, which gives the percentage of mycorrhization.

Another technique that could be used in the mycorrhizal control is the Near Infra-Red Spectroscopy (NIRS). This technique is used extensively as a rapid, eco-friendly and cost-effective analysis system. A recent smart device, a hundred times cheaper than classical instruments, it became available wherever there is a fast internet connection. It has been shown that the usual preparation and fixation of fine roots in lactic acid can be scanned orthogonally across the tube, in trans-reflectance mode, and the reflected 740-1070 nm spectra can be used to enhance the expression of intrinsic traits, such as the percentage of mycorrhization.

Materials and Methods:

The usual production of AM fungi in CCS Aosta is prepared in the following way: a mixture of peat, inert mineral elements and organic substance was sterilized with steam and used as a substrate in the pots. For each pot 10 liters of mixture were used. The mycorrhizal inoculum was added to the amount of 500 g in each pot. In each pot 4 sorghum seeds were sown; the pots were placed in the greenhouse and watered daily. After 40-60 days irrigation was eliminated to create a stress condition that allows the production of resistance spores.

The crude inoculum of mycorrhizal fungi is prepared by CCS Aosta once every two years. The pots containing the roots of mycorrhizal sorghum, the spores and all the elements that will act as inoculum are collected and ground very finely so that they can subsequently be inserted into the final product.

Currently, CCS Aosta doesn't have yet an adequate system of grinding and cutting for the mycorrhizal roots, therefore they are sent to an external company that takes care of this.

Various tests were carried out to improve the growing substrate and make it more and more suitable to the business needs both from the point of view of the percentage of mycorrhization and from the point of view of consistency, so as to make the grinding and therefore the business management easier.

Different cultivation substrates have been tested to improve the mycorrhiza cultivation production.

Three different theses with three different substrate compositions have been tested. As the usual mycorrhizal production, for each thesis a different mixture of peat, inert mineral elements and organic substance was prepared and sterilized with steam and used as a substrate in the pots.

For each pot, 600 g of mycorrhizal inoculum of the GU53 strain were used. This is a *Glomus coronatum* belonging to the collection of mycorrhizal fungi of CCS Aosta but which is not part of the Micosat F UNO product; it is used in other commercial formulations.

In each pot 4 sorghum seeds have been sown; the pots were placed in the greenhouse and watered daily.

For each thesis have been prepared 9 pots and 3 control pots (without mycorrhizal inoculation). Pots used had a capacity of 10 (±1) liters and were in earthenware.

After 40-60 days irrigation was eliminated to create a stress condition that allows the production of resistance spores.

SubstrateN1:
20 liters of silt (supplied by Dual)
5 liters of Organic Compost (supplied by Carlo Vanzetti)
35 liters of 0/20 mm Blond Peat (supplied by the Perlite company) (product No. 2)
50 liters of Fine Vermiculite (supplied by Perlite) (Product No. 3)

SubstrateN2:
20 liters of silt (supplied by Dual)
5 liters of Organic Compost (supplied by Carlo Vanzetti)
85 liters of Fine Vermiculite (supplied by the Perlite company) (Product No. 3)

SubstrateN3:
20 liters of silt (supplied by Dual)
5 liters of Organic Compost (supplied by Carlo Vanzetti)
35 liters of 0/20 mm Blond Peat (supplied by the Perlite company) (product No. 2)
50 liters of Fine Perlite (supplied by the Perlite company) (product No. 1)

Product no. 1 Perliter taleaggio
Product no. 2 Perliter vermiculiter
Product no. 3 Fine Vermiculite

After 90 days from inoculation a sample of roots from each pot has been collected, cleaned, colored and analyzed with the optical microscope as the method described in the Official Gazette to evaluate mycorrhization percentage.

Preparation and stainingof roots for observation under a microscope

Forty samples, approximately 1 cm long, are taken from the root system and fixed in 70% alcohol. The root pieces are immersed in 10% KOH in 10 mL tubes. Tubes are heated in a water bath at 60° C for about 30 minutes. The pieces of root are rinsed in distilled water and then, after having dried them with absorbent paper, they are immersed in blue lactic for 1 min. The excess of dye is dried with absorbent paper, and then the roots are fixed in lactic acid and left to incubate for a few minutes. Step was repeated in lactic acid two more times, always drying the roots between one passage and the other; the colored root pieces are transferred to a test tube and left to soak for 1 hour in lactic acid. The root samples are taken from the test tube and mounted, 30 pieces, on a glass slide. Put 3 drops of lactic acid on the slide, and mount with the coverslip, pressing gently so as not to damage the roots.

Samples observation by optical microscope for the calculation of the mycorrhizal index

A class index based on the following scheme: class 0 (absence of infection), class 1 (traces of infection), class 2 (less than 10%), class 3 (from 11% to 59%), class 4 (from 51% to 90%), class 5 (over 90%) is arbitrarily assigned to each piece of sample mounted on the slide. The mycorrhization index (M) is calculated with the

following formula: M%= (95n5 + 70n4 + 30n3 + 5n2 + n1)/N where n1, n2, n3, n4, n5 are the numbers of pieces belonging respectively to classes 1, 2, 3, 4, 5 and N is the total number of pieces examined.

NIR technique

In a one-year study, the usual root preparations, derived from the routine analyses of *Sorghum sudanensis* (No. 251) and other species (No. 11), were examined according to the official ministerial provision for the assessment of the mycorrhization index (0-100%) and were divided into four almost equal frequency classes: C (<25%); M1 (26-33%; M2 (34-37%) and M3 (>37%). The tubes were then scanned by means of smart NIR-SCÏOTM 2.0. The collected spectra were then elaborated, using the proprietary Lab-SCIO software, according to a qualitative Random Forest algorithm. The fingerprinting of the four classes, based on the AKA (As Known As) recognition matrix, were 72, 67, 76 and 68%, respectively, for the four classes (P>0.0001 vs. 25% random).

Results:

The mycorrhization index M, expressed in%, determines the efficacy of the product in terms of taking root or infection of the treated plants. In the specific case it must not be less than 30% (tolerable minimum value 25%) with a 5% tolerance.

Mycorrhization rates were calculated and the average for each thesis was reported with corresponding control, as well as the differential of mycorrhization between the thesis and its negative control.

	Myc	Negative control	Δ mycorrhization
N1	61,83%	5,90%	55,93%
N2	55,73%	0,53%	55,20%
N3	39,57%	1,40%	38,17%

Table 2: Results of mycorrhization in percentage of three different theses with three different substrate compositions

Mycorrhizal analysis showed that the highest value was the one related to the substrate N1.

Substrate N3, instead, turned out to be the less mycorrhized.

Observing the differential calculus, it was possibleto observe that the first two theses were similar, unlike the third one, which had lower values both in the percentage of mycorrhization and in the differential calculation.

Considering these results of NIR tests, it can be calculated that a 95% probability of correct memberships per lot can be attained by repeating the sampling three to four times. It has been concluded that the use of lactic acid-fixed and colored tissues, coupled with vibrational spectroscopy, to perform multiple tests, can be as much as 95% time saving for the micro-reading of samples **(Volpato et al., 2019 submitted).**

3.4 MYCORRHIZAL INOCULANTS AS BIO-FERTILIZERS: APPLICATIONS AND EXPERIMENTS IN OPEN FIELD

Since mycorrhizal plants are more efficient in the absorption of specific nutrients **(Smith and Read, 2008)**, the use of AM fungi as an inoculum offers the possibility of reducing fertilizer applications. Therefore, AM fungi has gained popularity as "bio-fertilizers" both in the field **(Heppell et al. 1998; Daei et al., 2009)** and containers **(Sylvia, 1999; Neumann and George, 2010)**.

Pellegrino et al. (2015) carried out a meta-analysisof 38 published field trials with 333 observations to determine the effects of inoculation and root colonization by inoculated and non-inoculated (resident) AMF on P, N and Zn uptake.

Thanks to the growth and yield of the grain, the meta-analysis demonstrated that, in-field, AM inoculation increases above-ground biomass, grain yield, harvest rate, concentration and P content of surface biomass, the content of P in straw, the concentration and the content of N in surface biomass, the content of N in wheat and the concentration in wheat of Zn.

The positive correlation between grain yield and root AM colonization rate was also demonstrated, on the contrary was observed a negative correlation between AM colonization and straw biomass.

The most important drivers of wheat growth response to AM have been shown to be soil inorganic matter, pH, total N and available P concentration, texture of soil, as well as climate and the AM species inoculated.

The meta-analysis shows that the use of AM as wheat inoculum in field conditions can be an effective agronomic practice, although profitability has not yet been analyzed in economically viable large-scale farming systems.

The industry of mycorrhizal inoculants production is expanding around the world (e.g., **http:// mycorrhiza.ag.utk.edu/**). Although some studies indicate that inoculation with more than one AM isolate may not bring more benefit to the host

plant **(Edathil et al., 1996)**, a mixture of AM with complementary functions appears to be more beneficial to the plant than a single isolate **(Pellegrino et al. 2012; Gustafson and Casper, 2006)**.

A comparison was made between the performance of *Trifolium alexandrinum* in the field **(Pellegrino et al., 2011)** inoculated with exotic AM (both single and mixed) with those of *Trifolium alexandrinum* inoculated with a native AM. It was shown that the native inoculum was more effective than the exotic AM fungi isolates and that in-field AM inoculation increased crop productivity and quality.

The persistence of the beneficial effects of AM until the second year after inoculation was also demonstrated, with yield increases in the following crop (maize).

In a second field study, **Pellegrino and Bedini (2014)** tested the agronomic relevance of field-inoculated locally sourced and foreign inocula on chickpea (*Cicerarietinum* L.), one of the most important worldwide grain legumes, evaluated not only the yield but also the improvement of the nutritional value of chickpea grain by protein, Fe and Zn bio-fortification and showed in the field the role of AMF as bio-fertilizers of crops and bio-fortification tools. Recent advances showed that a microbial consortium containing mycorrhizal inocula is more effective **(Masoero and Giovannetti, 2011; Masoero and Giovannetti, 2015; Nuti and Giovannetti, 2015)**.

3.5 DISCUSSION

Mycorrhizal fungi, due to their biological characteristics of biofertilizers are considered organisms of priority importance for the quality of the environment and for the development of an agriculture based on a decrease in chemical input. This importance makes it possible to consider the insertion of a mycorrhizal component during the formulation of a commercial product with microbes.

Techniques to detect and quantify AM fungi in roots are essential tools. These methods are primarily used to identify mycorrhizal associations and measure the degree ofroot colonization.

Positive aspects of the plants brought about by mycorrhization are evident and well-known, but several problems still need to be resolved for effective and optimized production management. Problems due in part to the need for *in vivo* cultivation, which involves the use of greenhouses and pots that must be prepared and cured daily, a highly expensive aspect for a company; partly due to the difficulty in managing the contents of the pots and their inclusion in a final commercial product.

In addition to these problems of business management, it is also essential to study the possible fungal mixes to be included in a product, if there are differences based on the crops to be treated or if mycorrhizal inocula act indistinctly on all the plants, whether is better to insert: native species or not, and in what quantity.

This field, although increasingly arousing the interest of farmers and researchers, is still much to explore and studied.

4 COMMERCIAL MICROBIAL CONSORTIA

A microbial consortium is a group of identified microbial strains that work together in a single product to carry out an overall reaction or process **(Brahmaprakash and Pramod, 2012)**. More recently, the microbial consortium is receiving increasing attention for use in agriculture and agro-industry as either bio-fertilizers/bio-effectors or plant protectants **(EFSA, 2015).**

During the last two decades, dozens of plant protection products and a few bio-fertilizers have been used based on a single-microbial-strains as the active substance. One way to improving biocontrol in the rhizosphere may be to add mixtures of combinations of biocontrol agents, particularly if they exhibit different or complementary modes of action or abilities to colonies roots microsites. Such multiple interactions are the normal situation in the rhizosphere. Numerous permutations have been considered, including combinations of different bacteria, fungi or both bacteria and fungi.

Currently, it is possible to find on the market many products composed of microbial consortia, including Micosat F, a biostimulant produced and marketed by CCS Aostaon which my research is based.

The success of the inoculation process depends on two factors, such as the microbial strain and inoculants formulation. In practical terms, formulation determines potential success of inoculants **(Fages, 1992)**. Thetechnical optimization of an inoculant formulation is independent of strains used, since most of the strains of same bacterial species share many physiological properties, it may be assumed that a technological progress developed for a particular strain is readily adaptable to another strain of same species with only minor modifications **(Bashan, 1998)**.

The most questionable characteristic, common to most of biofertilizers, is unpredictability of their performance. In order to harness the benefits of biofertilizers in agriculture, the consistency of their performance shouldbe

improved **(Wani and Lee, 2002)**. Bacteria introduced to soil may fail to establish in sufficient numbers in the rhizosphere because of competition from native numbers, and little is known about the factors controlling competitiveness of bacterial strains, more so under field conditions.

However, bacterial inoculants may have greatest contribution if inexpensive and easy to use formulations can be developed **(Bashan, 1998).**

4.1 ELEMENTS THAT DETERMINE THE QUALITY OF A MICROBIAL COMMERCIAL CONSORTIUM

It's important, when considering the use of mixtures or combinations of strains that no member of the mixture has an inhibitory effect towards another or interferes excessively with the existing, normal and non-pathogenic microbiota associated with the roots.

Clearly, the complex interactions that can take place in the rhizosphere between biocontrol agents and the indigenous microbiota needs to be considered during development of commercial microbial products **(Whipps, 2001)**.

Then, in order to produce and market a quality microbial consortium it's important not only to guarantee the quality of all the elements that make it up but also the quality of the biota considered as a whole.

4.1.1 Quality of individuals microorganisms present

To produce a single-microorganisms quality microbial consortium, the consortium should satisfy the quality conditions: this has a fundamental importance.

Here, we refer to chapters 2 and 3 in which we have already explained how to guarantee the quality of the individual microorganisms and mycorrhizal fungi that which are intended to be included in a microbiological consortium to be used in agriculture.

4.1.2 Quality of the consortium: composition, characterization and formulation

How should a microbial consortium be composed? Which microorganisms do I choose? How do I put them together?

These questions are the ones we should answer, when we decide to produce a quality microbial consortium.

The interactions that are established between the various microorganisms, are mainly of two types:

- **POSITIVE**: in which both individuals benefit from the establishment of the association;
- **NEGATIVE**: in which one or both individuals have adisadvantage from the association.

Negative interactions include parasitism, antagonism and competition. The latter type of negative interaction occurs when two bacteria use the same resources. The degree of competition increases as the concentration of resources decreases.

It is therefore important in the choice of the formulation of a microbial consortium that microorganisms within them synergistically interact. In synergy, both populations benefit from interaction, but the association is not obliged and is not specific. The two partners can, in fact, proliferate independently in different environments or be replaced by another partner capable of performing the same action.

The Micosat F UNO microbial consortium contains six different microorganism strains: *Agrobacteium rhizogenes* and *Bacillus subtilis* (AR39 and BA41, respectively), one *Streptomyces* spp. (SB14), one *Trichoderma harzianum* (TH01), one *Pochonia chlamydosporia* (PC50), one yeast, *Pichia pastoris* (PP59) and AM fungi.

The microbial community of Micosat F UNO is a complex system consisting of:

- Mycorrhizal fungi (mycelia, hyphae and spores), also called symbiotic fungi, which associated with plant roots give rise to mycorrhizae that amplify the root system. Through this enhancement the plant it feeds better and achieves a better yield, moreover an increase is obtained direct and / or indirect tolerance of abiotic stresses (thermal, water, nutritional, etc.).

- Growth promoter rhizosphere bacteria (PGPR), able of promoting and regulating the absorption of nutrients and to correct the physiological anomalies **(D. L. 04-29-2010, No. 75; G.U. n. 121 of the 26-05-2010)**.

- Saprophytic fungi, which act as a preventive measure by subtracting space and nutrients to widespread radical adversities.

- Actinomycetes that carry a large number of relevant activities. Among them we mention: the active participation in the decomposition of some components of the animal and vegetable fabrics; the formation of humus through the conversion of organic residues in compounds typical of the organic fraction of the soil; the role in the regulation of the microbiological balance of the soil; the possibility of becoming endophytes.

- Yeasts perform fundamental actions in the biogeochemical cycles of the soil.

In order to prepare a very effective consortium, tests on microbial compatibility (microbe-microbe interaction) is necessary: *in vitro* assays were performed to assess what effect the different microorganisms have between them and the type of interaction. All microorganisms contained in the product were therefore crossed two by two.

4.1.3 Quality of the carrier substrate

Microbial inoculants are usually prepared as carrier-based inoculants containing significant and effective microorganisms. Incorporation of microorganisms in carrier material enables easy-handling, long-term storage and high effectiveness of biofertilizers. Wide range of materials has been used as carriers to improve the survival and biological effectiveness of inoculants by protecting the microbes from various biotic and abiotic stresses **(Van Veen et al., 1997)**.

The material to be used must be suitable for transport, economical, easy to use, mixable, packable and available. Furthermore, the carrier must allow the exchange of oxygen and others gas, have a high content of organic substance and a high capacity to retain water **(Bashan, 1998; Ben Rebah et al., 2002)**. According to Somasegaran and Hoben **(1994)**, the effective carrier material must be non-toxic either to the bacterial inoculants or to the plant or even to soil itself. Furthermore, Stephens and Rask **(2000)** and Ferreira and Castro **(2005)** stated that the carriers should have an almost neutral or easily adjustable pH, be easily sterilized, readily available locally at a reasonable cost. These properties only indicate potential for a good carrier, while final selection of carrier shouldbe based on the characteristics linked to the microbial multiplication capacity in the inert substrate and to them during storage. Among carrier materials that can sustain high concentration of microbial load, peat is considered the most widely used carrier **(Burton, 1967; Peterson and Loynachan, 1981),** but it is not available worldwide **(Tilak and Subba Rao, 1978)**. Alternatively, other materials such as industry by-products, organic wastes, mineral soils, plant by-products, coal, perlite, talc powder and agro-industrial wastes have been tested as culture media for the microbial growth **(Brockwell and Bottomley, 1995; Stephens and Rask, 2000)**.

CCS Aosta, following several studies of applicability, evaluated the best carrier for the microorganisms that produces: this carrier is a micronized zeolite.

Zeolites are microporous, aluminosilicate minerals commonly used as commercial adsorbents and catalysts **(Grace et al., 2006)**.

The term *zeolite* was originally coined in 1756 by Swedish mineralogist Axel Fredrik Cronstedt, who observed that rapidly heating the material, believed to have been stilbite, produced large amounts of steam from water that had been adsorbed by the material. Based on this, he called the material *zeolite*, from the Greek ζέω (zéō), meaning "to boil" and λίθος (líthos), meaning "stone" **(Cronstedt AF, 1756)**. The classic reference for the field has been Breck's book *Zeolite Molecular Sieves: Structure, Chemistry, And Use* **(Breck DW 1973)**.

They are aluminosilicates with crystalline structures consisting of TO_4 tetrahedrons (T = tetrahedral species, Si, Al, P, etc.), whose oxygen atoms are shared with adjacent tetrahedrons; in fact, these tetrahedral units $[SiO_4]^{4-}$ and $[AlO_4]^{5-}$ share oxygens to form non-linear bridges.

It's possible to imagine the zeolitic structure as a set of SiO_4 and AlO_4 tetrahedrons that bind to each other, according to simple geometric shapes, which join together to form complex units such as chains, rings or cages of low potential energy **(www.chimdocet.it).**

An important class of zeolites has a cage structure. Since cages possess crystalline symmetry, zeolites represent a class of molecular sieves with high selectivity with respect to silica or activated carbon **(AA.VV., 2018)**.

Inside the structures, various cavities are formed that can be filled with water. The mineral can lose H_2O with exposure to air, by heating or by substitution with metals present. Pore size is important, as the catalytic action is related to them: the molecules selectively enter these pores and undergo cracking and isomerization reactions, for example. Moreover, the type of cation present inside the zeolitic structure influences the ion exchange kinetics **(www.chimica-online.it).**

The microporous structure of zeolites allows to absorb or filter liquid or gaseous molecules; in fact, in soils they absorb water and release it slowly, thus avoiding excessive dryness of the soil during hot periods. The zeolites retain the nutrients avoiding that the latter are washed away with rain, a soil rich in zeolites needs less fertilizer. These minerals also act as pH correctors and temperature stabilizers. For these reasons, CCS Aosta has adopted it as an inert substrate and stabilizes the microorganisms it produces.

4.1.4 Quality control of zeolites

The main feature for zeolite to be considered usable is the granulometry and the sterility. In fact, to obtain a final product that is qualitatively valid and that meets the standards indicated on the label, the grain size must be less than 75 μmmoreover it must not contain pathogenic microorganisms or which compete with those that are stabilized on the inert

Therefore, in addition to the prior request to the Company that provides zeolite to CCS Aosta (grain size of less than 45 μm), checks are made on the incoming material.

The inert is delivered in 900 Kg big bags, after an initial visual and tactile examination, samples are taken from each big bag and granulometric checks are carried out through the rot-ap, using 75 μm, 100 μm and 350 μm sieves. 80% of the product must be less than 75 μm to be acceptable.

For the evaluation of sterility of the product, during the evaluation of the supplier, a calculation of the C.F.U. on non-specific medium was carried out, it revealed the absence of pathogens and an acceptable amount of microorganisms present. Furthermore, to be sure that microorganisms do not develop during storage in big bags, the zeolite is sterilized with steam before use.

4.1.5 Conservation, manteinance, shelf life

In order for the marketed products to be qualitatively valid, it is essential to evaluate their vitality over time, storage and conservation methods.

One way to ensure greater longevity, microorganisms are stabilized on an inert substrate. In CCS Aosta case this is a micronized zeolite. It is also produced a granular formulation that is composed by microorganisms dried on zeolite, bentonite and glue that form very stables microgranules. This guarantees a product shelf life of up to two years.

Shelf life evaluation is part of quality controls of microbials. This is done as follows:

Every year a random pack for each type of commercialized product is stored to carry out analyzes at a distance of 15 days, 1 month, 3 months, 6 months, 12 months, 1 year and 2 years in order to evaluate both the product upwards, the vitality and duration of microorganisms. Visual checks are therefore carried out, relative humidity is calculated using a special laboratory instrument and microbial counts are carried out on selective media for fungi and bacteria.

In order for the product to maintain good quality over time, it is important that the conservation of the finished products is also adequate, in particular the temperature and humidity parameters must be considered. If the temperatures and humidity are too high, there is in fact the possibility that the microorganisms kept inactive in the inert substrate are activated or suffer and die, making the product less effective in the use phase.

Materials and methods:

Plates were prepared with MA and PCA media as follows to test the compatibility between microorganisms in the same product:

MA - Malt Extract Agar: malt extract: 12,75 g/l; dextrin: 2,75 g/l; Glycerol: 2,35 g/l; Gelatin peptone: 0,78 g/l; Agar: 15 g/l.

PCA - Standard plate count agar: yeast extract: 2,5 g/l; pancreatic digest of casein: 5,0 g/l; glucose 1,0 g/l; agar: 15 g/l.

The media were autoclaved at 125 ° C for 25 min and subsequently plated.

Crossings were made under a sterile hood.

Interaction *bacterium - bacterium*: with a sterile strain, equidistant waves were drawn by taking the two bacterial strains from the plate with the pure culture previously selected. PCA medium was used.

Interaction *fungus - fungus*: a rod was drawn for each of the two fungi considered and placed in the center of the two halves of the plate. MA medium was used.

Interaction *bacterium - fungus*: a fungal colony of about 4 mm was taken from a previously prepared plate on which the strain to be analyzed was grown and placed in the center of the Petri dish. By a sterile loop, a bacterial strain was inoculated on the same plate where the fungal colony was previously placed, drawing strips that from the proximity of the fungus until the edge of the plate.

Both PCA and MA media were used.

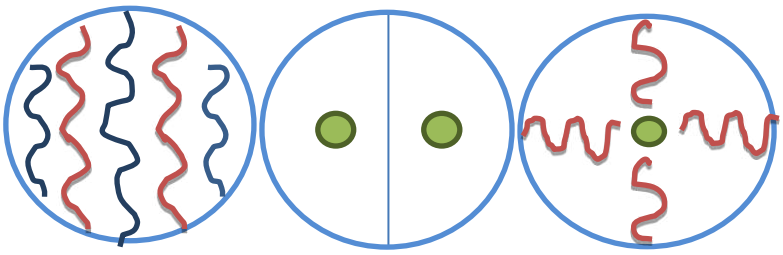


Figure 14: Method applied during plating: they are three types of interaction. In the first the two bacteria were plated alternately, in the second the two fungi were arranged in approximately equidistant rods and in the third the central fungus and the bacterium was placed around it.

At the same time, the viable microbial load of the micro-organisms present in the Micosat F UNO product was determined through serial decimal dilutions and tiling on different types of agarized medium.

The following media were used: TAM, MA, PDA, TSA, PCA.

The calculation of C.F.U. on serial dilutions is carried out on a sample of 10 g of product dissolved in 90 ml of water through a 10-minute stirring.

The 1:10 suspension of the sample was used to prepare subsequent serial dilutions up to the 10^{-6} dilution and aliquoted 100 μl of suspension on the plates with selective medium suitably prepared for dilutions from 10^{-3} to 10^{-6}. Two plates are inoculated for each dilution.

The plates were placed in an incubator at a temperature of 27 ° C for 3-5 days and the grown colonies were quantified.

Results:

From the tests carried out, it was possible to see that some microorganisms contained within the Micosat F UNO inhibit the growth of others. In some case the effect may be due to the use of selective terrain.

The most significant effect was observed with bacillus against fungi, especially Trichoderma. As shown in Fig. 15, both BA41 and AR39 strains inhibited the growth of TH01. Similarly, both BA41 and AR39 partially inhibited the growth of PC50.

Figure 15: BA41-TH01 interaction. In the left plate the microorganisms grew up on MA and it's possible to see how the strain TH01 grows like a star around strain BA41, but fails to grow above it.

'In the right' plate the microorganisms grew up on PCA and it's possible to observe how the BA41 prevents the growth of the TH01.

Some microorganisms showed different effects based on the medium they have grown on. For example, strainSB14 had an inhibiting effect on TH01 and PC50 only on PCA medium (Fig. 16).

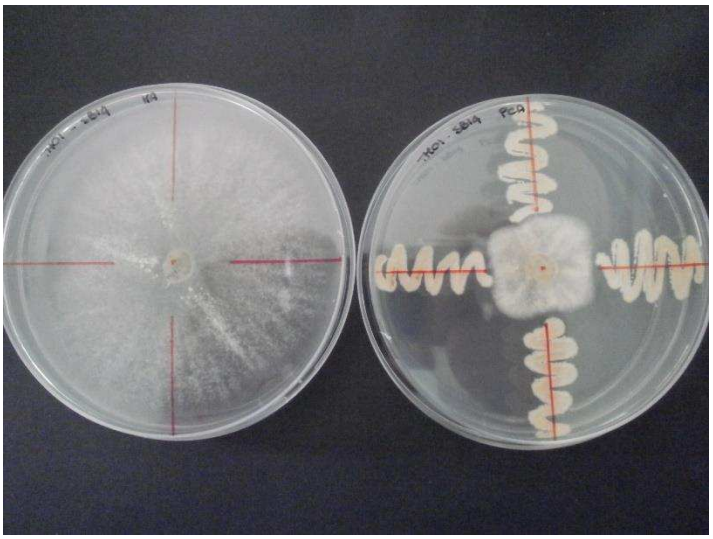


Figure 16: SB14 -TH01 interaction. In the left plate the microorganisms grew up on MA and it's possible to see how strainTH01 grows on the whole surface of the plate, inhibiting strain SB14. In the right plate the microorganisms grew up on PCA and it's possible to observe how strainSB14inhibits the growth of starinTH01.

In other case it didn't seem to be any effect on the growth of different strains on the same plate, as shown in the Figure 17.

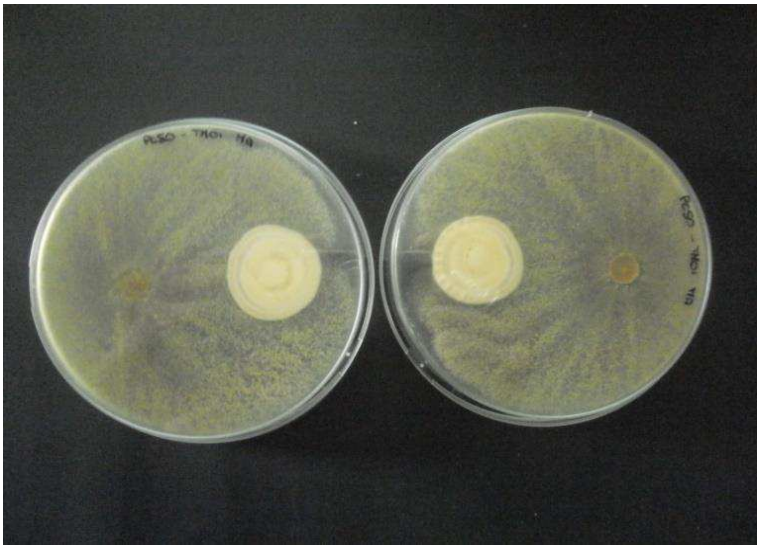


Figure 17: TH01-PC50 interaction. In both plates the microorganisms grew up on MA. It's possible to observe that there's no inhibition.

Single strain C.F.U. was lower: AR39: 4,7E+08; BA41: 4,67E+08; SB14: 1E+08; PP59: 2E+08; TH01: 1E+06.

We observed the inhibition of strain TH01 due to the presence of the AR39 strain and the growth of the PC50 was not found.

4.2 STANDARDIZATION OF PRODUCTION PROCEDURES

The industrial process of producing a biostimulant shouldbe properly studied and standardized, in order to guarantee the chemical characteristics in terms of concentration and type of bioactive compounds. As we have already seen, standardization should begin with the selection of the raw materials used.

If the quality of the raw material is guaranteed, also every step leading to the final product to be marketed must be standardized, controlled and repeatable.

CCS Aosta uses the ISO system as a guarantee of the production standard. It consists of a series of regulations and guidelines developed by the International Organization for Standardization that define the requirements for the realization inside of an organization of a quality management system, in order to conduct business processes, improve the effectiveness and efficiency and in the realization of the product and in the provision of the service, obtaining and increasing customer satisfaction.

An International Standard provides rules, guidelines or characteristics for activities or for their results, aimed at achieving the optimum degree of order in a given context. It can take many forms. Apart from product standards, other examples include: test methods, codes of practice, guideline standards and management systems standards.

The ISO 9000 family addresses various aspects of quality management and contains some of ISO's best-known standards. The standards provide guidance and tools for companies and organizations who want to ensure that their products and services consistently meet customer's requirements, and that quality is consistently improved.

ISO 9001 specifies requirements for a quality management system when an organization:

- Needs to demonstrate its ability to consistently provide products and services that meet customer and applicable statutory and regulatory requirements.
- Aims to enhance customer satisfaction through the effective application of the system, including processes for improvement of the system and the assurance of conformity to customer and applicable statutory and regulatory requirements.

All the requirements of ISO 9001 are generic and are intended to be applicable to any organization, regardless of its type or size, or the products and services it provides **(http://www.iso.org)**.

4.3 STANDARDIZED PRODUCTION OF A MICROBIAL CONSORTIUM

The CCS Aosta produces microbial consortia for agriculture in different formulations: liquid, microgranular and wettable powder.

The making of these three different products has many common procedures, but it differs at the end of the processing, in the stabilization phase. Following, the various standardized production phases leading to the production of Micosat F are described.

The ISO standards during the production of Micosat F products are respected through the registration of every production and management step. Thanks to a computerized support system, the products in the warehouse are counted and when a product runs out, it is reported, and the whole production system is activated. The kilos to be produced are calculated, the necessary material (and in case the raw materials to be bought back because they are finishing) and the quantity of fermentation broth to be prepared.

In addition to this, the consumptions made for each production are included in the system, both for consumables and for the energy used and the values for the checks carried out in each production step to determine whether the semi-finished product is acceptable or not.

Furthermore, thanks to the ISO system, suppliers are checked periodically, non-conformances related to incoming products, production processes, outgoing products and returns are recorded.

All this allows an extremely controlled management of industrial processes and continuous improvement and planning.

The various standardized production phases that lead to the production of Micosat F, begin in the laboratory with a prefermentation that will serve as a subsequent inoculation for the bioreactor.This term means any manufactured device or system that supports a biologically active environment **(IUPAC, 1997).** This is usually a container in which a chemical reaction takes place carried out by micro-organisms or substances derived from them. This type of bioreactor is usually cylindrical, composed of stainless steel and can reach sizes between a few liters and several hectoliters. This concept of bioreactor is akin to that of cell culture and is to be considered for the production of large-scale microorganisms.

As is well known, microorganisms are able to perform their functions optimally only under suitable environmental conditions. For this reason it is necessary to constantly monitor gas concentrations (such as O2, N2, CO2), temperature, pH and the mixing speed of the bioreactor content.

Most industrial bioreactors are equipped with sensors to monitor each parameter and a software capable of handling this information in order to supply it to the operator.

To avoid damage, the bioreactor must be easily cleanable and composed of smooth surfaces (for this, the most common form is the cylindrical one). Furthermore, bioreactors are equipped with a heat exchanger, necessary to maintain the bioprocess at a constant temperature. Since biological fermentations are a great source of heat, bioreactors are equipped with a cooling source. This device may consist of a sort of external insulating interspace or of an internal refrigerant coil.

Industrial bioreactors generally use bacteria or other simple organisms, which are preferred over other more complex organisms because they have simpler nutritional requirements and a higher reproduction rate.

The fermentation products are immediately used for subsequent production steps or, alternatively, they are stored in sterile bins in a cold room at a temperature of 2-

4° C. An ideal formulation is expected to facilitate the delivery of the living biocontrol agents in its active state, at the right place and at the right time. While the formulated microbial products must be effective at the site of action and compatible with agronomic practices, they should be easy to apply to and adhere to plant parts such as seeds, tubers, cuttings, seedlings, transplants and mature plants or be available in the soil medium for longer period even on adverse biotic and abiotic stress conditions. To make this possible, the stabilization phase of the microorganisms is of fundamental importance. There are different types of commercial formulations, and CCS Aosta commercializes three fundamental formulations: liquid, wettable powders and micro-granules. The liquid is the simplest to obtain: microorganisms deriving from fermentation in bioreactor are mixed in different percentages and are stored in jerry cans. The most problematic aspect of this formulation is its shelf life. Shelf life is, in fact, shorter and the product must be kept refrigerated. The last part of the industrial production of microbial consortia consists of packaging and preserving the finished product.

Laboratory production inoculum

The first step in the production of Micosat F is the laboratory preparation of flasks containing individual cultures of strains to be multiplied and which will be used as bioreactors inoculum.

Specific selective media are prepared for each microorganism to be produced and 1.5 liter of liquid medium is added to each flask. Three flasks are prepared for each strain to be produced. Flasks are then sealed and sterilized in autoclave for 20 min at 125° C, after the cooled time at room temperature (20±2° C) for about two hours they are inoculated under a hood to ensure sterility and grown in a shaker with controlled temperature and agitation (based on the organism to be multiplied). At the growth end time, flasks are controlled (see chapter 5 for the detailed control method) and used as inoculum of the bioreactor.

Bioreactor production inoculum

In the large-scale production of microorganisms, CCS Aosta uses the 2 liters content grown in flasks prepared in the laboratory as inoculum of bioreactor.

The bioreactor was previously filled with the specific liquid medium for the microorganism multiplied and sterilized already inside the bioreactor by means of steam. Each microorganism has a different growth time and during the whole time of reproduction the parameters of temperature, pH and oxygen are monitored and adjusted. At the end of the fermentation a sample is analyzed to control purity quality and growth of the produced microorganism.

At the end the fermentation is centrifuged through an industrial centrifuge to remove the culture medium liquid and increase the concentration of the microorganism by 10 times.

Drying and assembling: the industrial production

Powders are easily obtained by mixing the microorganisms produced in the bioreactor and the mycorrhizal inoculum with an inert compound. As mentioned earlier, CCS Aosta to do this with micronized zeolite (less than 75 μm granulometry). Instead, obtaining a granulate from the cultivation composite of microbial components is a more complex procedure. The machine currently used by CCS Aosta derives from a specific adaptation project of an industrial granulator. It was necessary to carry out a study aimed at researching the fundamental parameters to obtain a stable granule, which does not come apart and which has a precise humidity. The inert component (zeolite or bentonite) neededfor the transmission of microbes deriving from fermentation in bioreactor and VAM fungi obtained from the roots of inoculated sorghum, the type and quantity of adhesive were identified. At the end of the industrial processing process a microgranular product is obtained which is easy to manage.

Packaging and conservation

As far as packaging is concerned, there are important characteristics for the optimal preservation of the product and the necessary characteristics from a legislative point of view. From a legislative point of view, the package containing microbial consortia must have some features. The package must show the formulation of the product and its fundamental characteristics, the methods of use and the field of application. The non-hazardous nature of the product for humans, animals and the environment must be reported and finally must contain any warnings. On the packaging must also be reported information on storage, lot, the date of production and the expiry date.

Through the production process described above, it is possible to obtain a stable and easily conservable commercial microbial consortium.

4.4 DISCUSSION

The success of the inoculation process depends on two factors, such as the microbial strain and inoculants formulation.

In vitro tests confirmed what has already been known and can be found in the literature: producers should not insert in the same consortium fungi and bacilli,sincethey could establish a competitive relationship and maycancel or reduce the effect that both can bring to the crop, but the activity of several microorganisms together can lead to a synergy of action.

However, it should be remembered that the *in vitro* test could have different results compared to an *in vivo* test.

The manufacture of good-quality microbial consortia products requires both safe production and safe use in agriculture. This demands that the manufacturing procedures might considering the following factors:

All biofertiliser microorganisms used should be known to be non-pathogenic to humans, animals or plants. Obviously, this requirement demands that they be identified by genus and species as already described. In normal practice, this will mean that if there is significant doubt about the identification of any microbial strain, this strain should not be used, irrespective of how successfully it promotes plant growth and crop yield.

As we have observed, all microorganisms to be included in microbial consortia products should be shown to be compatible with each other, even if they are grown separately in production.

Information on whether there are incompatibilities between biofertiliser microorganisms and other beneficial microbes that farmers may need to use should also be prominently displayed, preferably on the label.

Another aspect to be considered in the formulation phase of a microbial consortium is the production system to which it is connected: it is in fact unlikely that the same microorganisms can function in the same way on different crops.

As we'll see next, inert carrier materials should also be shown to be of good quality, free from contaminants, or other undesirable properties (e.g. granulometry).

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5 QUALITY CONTROL

The quality is the standard of something as measured against other things of a similar kind; the degree of excellence of something.

Quality of inoculants in the biofertilizer pack is one of the most important factors resulting in their success or failure and acceptance or rejection by the farmers. Basically, the quality means the presence of right type of microorganism in active form and in desired numbers **(Sethi and Adhikary, 2012).**

It is very important to have a good product that the quality control process is standardized and that all the phases of the production process are controlled.

5.1 QUALITY CONTROL IN THE PRODUCTION PROCESS

Quality includes the total process whereby the quality of a finished product can be guaranteed.

The key steps in the production process that require quality controls are:

1. pre-fermentation process in the laboratory,
2. fermentation process,
3. carrier preparation,
4. mixing of bacterial broth and carrier,
5. packaging.

In the pre-fermentation and fermentation process, the identity and purity of the strains must be controlled, as must the absence of contaminant strains. The carrier should have the right granulometry, the crude inoculum must contain a right percentage of fungi and both the mixing of bacterial strains and the crude inoculum with the carrier and the packaging control must be performed (fig.18).

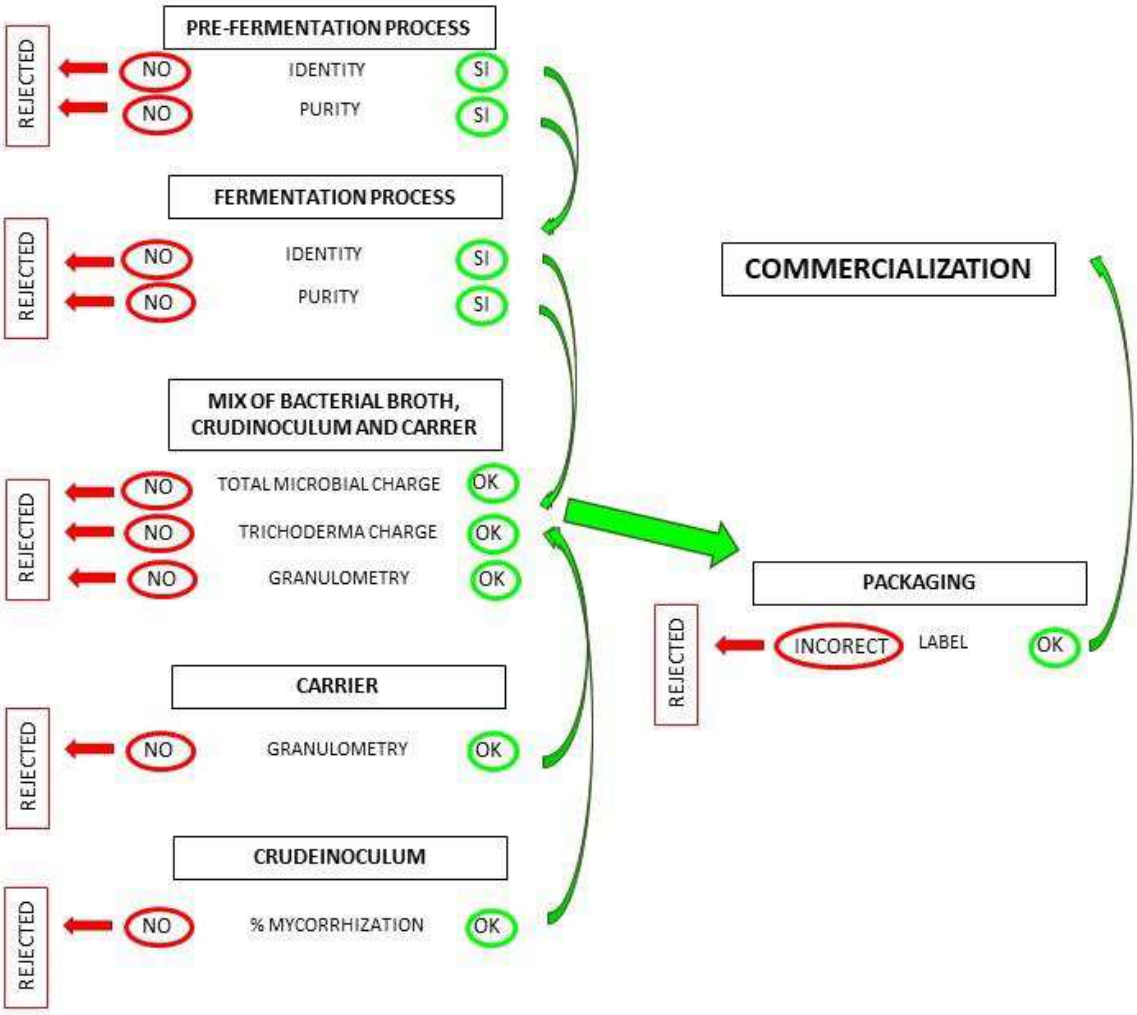

Figure 18: The key steps of the quality controls in the production process of CCS Aosta.

Following, all quality control steps in place at CCS Aosta are described.

5.1.1 Quality control in the laboratory pre-fermentation process

The first step of quality control in the production process of a microbial consortium is the assurance of the quality flask inoculum.

In the preparation of the single strain in the flask the purity and the identity of the cell culture is very important. To obtain this, some laboratory tests are made. A strip is made on a Petri dish with a PDA medium during the inoculation of the laboratory flask with the strain to be pre-fermented. The Petri dish is then placed in the oven at 27° C, if on the plate only the inoculated strain grows, this means that the inoculum is good and is not polluted, otherwise it means that there is a contamination, therefore the flask will be rejected.

There's a second control point in the pre-fermentative inoculum, it is at the end of the growth of the strain in the flask. At this point, an aliquot of the solution is taken under the hood and a slide is set up for observation under the microscope (to visually check that the strain is actually that and that there are no contaminations), the pH, sugars and PMW are also checked.

5.1.2 Quality control during the fermentation process

The fermentation process is the multiplication of microorganisms in bioreactor. During this processing phase many parameters are continually controlled: the air, the temperature, the pH, the pressure… but only at the end of the period of growth there's a laboratory control. An aliquot of this is taken and the C.F.U. is calculated using serial dilutions which are plated on selective media. The number of living cells is counted by spread plate or drop plate methods. Doing spread plate by making serial dilutions from 10^{-1}–10^{-7} or 10^{-8} (depends on concentration) then three replicates of 100 μl of broth from 10^{-8} and 10^{-5} are spread over the agar plates supplemented with antibiotics to avoid any fungal contaminations. CCS Aosta

mainly uses three different media: TAM medium with the addition of *cycloexemide* for the bacterial strains, PP59 medium with the addition of *steptomycin* for the fungal and yeast strains and KING B medium for the pseudomonas strains.

Plates are incubated at 28±2 °C for 2-7 days depending on the type of microorganism and if they don't show contamination from other micro-organisms and the concentrations are acceptable, then the product resulting from the fermentation can be used in the subsequent steps of preparing the microbial consortium. Otherwise it is not used and you will have to carry out the work from the beginning.

5.1.3 Quality control of the carrier

The inert material that is used for the stabilization of the microorganisms should be controlled.

It is therefore necessary to carry out a granulometry check through a ro-tap sifter with graduated sieves. The granulometry of commercial biofertilizers depends on the type of formulation and of the carrier used. Different companies use a system for calculating the different particle size for wettable powder and granular products. In the first case 50 g of powder was dissolved in 500 ml of water. The mixture is filtered through filter paper and the residue is dried in a fan oven and weighed. In the second case a Ro-Tap system is used with sieves of different granulometries. After 10 minutes of vibration the residue is weighed for each sieve and the granulometric percentage is calculated.

If it meets the required characteristics (in the case of CCS Aosta, for the product to be acceptable, at least 90% must have a granulometry of less than 75 μm) then it can be used as an inert on which to stabilize the micro-organisms, otherwise it will be sent back to the mother house.

5.1.4 Quality control of the mycorrhizal inoculum

As described in chapter 3.4.1 mycorrhizal inoculum is checked in a way to guarantee its viability and efficiency.

At the end of the production of *crude inoculum* in pots, randomized sampling is performed on the produced vessels and the percentage of mycorrhization is calculated with the method deposited in the official gazette.

Subsequently the product is sent to grind at an external company and when it comes back to check that the grain size is less than 75 μm a granulometry through a rotap sifter is carried out.

5.1.5 Quality control of the finished commercial product

After the processing industrial phase, to ensure the quality of the final product is very important.

The microbial consortium should meet the requirements of microbial charge, presence of all the microorganisms declared on the label, grain size and relative humidity.

Therefore, a sample is taken for each production lot on which these tests are carried out. For granulometry tests a ro-tap sifter instrument with graduated sieves is used. For relative humidity tests, a humidity analyzer balance is used which returns the dry weight of the analyzed sample. For the presence of microorganisms and their amount and proportion in the sample a C.F.U. count is done. It is a unit used to estimate the number of viable bacteria or fungal cells in a sample. Viability is defined as the ability to multiply via binary fission under controlled conditions. Counting with colony-forming units requires culturing the microbes and counts only viable cells, in contrast with microscopic examination which counts all cells,

living or dead. The purpose of plate counting is to estimate the number of cells present based on their ability to give rise to colonies under specific conditions of nutrient medium, temperature and time.

In order for the product to be acceptable it should have higher values than those stated on the label. This is because a shelf life of two years is given, and it is good to consider that over time the colonies present in the finished product receive a reduction in microbial load, but in order for the product to be marketable they should maintain the declared charge for as long as stated on the label.

In Figure 19 the shelf life of a commercial product, Micosat F UNO, is shown, in a time of 177 days. This is calculated by counting the C.F.U. on serial dilutions carried out on a sample of 10 g of product dissolved in 90 ml of water through a 10-minute stirring. 20 µl of the dilutions -4, -5, -6 and -7 are plated on selective media and then placed to grow in a small oven. Two plates are inoculated for each dilution.

For some random lots, the total C.F.U. is calculated both at the end of the product processing and at defined time intervals, up to two years. This is done to ensure the effective presence of the declared microbial load for the entire shelf life of the product. It is possible to observe by the graph a slight decrease in the microbial load (viable) over time.

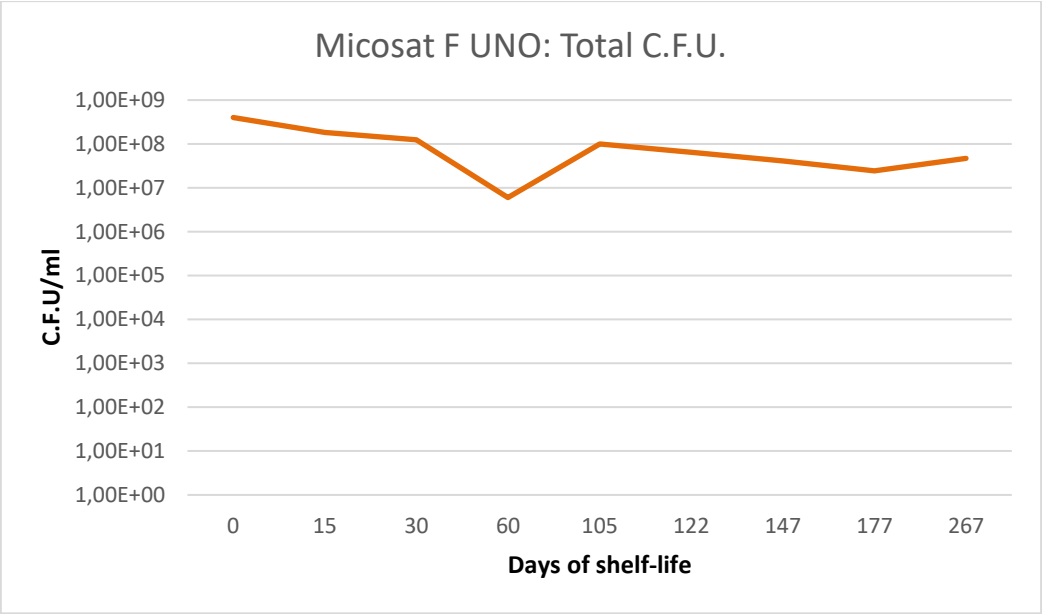

Figure 19: Total C.F.U. charge over time of the commercial product Micosat F UNO.

5.1.6 Quality control of packaging

It is very important for the marketing of a microbial product that the information contained on the label comply with Italian and European legislation and that they contain the actual contents of the package.

Control of this occurs during the drafting and realization of the label and before being sent to print. A second check is made following the molding of the packages.

Another element that should appear on the label is the lot number and the date of production. These are checked and inserted during product packaging.

5.1.7 Commercialization

If all the quality controls carried out during the making of the product are positive, the product is ready to be marketed.

5.2 QUALITY IN THE FIELD

For their acceptance as new technology, microbial consortia should demonstrate their efficacy, robustness and reliability in the field. Effectiveness in laboratory conditions is insufficient; farmers need to see improved productivity of their crops before they can be expected to adopt and continue to use these products.

If we want a microbial-consortium to be qualitatively valid, we should ask ourselves some questions about its use in the field, which crops work best, the dosages to be used and the compatibility with other treatments.

Which are the more suitable application doses of each product? The application doses required for a positive response to plant growth will depend on the physical, chemical and biological conditions of the soil at each site and on each crop. The recommended application doses should provide the number of organisms that will most likely give a response in a variety of conditions.

Can other fertilizers be used? Which ones? Can their use be reduced? The general principle is that the use of microbial consortia should allow a reduction in fertilizer application rates. It is important to underline that the products could have a reduced effectiveness if high doses of chemical fertilizers are maintained, due to the possible repression of beneficial organisms.

What is the effect of agricultural chemicals, such as herbicides and fungicides, on the effectiveness of microbial consortia? Information on possible interference must be provided with the product. The answers to these questions depend on the organisms contained in the product and on the specific conditions of the soil and the environment at the application site.

It will be also important to adopt specified and targetd protocols to confirm the plant growth-promoting effect of the product in the field, and to verify its effect on yield through strip trials. There is no satisfactory substitute for a field test when assessing strains or when determining the best way to integrate them into an

agricultural system. Field tests are usually expensive and so must be carefully planned. They may differ in terms of complexity and the demand made on resources.

5.2.1 Quality in green house tests

In order for a microbial consortium to be of quality, it's necessary that it will be test in the soil, with reference plants and crops. The product should be tested first on a small scale, to be sure that it will work as guaranteed and guarantee good economic return. To do this, greenhouse tests with the product to be tested on target crops are essential, accompanied by a check without addition of the product.

As an example, seedlings might be treated pouring a suspension of the microbial consortium into the soil. After a specified time of growth, the seedlings are gently removed off the ground and washed with water. A visual comparison should reveal significant differences between control and the treated plants. Furthermore, differences in roots or shoots development can also be verified based on fresh or dry weight.

5.2.2 Quality in open field tests

Timing and application doses

Microbial consortia are often used to increase the growth or tolerance of crops to abiotic stresses. The effectiveness of microrganisms depends not only on the composition but also on the timing of application depending on the occurrence of the stressful event.

The response of the crop could be very different if the distribution of the product is carried out before, during or after the occurrence of stress. Since biostimulants may activate specific physiological and biochemical processes in plants, it is essential to identify the best application time to maximize their effectiveness. The optimal dose is also another very important factor because within a certain concentration range the crop can respond positively to the application of microbials, while at lower or higher elevations it may not have effects **(Vernieri et al., 2005).**

Selecting and preparation of the site

It is important to select a site where the conditions are as uniform as possible.

Each replicate should also be placed in a uniform area. The type of soil and the pH should be identified and determined. The country notebooks with the history of the previous harvests and the treatments carried out must be present. It is desirable to have good access to facilitate the withdrawal of samples.

When preparing experimental sites that involve microorganism is essential to consider the problems of contamination between the treatments and consequently adequately prepare the experimental field. This requires care in the experimental design, with the insertion of adequate border areas that guarantee a good separation of the tests and that prevent contamination from one treatment to another.

Applying the treatments

In addition to the aspects related to contamination, it is important that the treatments carried out are recorded both as regards the application dosages and as regards the dates and climatic conditions at the time of treatment. In fact, different climatic conditions could have different effects on the application of microorganisms in the field.

Moreover, the applications can vary considerably from one cultivar to another, therefore it is important to report on which cultivars the experiments have been carried out.

Sampling and evaluation

Even in this sampling phase, small precautions are necessary to limit possible contamination. The sampling time and the samples taken may vary according to the objectives of the trial.

Both visual evaluations and instrumental measurements, such as height of the plant, the fresh and dry weight of the plant, the fresh and dry weight of the roots are therefore carried out.

5.2.3 Pratical applications

The patented commercial product "Micosat F" (MF) contains a mixture of AMF (*Glomus coronatum*, *Glomus caledonium*, *Glomus intraradices*, *Glomus mosseae*, *Glomus viscosum*) and helper bacteria (*Pseudomonas* spp., *Bacillus* spp., Actinobacteria *Streptomyces* spp. and the saprophytic fungi *Trichoderma* spp.). Delivery of the inoculant is done via roots or seed coating, and for trees through localized soil treatment with granular formulations. The use of the AM alone and the microbial consortium of MF was comparatively studied on some major crops to measure the quantitative response and final quality of the epigeal parts. The quantitative response on average was higher for MF: for maize 19% in cut up, 12% in spikes with bracts and 6.4% of grain yield; 13% for wheat grain; 11% for total yield of tomato, due to an increase of 6% of the fruit mass; 11% for cucumber; 8%-20% in the development of the olive trees; null in melon a normal mycotrophic species

(Masoero and Giovannetti, 2015). The rapid scan by UV-Vis-NIR rays from 350 nm to 2,500 nm of the leaves, flower and fruit parts, which was associated with a rapid examination by an electronic nose (EN) for a total of more than 1,400 analyses, revealed that the cultures submitted to the microbial treatments appeared different from the control samples, with linear regression *R* values of 0.40-0.70, but with oscillations between the different species and run-test.

Grain- and forage-maize, aromatic plants, camellia, apple (flowers and leaves), melon and water melon, ryegrass *Lolium* spp., oat and clover are strongly responsive to the treatment with the microbial consortium. Tomato was medium respondent, while alfalfa and vetch were lowly respondent in an EN test. In some cases, the results of the rapid methods were fairly corroborated by fine chemical analyses. The modern wheat cultivar "Blasco" treated with MF gave consistent results, predicted by the EN test, in a bread-making panel test: the panel appreciated the bread from the treated "Blasco" flour as very similar and as good as the bread obtained from the ancient wheat cultivar "Sieve", "Inallettabile" and "Gentil Rosso" **(Masoero and Giovannetti, 2015)**.

Another study on tri-trophic consortium *Azospirillum-Pseudomonas-Glomus* **(Couillerot et al., 2013)** showed that the three-component inoculants may be useful in promoting maize growth. Application of a consortium of AM and the plant growth-promoting rhizobacteria was studied by Mäder et al. **(2011)** and found to positively affect crop yield, grain, soil quality and nutrient uptake of the staple food crop wheat (*Triticum aestivum*(L.)) in a rotation with either rice (*Oryza sativa* (L.)) or black gram (*Vigna mungo* (L.) Hepper). Recently, Berta et al. **(2013)** showed that the inoculation with MC containing bacteria and AM promote the growth of maize cultivated in field conditions and differentially affect the grain nutritional content. The induction of a healthier status of crop plants (e.g., increased content protein, starch and microelements) due to the use of microbial consortium containing AM may encompass a natural, decreased susceptibility of the plants to pathogens. It has

been proposed to call this trait "mycorrhiza-induced resistance (MIR)" **(Pozo and Azcòn-Aguilar, 2007; Tataranni et al., 2012; Cameron et al., 2013),** providing systemic protection against a wide range of pests and pathogens and sharing characteristics with systemic acquired resistance after pathogen infection and systemic induced resistance following root colonization by non-pathogenic rhizobacteria. It is commonly assumed that fungal stimulation of the plant immune system is solely responsible for MIR. However, the latter could be the result of a cumulative effect of direct plant responses to mycorrhizal infection and indirect immune responses (ISR) to ISR-eliciting rhizobacteria in the mycorrhizosphere. The mycorrhizal MF-induced resistance has been verified in the case of flavescence dorée of grapevine in the Piedmont area in Italy **(Giovannetti, 2014)**. Continuous cropping of grapevine in the same soils during the last 70 years and over-use of chemical fertilizers produced the well-known soil degradation effects on one side **(Nuti, 2015)** and a generalized impairment of grapevine towards phytoplasms known as causal agents of flavescence dorée **(Giovannetti, 2014; Nuti and Giovannetti, 2015)**.

5.3 PRODUCT CHECK

It is important to carry out checks on the products from time to time so as to understand at the molecular level what is actually present in the product being sold.

In 2017, we conducted a study on the bacterial and fungal composition of Micosat F biofertilizers and their components.

Materials and methods

Sampling of studied substrates was carried out in May 2017. Eight samples of substrate present in the sorghum vessels used by were collected for the propagation of many isolates of arbuscular mycorrhizal fungi (called "crude inoculum"; codes GU53, GC41, GC31, GB67, GP11, GM24, acid / fruits, acid / vine), three samples of composted soil improver used for the cultivation of sorghum, three samples of quarry earth used for the cultivation of sorghum or as carrier in some finished products, three samples of the finished product Micosat F UNO, three of Micosat F MO and three of Micosat F WP TAB PLUS. Each of the above samples was composed of portions of substrate taken from three different vessels, bags or areas.

Subsequently, 500 mg of substrate/soil from each of the 23 samples were weighed, stored in 2 ml plastic tubes with screw caps, deposited for a short period of time at -20 ° C and sent by courier to the company BMR Genomics (Padova) for the extraction of metagenome, amplification and sequencing of the V3-V4 region of the gene 16S bacterial ribosomal and a hyper-variable region of fungal ITS2.

Sequences obtained by sequencing using Illumina MiSeq technology 300bp paired-ends were processed with the open-source program mothur v1.33 for Mac. After assembling the contigs using the sequences in the run forward and in reverse run, bacterial and fungal sequences have been filtered based on specific quality requirements (minimum length, length of homopolymers, absence of ambiguous nucleotides). After grouping of identical sequences, singleton and chimeric

sequences have been removed using the open-source algorithm UCHIME according to the "de novo" method. The remaining sequences were again grouped in Operational Taxonomic Units (OTU) using a similarity cutoff of the 97% using the OptiClust algorithm. The OTUs with a low number of sequences (<10) have been removed from every sample in the dataset, so as to reduce any problems related to cross-contamination and the presence of OTUs artifact (caused by PCR or sequencing errors). The most abundant sequence of each OTU has been chosen as representative. The taxonomy was assigned through the consensus created by a research on the Greengenes databases 13_5 e UNITE + NSCD, respectively for bacterial and fungal sequences, using Blast v 2.2.29 and the Bayesian method RDP classifier. The non-specific sequences belonging to chloroplasts, mitochondria, chromists, protists, protozoa and metazoans have been removed from the dataset. The dataset was later normalized by correcting the abundance of the OTUs bacterial based on the number of copies of 16S using the PICRUSt package e sub-sampling each sample at about 90% of the sample size minus plentiful. Finally, using the PICRUSt and FUNGuild packages were predicted the functional characteristics of the bacteria and fungi communities.

Results

Regarding the finding of bacterial and fungal strains (except fungi AM) intentionally introduced by CCS Aosta into the finished product, starting from *in vitro* culture or bioreactor, a general situation of success was found in the inoculation process. In fact, in the three products tested (namely UNO, MO and WP TAB PLUS), almost all the inoculated strains have been found. In the samples of Micosat F UNO, that should host *Bacillus subtilis* BA41, *Streptomyces spp*. SB14, *Agrobacterium radiobacter*AR39, *Trichoderma harzianum* TH01, *Pochoniaclamydosporia* PC50 and *Pichia pastoris* PP59, sequences of *Streptomyces sp*. (8.8%), *Bacillus subtilis* (2.8%), *Agrobacterium sp*. (0.4%) were actually found in the bacterial community and

of *Pichia pastoris* (44.6%), *Pochonia clamydosporia* (12.3%) and *Trichoderma harzianum* (8.0%) in the fungal community. In the samples of Micosat F MO, which should host *Bacillus subtilis* BA41, *Streptomyces spp*. SB14, SA51 and ST60, *Pseudomonas fluorescens* PN53, *Pseudomonas spp*. PT65, *Trichoderma harzianum* TH01, *Trichoderma viride* TV03and *Pochonia clamydosporia* PC50, sequences *of Streptomyces sp.*(16.9%) and *Pseudomonas sp*. (7.4%) were actually found in the bacterial community and *Pochoniaclamydosporia* (25.6%) and *Trichoderma harzianum* (15.3%) in the fungal community. The very low presence of *Bacillus subtilis* (0.16%) is noted in this product.

In samples of Micosat F WP TAB PLUS, which should host *Bacillus subtilis* BA41, *Streptomyces spp*. SB19, *Trichoderma harzianum* TH01 and *Trichoderma viride* TV03, were actually found *Streptomyces sp*. (4.0%) and *Bacillus subtilis* (6.5%) in the communitybacterial and *Trichoderma harzianum* (29.4%) in the fungal community. This product showed the unexpected presence of discrete quantities of *Pochonia clamydosporia* DNA (4.8%), which theoretically should not have been inoculated.

All three products showed the presence of an unexpected abundance and often

dominance of some lactic bacteria such as *Lactobacillus sakei* (24.8% in Micosat F ONE and 4.5% in Micosat F WP TAB PLUS) and *Lactococcus lactis* (35.6% in Micosat F MO and 7.8% in Micosat F WP TAB PLUS).

The bacterial communities of finished products showed, instead, the appreciable presence of some taxa potentially coming from the various components used for the production. These were *Frigoribacterium sp*., ubiquitous in compost, in crude inocula and in the cave soil, *Sinobacteraceae sp*., ubiquitous in crude inocula, *Devosia sp*., ubiquitous in compost and crude inocula, but rare in the quarry earth, *Sphingomonas sp*., ubiquitous in compost, in crude inocula and in quarry earth, and *Arthrobacter sp*., rare in the crudinocula, but ubiquitous in the quarry earth. None of these taxa have however been found in high quantities in the finished products, since the

presence of DNA belonging to these species never exceeded 3% of the total bacterial DNA of the samples.

There was also a presence of sequences possibly derived from an environmental origin and with potential pathogenicity (even very low), in comparisons to plants and animals close to *Thanatephorus cucumeris* (anamorph of *Rhizoctonia solani*), *Acremonium persicinum* (characterized by different types oftrophism, also isolated from lichens), *Fusarium oxysporum* (also found in low quantities in the crude inoculum GP11), *Aureobasidium pullulans* and *Trichosporon sp*.(found mainly in WP TAB PLUS; ubiquitous yeast that causes infections opportunistic on man).

Regarding the crude inoculum, the AM fungal propagules are not effectively included in the final products, in which a negligible presence (< 0.5%) of DNA belonging to members of the phylum *Glomeromycota* was detected. The single crude inoculum doesn't contain a single species of fungus AM, but more species; most of the crude inocul (GC31, GC41, GM24, GP11 and GU53) mainly contains all two sequences close to *Kamienska perpusilla* and *Claroideglomus etunicatum.*

Some sequences close to potential plant pathogens that have been abundantly characterized in the quarry land as *Mycosphaerella tassiana* (synonym of *Davidiella taxiana* and synonym of anamorph *Cladosporium herbarum)* was also present in the crudinoculum GP11; colonizers of organic substrates and plant pathogensas: *Alternaria alternata* (also saprotrophic and endophytic potential), *Epicoccum nigrum* (also a potential parasitic fungus, endophyte, saprotrophic, used to support root growth and to control pathogens of sugar cane), *Chalastospora ellipsoidea* (whose pathogenicity seems uncertain) and *Ulocladium chartarum* (saprotroph active in the deterioration of organic substrates and inorganic, due to leaf necrosis in *Quercus pubescens*) have also been found insignificant amounts in finished products.

The inference of the functions of the bacterial metagenome in the finished products carried out with the PICRUSt package demonstrated the potential presence of functions of biodegradation of xenobiotics (including naphthalene, chloroalkanes,

chloroalkenes, caprolactam, PAH, PAH, bisphenols, toluene, chlorocyclohexanes, chlorobenzene, styrene, xylene, dioxin, nitrotoluene, ethylbenzene, atrazine, fluorobenzene, benzoate and aminobenzoate), without highlighting particular differences between the three products tested (Micosat F UNO, Micosat F MO, Micosat F WP TAB PLUS). The study of fungal trophies present in finished products highlighted a significant difference in the percentage of saprotrophs (69% in Micosat F ONE, 34% in Micosat F MO and 84% in Micosat F WP TAB PLUS), patotrophs (16% in Micosat F ONE, 32% in Micosat F MO and 12% in Micosat F WP TAB PLUS), and symbiotrophs (in particular AM fungi; 0.3% in Micosat F ONE, 0.06% in Micosat F MO and 0.3% in Micosat F WP TAB PLUS).

5.4 REGISTRATION AND LOW

Microbial inoculants have been practicing commercially in global agriculture for over 120 years **(Nobbe and Hiltner, 1896; Deaker et al., 2004)**, but today their use received increased attention due to an increased use of chemical inputs. There are currently over 149 registered microbial strains for agricultural use **(Copping, 2009)**. A special publication by the American Society for Microbiology (ASM) suggested that microbes may be, at least in part, a sustainable solution to increasing agricultural production and outlined current shortcomings of microbes in helping to feed the world **(Reid and Greene, 2013)**. The regulatory situation is very complex today and one of the main reasons for this is the lack of formal definition and acceptance of the concept by regulatory bodies. At present in Europe, biostimulants are placed on the market by following either of two routes: one is the national regulations on fertilizers, the other one is the European pesticides law, which combines both supranational and national provisions for introducing plant protection products on the market **(Woo, 2018)**.

The new regulatory framework for plant protection products is laid out in Commission Regulation (EC) No. 1107/2009 **(European Parliament and the Council. 2009)** and Commission Regulation (EU) No. 283/2013 **(Commission Regulation. 2013),** and explicitly requires consideration of impacts on non-target species, their ongoing behavior and the biodiversity and ecosystem, including potential indirect effects via alteration of the food web. Single microbial strains and there of products used as bio-fertilizers or bio-effectors are present on the EU market as high cell density commodities and are subjected to risk assessment in the framework of national legislations. These products generally do not require the same extensive risk assessment as for plant protectants and are therefore marketed with more limited registration requirements **(Nuti and Giovannetti. 2015).**

Manufacturing companies should critically evaluate the properties and mode of action of the potential microorganism from the point of view of the most consistent reproducible effects and the marketability of the future product.

According to this regulation, which provides authorization of the active microorganism(s) and formulation(s), the registration portal consists of two parts: i) Active substance (microorganism) record and ii) Product record. Information on the identity/biology of microorganism, on the methods used for production of the microbes as well as of the final product, on analytical methods used for the active microorganism and formulation are parts of the registration dossier. Safety evaluation sheet contains information of both a microbe and the product (formulation) on human toxicology and ecotoxicology, fate and its survival in the environment **(Kamilova et al., 2015).** Preliminary tests should be performed by laboratories according to guidelines and standards of international organizations such as Collaborative International Pesticides Analytical Council (CIPAC), Organization for Economic Co-operation and Development (OECD) **(1996, 2002)** or United States Environmental Protection Agency Office of Chemical Safety and Pollution Prevention (US EPA OCSPP), and International Organization for Standardization (ISO) according to Good Laboratory Practice (GLP) **(Stevens, 2003)**. Other than Europe, these tests can be performed according to specific national standards. Microorganisms to be registered need to be recognized as safe by the EU: this involves that the applicant company, the relator Member State designated by the applicant, the national agencies and the European Food Safety Authority (EFSA) should communicate with each other to carry out a scientific assessment. This discussion between the partiesleads to publication of the scientific opinion by EFSA **(www.efsa.europa.eu)**. Based on this document, the European Commission decides on inclusion of particular microorganism into the list of safe active substances (Qualified Presumption of Safety - QPS list). Then, a submission of a product report should be done at the national level. In the EU, efficacy trials should be performed

by authorized institutions, according to Good Experimental Practice (GEP) in many respects based on EPPO standards in the intended geographical Zone **(www.eppo.int)**.

On March 27, 2019 the EU Parliament voted the new EU regulation on fertilizers. The new regulation is the 2019 /1009 of the European Parliament and of the Council and it has been approved the on June 5, 2019 **(Regulation (EU) 2019/1009 of the European Parliament and of the Council of 5 June 2019)**.

An important decision, intended to harmonize the entire sector and analysis, proposals, institutional sessions and technical meetings. This regulation (EU) establishes the rules concerning the making available on the market of EU fertilizer products (EC) n. 1069/2009 **(Regulation (EC) No 1069/2009 of the European Parliament and of the Council of 21 October 2009)** and (EC) n. 1107/2009 **(Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009)** and repealing the regulation (EC) n. 2003/2003 **(Regulation (EC) No 2003/2003 of the European Parliament and of the Council of 13 October 2003)**. The regulation establishes that certain substances, mixtures and microorganisms, referred to as plant biostimulants, are not as such inputs of nutrients, but nevertheless stimulate plants natural nutrition processes. Where such products aim solely at improving the plants nutrient use efficiency, tolerance to abiotic stress, quality traits or increasing the availability of confined nutrients in the soil or rhizosphere, they are by nature more similar to fertilizing products than to most categories of plant protection products. They act in addition to fertilizers, with the aim of optimizing the efficiency of those fertilizers and reducing the nutrient application rates. Such products should therefore be eligible for CE marking under this Regulation and excluded from the scope of Regulation (EC) No 1107/2009 of the European Parliament and of the Council. Regulation (EC) No 1107/2009 should therefore be amended accordingly. **(ST 7738, 2019).**

In United States of America (USA), registration of the active microorganism and the product is regulated by the Federal Insecticide, Fungicide, and Rodenticide Act **(FIFRA, http://www.epa.gov/agriculture/lfra.html)** and implemented by US EPA. In most aspects related to data requirements for the registration dossier there is not much significant difference between USA and EU regulations. However, one particular difference is that efficacy trials are an obligatory part in EU whereas in USA these studies were not required by EPA for a Product dossier. However, they are required by the regulatory authorities of some states e.g. California and Florida. African and South American countries, China and Mexico do accept data present in the European or the US dossiers **(Leahy et al., 2014)**. The Canadian Food Inspection Agency regulates biofertilizers via Fertilizers ACT and Regulations C.R.C.666 **(http://laws-lois.justice.gc.ca/eng/acts/F-10/)** providing much importance to the safety for human and animal health, and environmental at the same time quality of the product. In Canada there are no specific requirements for efficacy data for biofertilizers **(Fertilizers ACT, R.S.C., 1985, c. F-10)**.

In the countries of the Commonwealth of Independent States (CIS) the registration of microbiological fertilizers and PPPs were performed by the department of Phytosanitary and Veterinary Surveillance at the Ministries of Agriculture. Efficacy tests should be performed in the country of application. In general, all tests are required to be performed by the national institutions or specialized laboratories. However toxicological tests performed in the EU or in the USA after evaluation by the designated national institutions can be accepted **(Kamilova et al., 2015)**.

In India, it is obligatory to register biopesticides in order to ensure their safety to human health, beneficial non-target organisms and environment. The developed and developing countries have presented several dossiers for registering biopesticides **(OECD 1996, 2002; FAO 1988; Leahy et al., 2014)**. Countries promoting biopesticides set minimum dossier requirements for registration when compared to dossiers required for conventional pesticides such as chemicals, since

biological pesticides are generally less toxic. Different stage systems are adopted across countries for regulating and registering any substance or mixture of substances like biochemical and microbial consortium intended for controlling the pests **(Smyth and McHughen, 2012)**.

Another example of registration is the patent. In this regard, it is necessary to mention the patent filed by the CCS Aosta about the use of microbiological consortia of rhizosphere for pollutants elimination, mycotoxins and nitrates reduction and antioxidant compounds increase in agricultural products. The present invention relates to the use of microbiological consortia of the rhizosphere for elimination of pollutants from agricultural products, reduction of mycotoxins, as well as increase of anti-oxidants, Brix degree, forage unit and proteins in the agricultural products themselves. In particular, the knowledge acquired by the present applicant on the use of microbiological consortia of the root as fertilizers has led the present applicant to verify that the use of microbiological consortia of the root determines an improvement in the wholesome of foodstuffs through a reduction in carcinogenic substances and mycotoxins, an increase in antioxidant and aromatic substances and a reduction in the amount of nitrates in the agricultural products themselves. **(Application number: US20060295111 20060330)**

5.5 GENERAL DISCUSSION AND CONCLUDING REMARKS

Several aspects related to the quality of a commercial product composed by microorganisms should be considered.

After a detailed analysis of the various essential laboratory controls during the entire production process, the attention has been given to field tests that are carried out both before the product is released on the market and later as a check. The verification can take place on different levels, it can be a check on the formulation of the product on the market or on its effectiveness in the field.

Even the registration, whether at a national or European level, falls within this process that aims to guarantee a qualitatively valid and competitive product.

5.5.1 Considerations on the quality control of the production

The laboratory controls carried out throughout all the production process, are aimed to guarantee a standard and uniform commercial product, which maintains the declared qualities and characteristics over time.

An aspect that is not currently considered but which would be interesting and important to evaluate is a **post-market control**. This could be done through a random sampling of boxes already sold to distributors but not yet to final consumers in order to verify that the product leaving the company is the same and with the same qualities as the one the farmer receives.

It would also make it possible to assess whether transport and storage conditions in the dealers' warehouses are suitable for maintaining a good quality of the product and if the distributors might have special precautions.

5.5.2 Considerations on the use of microbial consortia in the field

One critical aspect of microbial consortia in agriculture is the lack of efficacy in different species or even within different cultivars of the same species. This can happen because the single bioactive compounds can have a threshold of biological activity variable from culture to culture. Therefore, different cultures could have a different sensitivity threshold for individual bioactive compounds which results in different physiological responses. Therefore, the research activities should be oriented to the determination of the sensitivity thresholds of the crops to the different compositions so as to be able to guarantee their effectiveness over time.

As has already stressed, it is not plausible that the same micro-organisms work and are effective indistinctly on all crops: it is therefore fundamental for a quality product that each formulation is connected to one or a few production systems on which the product should have been tested.

If, for example, a microbial grapevine consortium is put on the market, that pool of microorganisms should have followed an experiment on the grapevine.

Another aspect to be evaluated in the field tests is the duration of the experiments. In order for a trial to be valid it should be repeated in the field for at least three seasons. This because the environmental conditions can be very variable from year to year, and different problems may raise during such experimental phase that don't allow to obtain reliable results.

Furthermore, the field-testing phase should be carried out following scientific suggestions and standads from the international literature that have already proved their reliability in that context.

5.5.3 Check considerations

The check of the actual composition of some of the Micosat F finished products showed regarding both bacteria and fungi, the crude inoculum was found characterized by a higher level of diversity and equitability than the others components and finished products. Bacterial and fungal communities present in the various components and in the finished products were found to be quite different according to NMDS ordering. This means that, in terms of composition and structure, communities are very different.

The finished products showed to share a high number of bacterial taxa and fungal with the crude inoculum and a much lower number with the others components (composted soil conditioner and quarry soil). Nevertheless, none of the most abundant members of the fungal community of crude inoculum has been found in appreciable quantities in finished products (except for *Mycosphaerellatassiana* present in the crude inoculum GP11 and in all finished products).

Indications linked to the scarce presence of AM fungi in the finished product should be taken into consideration by the company, which should remedy the problem making some changes to the AM fungi production process.

Another interesting aspect of the check that isn't always taken into consideration is related not to the actual composition of the product but to its verification in the field. If it is properly used by farmers and if information and indications related to use and application are sufficient and clears.

5.5.4 Registration considerations

Although worldwide attention is increasingly focused on the interests on the environment and solutions that preserve the planet and one of these could be the use of microbial consortia used in agriculture instead of chemical products that

were much exploited in the past, the legislation linked to the use of microorganisms in agriculture and to their registration both nationally and internationally is still very confusing.

Whereas for the researchers and end-users of the microbial products this combination of beneficial properties looks very interesting and attractive, it seems to be complicated for the regulatory authorities worldwide. Regulatory authorities apply to agricultural microbial products the same approach as to chemical products: Plant Protection Products (PPPs) and fertilizers are regulated differently. So, even though many beneficial microbes combine more than one beneficial trait, from the regulatory point of view currently microbial inoculants can be assigned only as either biopesticides or as biostimulants/plant strengtheners/biofertilizers. This requirement plays a critically important role in the registration of microbial products and in the way the products can be placed in the market also because procedures are very different.

6 FUTURE PERSPECTIVES

Since the protection of the environment has become in recent years more and more a central topic for both Organizations and Administrations around the world and in view of the global increase in population and the subsequent lack of food, especially in the poorest Countries, the prospect of using microorganisms and in particular microbial consortia in agricultural practices should be increasingly widespread and used.

The costs are still too high due to the complicated production process to allow their massive use, they should be reduced to allow everyone, in all parts of the world, to use these natural micro-organisms which, as we have seen, improve plant health, soil conditions and indirectly also human health, because thanks to the food chain it is possible to find them in food.

Just for the ever-increasing interest in these new agronomic practices both by farmers and Institutions, companies producing these microbial consortia should increasingly aim at a high quality of their products, going to consider the quality of the individual microorganisms and their individual elements, the production processes that create the final products, and the consortia themselves as a whole.

From the in-depth study carried out on one of these microbial consortia, Micosat F UNO, the importance of having a well-known starting product emerged, this means that all the single microorganisms must be well studied and characterized, not only from the point of morphological and productive view, but also at the molecular and metabolic level, the characteristics of the microorganism must be studied, whether it acts as PGPR, resistance inducer or antagonist of other microorganisms (and in this case it is interesting to understand which and how it acts) and how it interacts with the other microorganisms with which the Company wants to associate it.

The in-depth knowledge of all these elements allows us to study and create targeted consortia of good quality. Although this study could be considered a non-relevant for companies because it is very laborious and expensive, it should instead be

included in the process of acquiring and evaluating a strain, also because having all this information, the company is also facilitated for a possible registration.

From this work emerged that, in order to obtain a quality microbial consortium, in addition to the intuitive importance of an optimized and controlled production process in all its passages, the aspect of product validation after the microbial consortium should be taken into consideration byCompanies and his verification of effectiveness in the field.

One of the means to do this, in addition to the tests in the greenhouse and in the field, could be a periodic feedback from the agronomic technicians who follow companies that use these products. In this way they can collect opinions, doubts, difficulties but also points of strength and the positive characteristics of the use in the field.

The major shortcomings that emerged from analysing the Micosat F consortium are reated to the crude inoculum, both from the point of view of production and quality. In fact, the management and reproduction of AM fungi is expensive and complicated, requiring external processing that probably deteriorates the crude inoculum, in fact is difficult to find it in the finished product.

The use of quarry soil to produce AM inoculum brings potential pathogens (although no worrying and / or alarming quantities of potentially pathogenic species in finished products have been found), so in perspective, the Company should try to further reduce the risk of contamination improving the quality of the quarry soil used. However, it should be noted that many of these species have an environmental dissemination and, therefore, they often come as "environmental contaminants".

The other criticality linked to the mycorrhizal component in the product is to solve the problem related to the introduction of fungal propagules AM in the finished product, or consider the differentiation of products in two categories: one

productive line, in which the crude inoculum is introduced into the finished product without being subjected to processes of pulverization/screening that can eliminate the propagules. In this case there would be some problems linked to the acceptability of the final product from the granulometry point of view. In fact, the insertion of propaguli and non-milled AM fungi would make the product incompatible with the irrigation systems currently used in agriculture, clogging them. Another line in which the AM propagules aren't introduced, thus not creating problems related to grain size and making the product compatible with agricultural irrigation systems.

Overall, Micosat F UNO can still be considered a quality microbial consortium, even if research and continuous improvement, focusing on the most deficient aspects and studying the lesser-known ones, can make it always better and more and more competitive.

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8 ANNEX

ANNEX 1

CODE	TYPE	NAME	GENERAL DESCRIPTION	SUPPLIER	YEAR OF ARRIVAL	M.O. DESCRIPTION																											NOTES
						ISOLATION METHODOLOGY							COLONY APPEARANCE												MICROSCOPIC APPEARANCE				PHOTOS					
						TERRAIN INSULATION	ADDED TO	QUANTITY [mg./lt.]	DILUTIONS	PLATES C/DIL.	INCUBATION °C	DAYS OF INCUBATION	COLOR	COLOR UNIFORMITY	SURFACE SHAPE	VISUAL ASPECT	COLONY THICKNESS [mm.]	DIAMETER [mm.]	EDGES	SPEED OF GROWTH [gg]	ISSUE OF PRODUCTS	DIFFICULTY IN THE PICK	POTTER COLOR	RESOLUTIONABLE	VEGETATIVE FORM	AGGREGATION	FORMS OF RESISTANCE	COLONY		COLONY MICRORGANISM	LIQUID CULTURE MICRORGANISMS		
AR39	RHIZOSPHERE BACTERIA	*Agrobacterium radiobacter*	It was brought to the company by dr. Giusto Giovannetti in 2004 with origins Nurseries Battistini. In the same occasion it was also given the recipe of the terrain to grow it in solid culture. It should be an especially active against the bacterial strain of the peach and cherry, therefore it is recommended to use in products used on fruiting plants.	Vivai Battistini	2004	NA	cycloheximide	100	8	6-8	26	1	white ice, sometimes verging on salmon color	no	irregular	slightly glossy	<1	60	very jagged	4	No	No	na	excellent	very elongated Sticks	Sometimes in chains	Yes, sticks spores	immaginiceppiastra\AR39.JPG					

PC50	SAPROPHYTIC FUNGI	*Poconia chlamydosporia*	Purchased from DSMZ in 2012. The strain (DSM No. 3153) was freeze-dried. His name on the DSMZ site was *Verticillium chlamydosporium*, but according to a recent reclassification appears to be *Poconia chlamydosporia*. It is a pathogenic fungus in respect of eggs and females of the phytopathogenic nematodes.It is a pathogenic fungus in respect of eggs and females of the phytopathogenic nematodes. The nematocita activity is	DSMZ	2012	Mepa	streptomycin	200	6	4-6	26	1	white	uniform	convex	Cottony	>1	150	smooth	7	No	yes	milky white	good	hyphae	NA	elliptical spores	imma gini ceppi piastra\PC50.jpg							

			probably executed via an alkaline peptide with protease activity that damages the eggs of those pests that have a high protein content in the shell. The colonies, have a shining white coloring; the mycelium is cottony. The colony edges are smooth; The colonies have a clear round shape. It is a slow strain to grow and its colonies do not exceed 15 mm in diameter although are lifted from the substrate (on the whole colony is slightly convex). From a supplier, the ideal ground																														

			to cultivate is the MALT EXTRACT AGAR PEPTONE (abb Mepa).																												
PN53	RHIZOSPHERE BACTERIA	*Pseudomonas fluorescens*	Ex *Pseudomonas fluorescens* type A [9/4]. He arrived at the nursery in spring 2006 by the University of Pisa (Prof. Marco Nuti). The strain appears to be a major producer of alginates, in fact it was used to make softer the agricultural land, as in the case of grassland or land used for the cultivation of vines. It grows well on King B agar where	Univ. Pisa	2006	King A gar B	cycloheximide	100	8	6-8	26	1	green fluorescent	darker central area	lemon-shaped	glossy	1	3	smooth	3	Dark green pigment	No	Na	good	Pasti na/hair	NO	Na	immaginiceppipiastra\PN53b.JPG					

			spreads a very dark pigment.																												
PP59	YEAST	*Pichia Pastoris*	The strain was arrived in the nursery in spring 2006, Manelli origin. This strain was initially used as a biosurfactant and subsequently to make soft soil in which it was distributed. It grows well on land PP59 TS	Monelli	2006	PP59 TS	streptomycin	200	-7	5-7	26	1	white ice	yes	roundish	pasty	1	3	smooth	5	No	No	Na	excellent	roundish	yes	No	immaginicippiastra\PP59.jpg	-				
PT65	RHIZOSPHERE BACTERIA	*Pseudomonas Spp.*	The strain was isolated in spring 2007 by truffle ODO (Angellozzi), levy in "pianello". *T. melanosporum* (Sample no. 2). Tests were carried out to assess the production of siderophores and this strain	Dr. G. Giovannetti	2007	King A gar B	cycloheximide	100	8	6-8	26	1	Yellow to green	No	roundish	glossy mucosa	>5; drops	>5	smooth	3	crystals	No	NA	good	thin sticks	No	No	immaginicippiastra\PT65c.JPG	-				

			was found to be the largest producer. During the experiment conducted by Mannitto on basil it gave the least good results compared to PT 68 and PT strains 67. It grows well on King.																														
SA51	STREPTOMYCES	*Streptomices Spp.*	Isolated autumn 2004 from rhizosphere olive (Arnasco); It was tested against pathogens, It gave outstanding results. Considerations of the strain morphology: when the strain was stabilized for more than two months, the colonies take on a different form, furrowed.	Rhizosphere of an olive grove in Arnasco	2004	TAM	cycloheximide	100	8	6-8	26	1	antique pink / cream	darker central area	rotunda	Cottony	1	6	smooth	7	No	soft	antique pink	good	hyphae	Na	conidia	immaginiceppipiastra\SA51.jpg						

SB14	STREPTOMYCES	*Streptomices Spp.*	Isolated from CCS AOSTA in 2002 from a soil sample that came by the farm Le Fracce. Thereafter, the strain was tested by the University of Pisa to test the activity against Fusarium. This strain has given the best results in terms of reduction of pathogens.	Az. Le Fracce	2002	TAM	cycloheximide	100	8	6-8	26	1	Hazelnut	yes	The colonies may crack	dry	1	3	tends to smooth	7	No	nothing	Beige	good	hyphae	Na	conidia	imma ginicep pipiastra\SB14a.jpg	imm aginicep pipiastra\SB14b.jpg				
SL81	STREPTOMYCES	*Streptomyces lydicus*	The strain was isolated from a commercial product Actinovate in the year 2013. It 'a strain normally present in soil that is normally used to combat numerous fungal infections including those made	Actinovate	2013	TAM	cycloheximide	100	6	4-6	26	1	Grey cream	hoops	conical	Cotton y	2	6	smooth	>7	No	No	Beige	average	hyphae	Na	conidia						

			by powdery mildew.																													
BA41	RHIZOSPHERE BACTERIA	*Bacillus subtilis*	It coming in the CCS Aosta in autumn 2004, it was provided by Arnold Linser, in an envelope calledXP 105, should have a good activity against late blight.(Bacillus subtilis)	Arnold Linser	2004	TSA	cycloheximide	100	8	6-8	30	3	white ice	colors alternating radial	Slightly rounded	Slightly glossy	<1	30	Slightly ragged	3	No	No	na	good	chopsticks	No	sticks spores	immaginicceppipiastra\BA41.jpg	-				
BB48	SAPROPHYTIC FUNGI	*Beauveria Bassiana*	Isolated from CCS Aosta in 2011 by BOTANIGARD BOULDER products of the Company 'DIVISION AGRO. Strain GHA.It is an endophyte fungus with entomopatogene properties and is capable of causing damage to various insects by acting as a parasite.	Dr. G. Giovannetti	2011	PDA	streptomycin	200	-6	4-6	24-26	10	white shining	Uniform	flat	The outer edges are very powdery	<1	30	Irregular	>15	No	No	milky	good	hyphae	no	elliptical spores	immaginicceppipiastra\BB48a.jpg	immaginicceppipiastra\BB48b.jpg				

			When the spores come in contact with the body of an insect, germinate, enter the body and grow there, killing the insect. After the death of the insect, a white foam develops on the body to produce new spores. Most of the insects that live on the ground have developed natural defenses against Beauveria bassiana, but many others are susceptible (from Wikipedia). It grows well on PDA. It 'a negative impact on growth from strain BA41																														

BR62	RHIZOSPHERE BACTERIA	*Bacillus Spp.*	Isolated from CCS Aosta in spring 2007 from the rhizosphere of rice plants grown in conditions of anoxia far enough. Tested against pathogenic fungal strains gave outstanding results in containing the growth of fungal mycelium.	Dr. ssa Brondolo	2007	TSA	cycloheximide	100	8	6-8	30	3	white ice	Radial alternating white ice and yellow	Raised in the middle	Rough in the central part, smooth edges	<1	20	Very jagged	3	No	No	milky white	good	chopsticks	Some vegetative sometimes aggregate to each other like bricks	sticks spores						

MA45	SAPROPHYTIC FUNGI	*Metarhizium anisopliae*	Purchased from DSmZ in year 2010. The stump, (DSM Code 1490) was lyophilized. It is a soil fungus with entomopatogene properties and can cause damage to various insects by acting as a parasite. When the spores of the fungus come into contact with the body of an insect, germinate and penetrate the cuticle of the insect causing death for what is called green disease, due to the color of the spores that remain on the corpse. The insect dies due to some toxic peptides	DSMZ	2010	PDA	streptomycin	200	6	4-6	24-26	7	brown	dot	Corrugated	rough	<1	10	jagged	>7	No	average	Brown/sepia	good	hyphae	No	elliptical spore	immaginiceppipiastra\MA45.jpg	-					

			produced by the fungus. The colonies have a non-uniform sepia coloring; the mycelium is very rarefied, and from place to uncoloured Brown dots. The borders of the colonies are highly rugged and not very well defined, as if they were sprayed and overall the colony has an irregular shape. This strain is quite slow to grow and its colonies do not exceed 30 mm remaining rather flat on the substrate. Grows well on PDAs. Purchased from DSmZ in year 2010. The stump, (DSM Code 1490) was																														

			lyophilized. It is a soil fungus with entomopatog ene properties and can cause damage to various insects by acting as a parasite. When the spores of the fungus come into contact with the body of an insect, germinate and penetrate the cuticle of the insect causing death for what is called green disease, due to the color of the spores that remain on the corpse. The insect dies due to some toxic peptides produced by the fungus. The colonies have a non-uniform sepia coloring; the																																			

			mycelium is very rarefied, and from place to uncoloured Brown dots. The borders of the colonies are highly rugged and not very well defined, as if they were sprayed and overall the colony has an irregular shape. This strain is quite slow to grow and its colonies do not exceed 30 mm remaining rather flat on the substrate. Grows well on PDAs. It is negatively affected by the strain growth BA41																														

PA29	RHIZOSPHERE BACTERIA	*Pseudomonas fluorescens (exchlororaphis)*	It was isolated from the CCS Aosta on October 3, 2003, from a liquid suspension deep blue colour contained in a test-tube Falcon type supplied to us by Arnold Linser. The falcon was marked CEDOMON (B.A. 3567). By molecular analysis performed by Biosearch Srl has emerged to be a "Pseudomonas clororaphis". The colonies are characteristics of Pseudomonas fluorescent green color that remains unchanged over time. Are uniformly bright, therefore assume	Arnold Linser	2003	King Agar B	cycloheximide	100	8	6-8	28-30	3	fluoresh ent yellow	uniform	slightly convex	polished	1	10	Slightly ragged.	3	crystals	No	NA	Medium	shortchopsticks	No	No	immaginiceppipiastra\PA29.jpg	-				

			amoeboid shape with time. The edges are slightly jagged edges and the colony takes on a slightly convex shape. The average diameter is approximately 10 mm. After a few days we observe the precipitation of crystals. Under the microscope the stump is shaped like an elongated and tend to grow free forms-chips from each other. Grows well on King Agar b.																																
AB86	RHIZOSPHERE BACTERIA	*Azospirillum brasiliensis*	Supplied to us in 2015 by Prof. Marco Nuti from the University of Pisa . The strain has good nitrogen fixer capacity, which is also confirmed by	Prof Marco Nuti	2015	NA	cycloheximide	100	8	5-8	24-26	5	Salmon	hoops	Slightly concave	glossy	<1	5	Smooth	7	No	good	Na	good	Simil Pst	No	No	[immaginicceppiapistra\AB86.jpg](immaginicceppiapistra\AB86.jpg)	-				

			the literature where the Azospirillum genus is listed as one of the most studied PGPR.																															
SA55	STREPTOMYCES	*Streptomices Spp.*	the microorganism was isolated from the rhizosphere of olive trees in autumn 2004 (Arnasco); tested against pathogens has shown excellent results.	rhizosphere of olive trees in Arnasco	2004	TAM	cycloheximide	100	8	6-8	28-30	5	white	Semitransparent outer ring	Round	cottony	1	8	smooth	7	No	Mild	Cream	good	hyphae	Na	conidia	[immaginiceppiastra\SA55.jpg](immaginiceppiastra\SA55.jpg)	-				
SB19	STREPTOMYCES	*Streptomices Spp.*	The strain was isolated from CCS AOSTA in 2002, from a soil sample (unknown root plant rhizos) from the Azienda Agricola le Fracce. Subsequently, the strain was analyzed by the University of Pisa to test its activity against Fusarium.	Az. La Fracce	2002	TAM	cycloheximide	100	8	6-8	28-30	5	hazelnut	yes	Colonies may break apart	dry	1	3	Tendently smooth	7	No	nothing	Beige	good	hyphae	Na	conidia						

			This strain gave the best results in terms of containment of pathogens.																																
SC43	STREPTOMYCES	*Streptomices avernichilis*	The strain arrived at CCS Aosta in the autumn of 2004, provided by Paolo Costa Pare, is particularly active against mites and nematodes, so it is very important for the red lightning and nematode fighting in the greenhouse. It was observed, in the greenhouse, good responsiveness especially in the fight against adult red spider.	Paolo Costa	2004	TAM	cyclohexamide	100	8	6-8	28-30	5	hazelnut	White, light hazel, white and finally the outer edge is semi-transparent	Convex	Slightly cottons	2	3	smooth	7	No	high	Beige	Medium	hyphae	Na	conidia									
TA28	SAPROPHYTIC	*Trichoderma Atro*	The strain arrived at CCS Aosta in autumn 2004, provided by Paolo Costa,	P.ssa Pico	2006	PDA	streptomycin	200	6	4-6	24-26	7	green	Yellow gold green (typical	planes	Cotton like tric	1	90	smooth	5	No	easy	green	good	hyphae	Na	conidia									

	FUNGI	*viridae*	seems to be particularly active against mites and nematodes, so it is very important for the greenhouse fight against red spider and nematodes. It was observed, in the greenhouse, good responsiveness especially in the fight against adult red spider.										color variations in relation to light); The line of demarcation between the individual colors is, however, just mentioned; Sometimes cotonous tufts appear.		odorms																			
TH01	SAPROPHYTIC FUNGI	*Trichoderma hartianum*	The strain arrived at CCS Aosta in 2001, provided by Paolo Costa. It is the first strain that was cultivated, studied and reproduced by CCS	Paolo Costa	2001	PDA	streptomycin	200	6	4-6	24-26	7	green	White green (typical color variations in relation to light); Sometimes	planes	Cotton like tricoderms	1	90	smooth	5	No	easy	green	good	lfe, fields	Na	conidia, clamidospores	[immaginiceppipiastra\TH01.jpg](immaginiceppipiastra\TH01.jpg)	-				

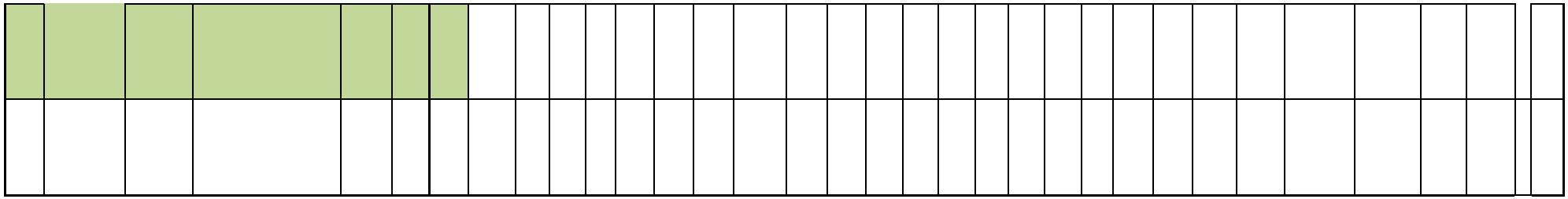
			AOSTA. Trichoderma harzianum is a ubiquitous saprophytic mushroom (probably the most commonly cultivable strain in the soil), which is used in the biocontrol of pathogens such as Botrytis, Fusarium and Penicillium (by competing for nutrients with strain antagonists and the production of antibiotic substances). It is capable of quickly colonizing the roots of a plant, creating a kind of symbiosis that leads the roots to grow faster and the plant to raise its defenses against										cotton buds appear, but are rare.																					

			pathogenic strains.																													
TH27	SAPROPHYTIC FUNGI	*Trichoderma Harzianum*	The microorganism arrived at CCS Aosta in 2006, provided by Fr. Anna Maria Picco of the University of Pavia. It was the subject of studies on cytochromes by the CNR of Pisa (Prof. Vincenzo Longo). During those studies it emerged as if it were an endophyte.	P.ssa Picco	2006	PDA	streptomycin	200	6	4-6	24-26	7	green	Yellow gold green (typical color variations in relation to light); Sometimes cotonous tufts appear.	Piane	Cotton like tricoderms	1	90	smooth	5	No	easy	Verde	Buona	hyphae	Na	conidia						
TV03	SAPROPHYTIC FUNGI	*Trichoderma Spp.*	The microorganism arrived at COS Aosta in 2004, provided by Professor Anna Maria Picco,	P.ssa Picco	2004	PDA	streptomycin	200	6	4-6	24-26	7	green	White green (typical color variations in relati	Piane	Cotton like tricoderms	1	90	smooth	5	No	easy	Verde	Buona	lfe, fialidi	Na	conidia, clamidospores						

			isolated from site Polluted by PCBs. Shipped to Longo for cytochrome verification.										on to light); Sometimes cotton buds appear, but are rare.																		
UO18	SAPROPHYTIC FUNGI	*Ulocladium Oudemansii*	IThe strain was isolated in 2005 by a powdery and water-soluble product. It is a fungus with strong action against Borytis cinerea, since it manages to successfully cover the niche and therefore restricts pathogenicity. Spores are also able to stick tensile to plant tissues, preventing their dilation by rain or irrigation. Grow well on solid PDA soil (colonies of intense gray	Dr. G. Giovannetti	2005	PDA	streptomycin	200	6	4-6	24-26	7	black	Sometimes a few white portions	Piane	Cotton, but with air mycelium that seems damp	1	70	Waves	7	No	difficult	dark gray	good	hyphae	Na	Large spores set at the cross	immaginiceppipiastra\UO18.jpg	-				

			and rapid growth), although in the bibliography it is indicated as a suitable crop medium Potato Carrot agar at 26 ° C.																																							
GU53	FUNGO SIMBIONTE	*Glomus Coronatum*	AM fungus provided to us by Prof. Giovannetti. The identification of the strain as coronatum was made by the supplier, therefore it should.	P.sa Giovannetti	1999	Terriccio																																			
GP11	FUNGO SIMBIONTE	*Glomus Mosseae*	AM fungus provided to us by Prof. Giovannetti. The identification of the strain as mosseae was made by the supplier, therefore it should.	P.sa Giovannetti	1999	Terriccio																																			
GC41	FUNGO SIMB	*Glomus Visc*	AM fungus provided to us by Prof. Giovannetti. The	Dr. G. Giovan	2001	Terriccio																																			

	ION TE	*osum*	identification of the strain as viscosum was made by the supplier, therefore it should.	nett i																																
GG31	FUN GO SIMB ION TE	*Glomus Intraradices*	AM fungus provided to us by Prof. Giovannetti. The identification of the strain as intraradices was made by the supplier, therefore it should.	P.ssa Giovannetti	1999	Terricio																														
GM24	FUN GO SIMB ION TE	*Glomus Caledonium*	AM fungus provided to us by Prof. Giovannetti. The identification of the strain as caledonium was made by the supplier, therefore it should.	Sconosciuto	2001	Terricio																														



ANNEX 2

MICROBION S.r.l.
Registered office:
Via IV Novembre 24,
I-37126 Verona, Italy
VAT No.: IT04045090232

Operational headquarter:
Via Monte Carega 22,
I-37057 San Giovanni
Lupatoto, Italy
e-mail: info@microbion.it
Contact: www.microbion.it

San Giovanni Lupatoto, March 15th, 2019

To whom it may concern,
CCS Aosta;
Fraz. Olleyes, 9;
11020 Quart (AO)

Subject: identification of the bacterial strain PT65 from CCS Aosta, presumptively belonging to the genus *Pseudomonas*, by analyzing the 16S rRNA sequence, *rpoB* and *rpoD* coding genes retrieved from the strain assembled genome (ID NGS: 7).

Dear customer,

We identified a bacterial strain from CCS Aosta, namely "*Pseudomonas* sp. PT65", by analyzing the 16S rRNA sequence (Annex A), *rpoB* and *rpoD* coding genes (Annex B), which were retrieved from the assembled genome provided for the strain (ID NGS: 7).

We then compared of the 16S rRNA sequence from the strain PT65 against a qualified database (Annex C). Moreover, we concatenated the nucleotide sequence of *rpoB* and *rpoD* genes for the strain PT65, which was aligned and was compared with the homologous sequences from the type strains of the most phylogenetically related species (Annex D).

According the analysis of the 16S rRNA sequence, the strain PT65 resulted related to *Pseudomonas fluorescens* group within the *Pseudomonas fluorescens* lineage (Annex C), which includes also *Pseudomonas gessardi*, *Pseudomonas fragi*, *Pseudomonas mandelii*, *Pseudomonas jessenii*, *Pseudomonans koreensis*, *Pseudomonas corrugata*, *Pseudomonas chlororaphis* and *Pseudomonas asplenii* subgroups (Gomila *et al*., 2015; Mulet *et al*., 2012).

The analysis based on the concatenated sequence of *rpoB* and *rpoD* genes revealed the correlation of the strain PT65 with the type strain of the species *Pseudomonas granadensis* F-278,770.

Above all considered, the bacterial strain PT65 from CCS Aosta belongs to the species *P. granadensis*.

Best Regards.

Annex A – Nucleotide sequence of the gene coding for 16S rRNA retrieved from the genome of the strain PT65.

>PT65_(7)_scaffold_9127_326790-328330_16S

TTGAACTGAAGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGATGAGA
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GAAAGGAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCT
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ACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTG
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ATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTAATGGTGGGCACTCTAAGGAGAC
TGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCT
ACAATGGTCGGTACAAAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAG
TCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGTGAATACGTTCCCGGGCCT
TGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGCTAGTCTAACCTTCGGGAGGACGGTTACC
ACGGTGTGATTCATGACTGGGGTGAAGTCGTAACAAGGTAGCCGTAGGGGAACCTGCGGCTGGATCACCTCCTTAAT

Annex B – Nucleotide sequence of the *rpoB* and *rpoD* genes retrieved from the genome of the strain PT65.

>PT65_(7)_scaffold_8997_10753-9645_rpoB

GAGCGTCTGTCGATGGCTGAAAGCGAAGGCCTGATGCCGCAAGACCTGATCAACGCCAAGCCAGTGGCTGCGGCGG
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CGAACTGGTAGCCGTACGTCACCTGAACGAATTCACCGTCAAGGCGCCTGAAGAAGTCACCTTGATGGACGTTTCGCC
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GTGTGGCCCGTGACACCAAGCTTGG

>PT65_(7)_scaffold_9070_26274-27035_rpoD

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CCATCGGTCGCGTGCAGCCGGACATCATTCGTTGCCAGCAGAAACTGATCGCGCTGGAAACCGAAACCGGTCTGACC
ATCGCAGAAATCAAGGACATCAACCGTCGCATGTCGATCGGTGAGGCCAAGGCCCGCCGCGCGAAGAAA

Annex C – Identification of the strain PT65 by comparing the 16S rRNA sequence (Annex A) against a qualified database of type strains sequences.

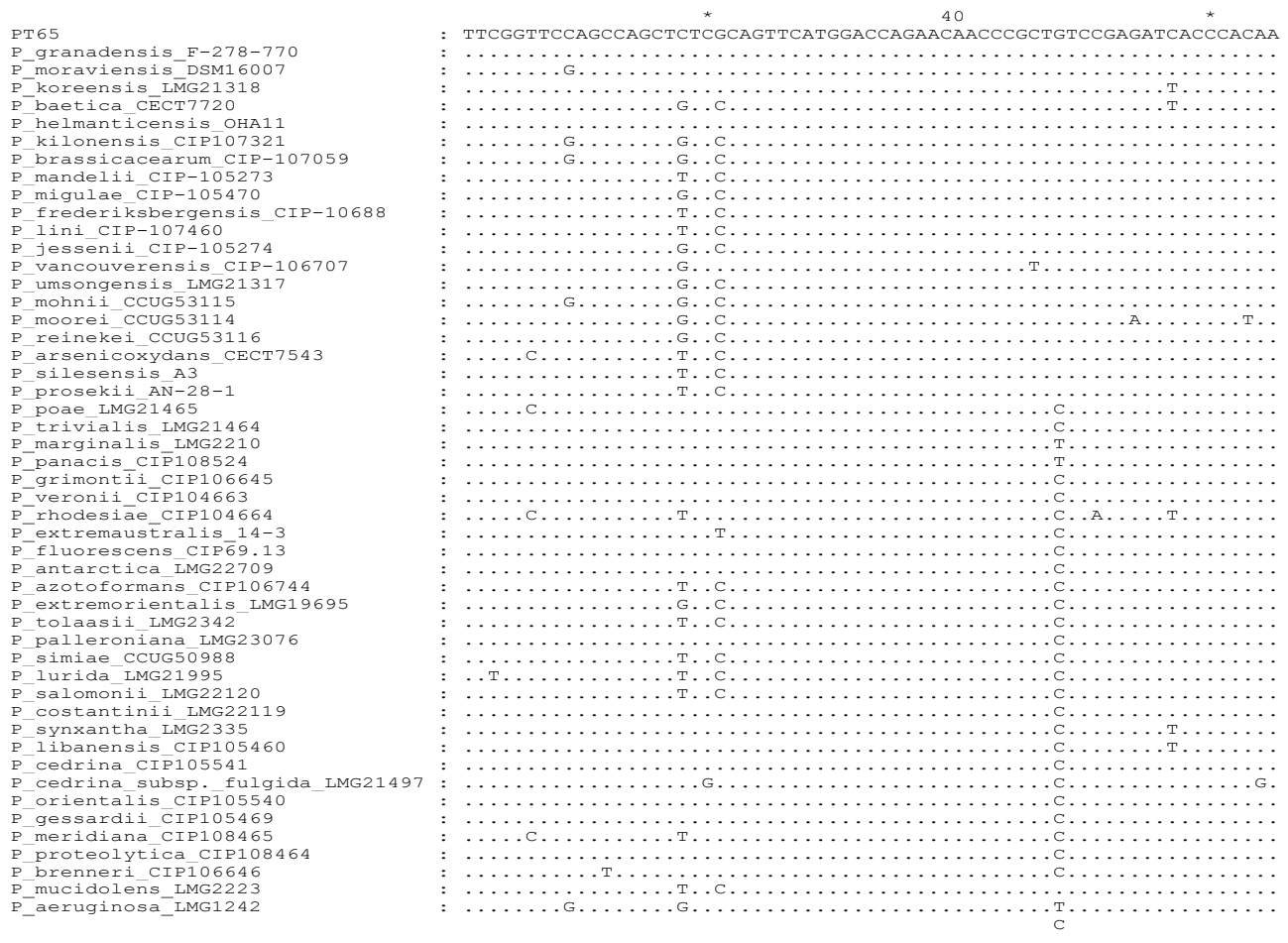
Target species (Type Strain)*	**NCBI Acc. No.**	**Similarity** **	**No. Diff.****
Pseudomonas granadensis	HG764746	99.86	2/1419
Pseudomonas koreensis	AF468452	99.38	9/1455
Pseudomonas reinekei	AM293565	99.33	10/1492
Pseudomonas moraviensis	AY970952	99.15	13/1525
Pseudomonas baetica	FM201274	99.13	13/1494
Pseudomonas vancouverensis	AJ011507	99.13	13/1494
Pseudomonas jessenii	AF068259	98.94	16/1515

*Best match of the query sequence with the sequences of the type strains belonging to the most phylogenetically related species.

** Similarity in terms of percentage, calculated as the ratio between the number of the matching nucleotides and the total number of the nucleotides of the compared sequences. The possible degenerated sites due to overlapping peaks in the electropherogram have not been considered in the similarity assessment.

*** Number of Single Nucleotide Polymorphisms (SNPs) between the query sequence and the most similar sequence in the database.

Annex D – Alignment of the concatenated sequence of *rpoB* and *rpoD* genes of the strain PT65 and the type strains of the most related species reported by Kaminski *et al*. (2018), von Neubeck *et al*. (2017), Kosina *et al*. (2013), and Pascual *et al*. (2015), which include: *P. kilonensis* CIP 107321 (NCBI Acc. No: AJ717472.1, AM084336.1), *P. mandelii* CIP 105273 (NCBI Acc. No: AJ717435.1, FN554482.1), *P. brassicacearum* CIP 107059 (NCBI Acc. No: AJ717436.1, AM084334.1), *P. aeruginosa* LMG 1242 (NCBI Acc. No: AJ717442.1, AJ633568.1), *P. migulae* CIP 105470 (NCBI Acc. No: AJ717446.1, FN554486.1), *P. jessenii* CIP 105274 (NCBI Acc. No: AJ717447.1, FN554473.1), *P. frederiksbergensis* CIP 106887 (NCBI Acc. No: AJ717465.1, AM084335.2), *P. lini* CIP 107460 (NCBI Acc. No: AJ717466.1, FN554478.1), *P. vancouverensis* CIP 106707 (NCBI Acc. No: AJ717473.1, FN554517.1), *P. koreensis* LMG 21318 (NCBI Acc. No: FN554737, FN554476.1), *P. mohnii* CCUG 53115 (NCBI Acc. No: FN554741.1, FN554487.1), *P. moorei* CCUG 53114 (NCBI Acc. No: FN554742.1, FN554489.1), *P. moraviensis* DSM 16007 (NCBI Acc. No: FN554743.1, FN554490.1), *P. reinekei* CCUG 53116 (NCBI Acc. No: FN554754.1, FN554508.1), *P. umsongensis* LMG 21317 (NCBI Acc. No: FN554763.1, FN554516.1), *P. baetica* CECT 7720 (NCBI Acc. No: HE800504.1, FN678357.1), *P. arsenicoxydans* CECT 7543 (NCBI Acc. No: HE800503.1, HE800488.1), *P. granadensis* F-278,770 (NCBI Acc. No: HG764747.1, HG764748.1), *P. helmanticensis* OHA11 (NCBI Acc. No: HG940518.1, HG940517.1), *P. synxantha* LMG 2335 (NCBI Acc. No: AJ717420.1, AB039550.1), *P. cedrina* CIP 105541 (NCBI Acc. No: AJ717424.1, FN554459.1), *P. marginalis* LMG 2210 (NCBI Acc. No: AJ717425.1, AB039575.1), *P. mucidolens* LMG 2223 (NCBI Acc. No: AJ717427.1, AB039546.1), *P. rhodesiae* CIP 104664 (NCBI Acc. No: AJ717431.1, FN554511.1*), P. orientalis* CIP 105540 (NCBI Acc. No: AJ717434.1, FN554493.1), *P. gessardii* CIP 105469 (NCBI Acc. No: AJ717438.1, FN554468.1), *P. grimontii* CIP 106645 (NCBI Acc. No: AJ717439.1, FN554470.1), *P. veronii* CIP 104663 (NCBI Acc. No: AJ717445.1, FN554518.1), *P. fluorescens* CIP 69.13 (NCBI Acc. No: AJ717451.1, AB039545.1), *P. libanensis* CIP 105460 (NCBI Acc. No: AJ717454.1, FN554477.1), *P. azotoformans* CIP 106744 (NCBI Acc. No: AJ717458.1, AB039547.1), *P. tolaasii* LMG 2342 (NCBI Acc. No: AJ717467.1, AB039561.1), *P. brenneri* CIP 106646 (NCBI Acc. No: AJ717482.1, FN554457.1*), P. antarctica* LMG 22709 (NCBI Acc. No: FN554727.1, FN554450.1), *P. costantinii* LMG 22119 (NCBI Acc. No: FN554732.1, FN554461.1), *P. extremorientalis* LMG 19695 (NCBI Acc. No: FN554733.1, FN554464.1), *P. meridiana* CIP 108465 (NCBI Acc. No: FN554740.1, FN554485.1), *P. palleroniana* LMG 23076 (NCBI Acc. No: FN554747.1, FN554497.1), *P. panacis* CIP 108524 (NCBI Acc. No: FN554748.1, FN554498.1), *P. poae* LMG 21465 (NCBI Acc. No: FN554751.1, FN554504.1), *P. proteolytica* CIP 108464 (NCBI Acc. No: FN554752.1, FN554505.1), *P. salomonii* LMG 22120 (NCBI Acc. No: FN554756.1, FN554512.1), *P. simiae* CCUG 50988 (NCBI Acc. No: FN554757.1, FN554513.1), P. trivialis LMG 21464 (NCBI Acc. No: FN554762.1, FN554515.1), *P. cedrina* subsp. *fulgida* LMG 21467 (NCBI Acc. No: HE586401.1, HE586449.1), *P. lurida* LMG 21995 (NCBI Acc. No: HE586402.1, HE586451.1), *P. prosekii* AN-28-1 (NCBI Acc. No: JN814370.1, JN814373.1), *P. extremaustralis* 4-13 (NCBI Acc. No: JN814371.1, JN589935.1). The sequence of the genes for *P. silesiensis* A3 was retrieved directly from the genome sequence (CP014870.1).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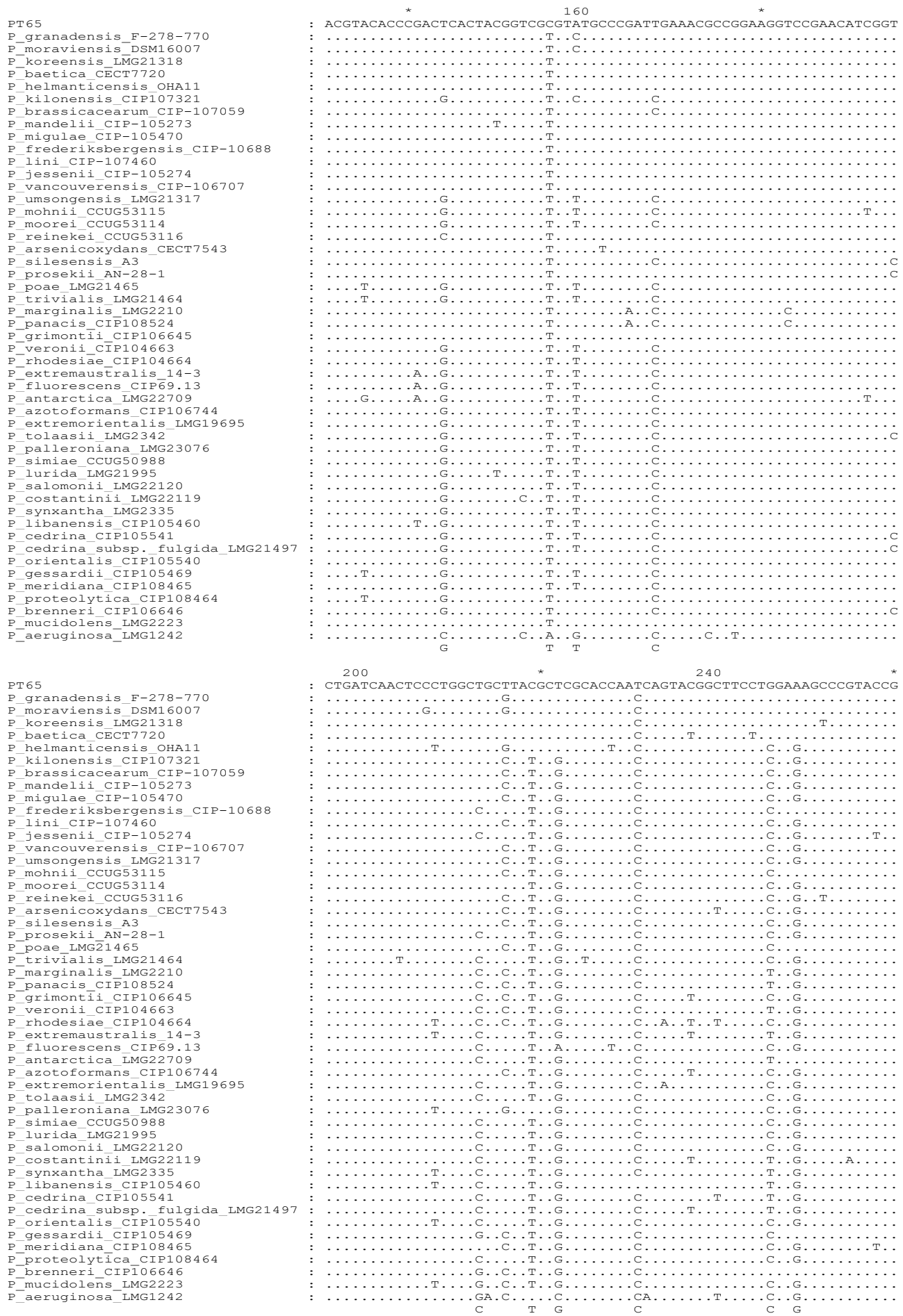
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P_baetica_CECT7720
P_helmanticensis_OHA11
P_kilonensis_CIP107321
P_brassicacearum_CIP-107059
P_mandelii_CIP-105273
P_migulae_CIP-105470
P_frederiksbergensis_CIP-10688
P_lini_CIP-107460
P_jessenii_CIP-105274
P_vancouverensis_CIP-106707
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P_mohnii_CCUG53115
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P_brassicacearum_CIP-107059
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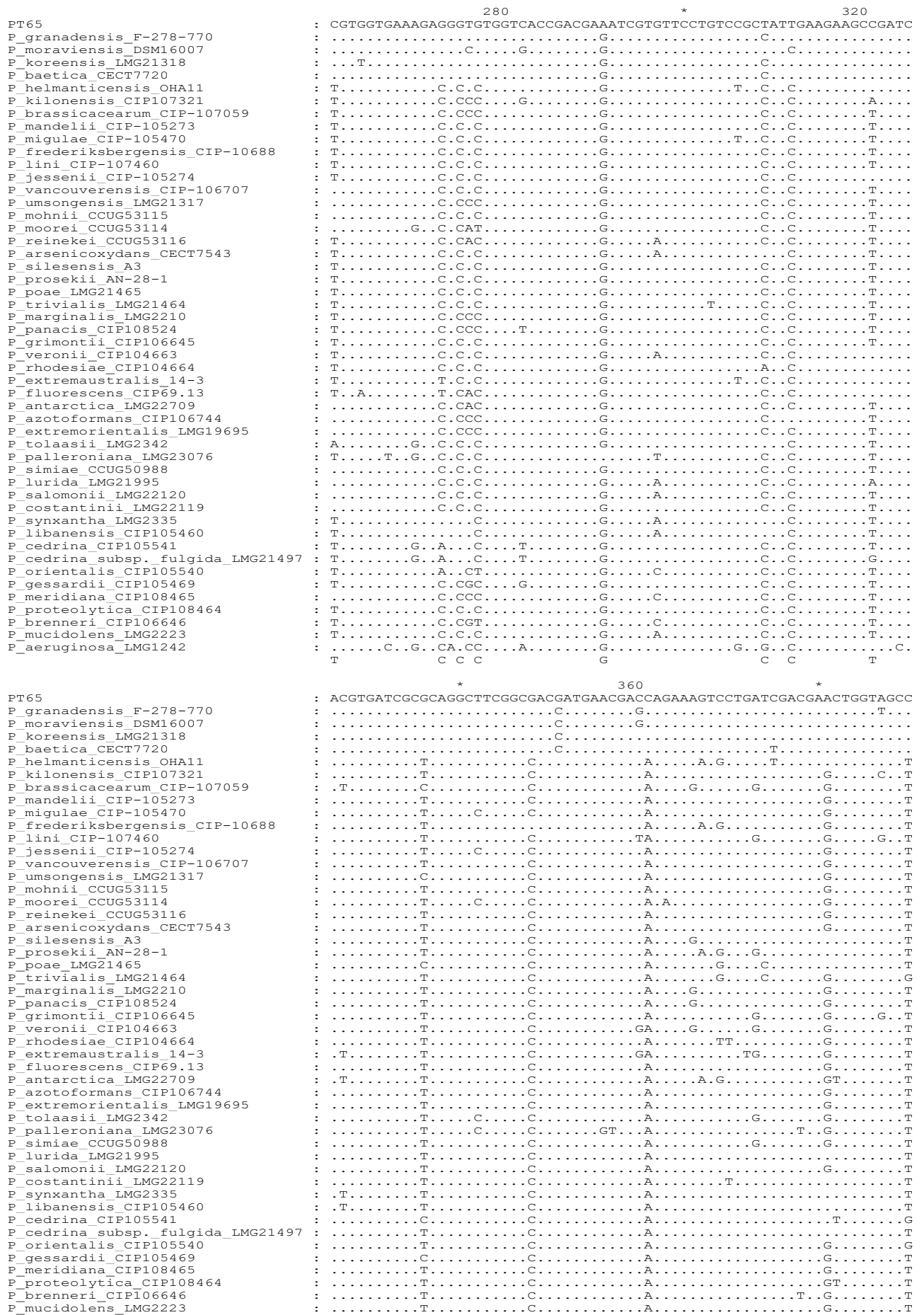
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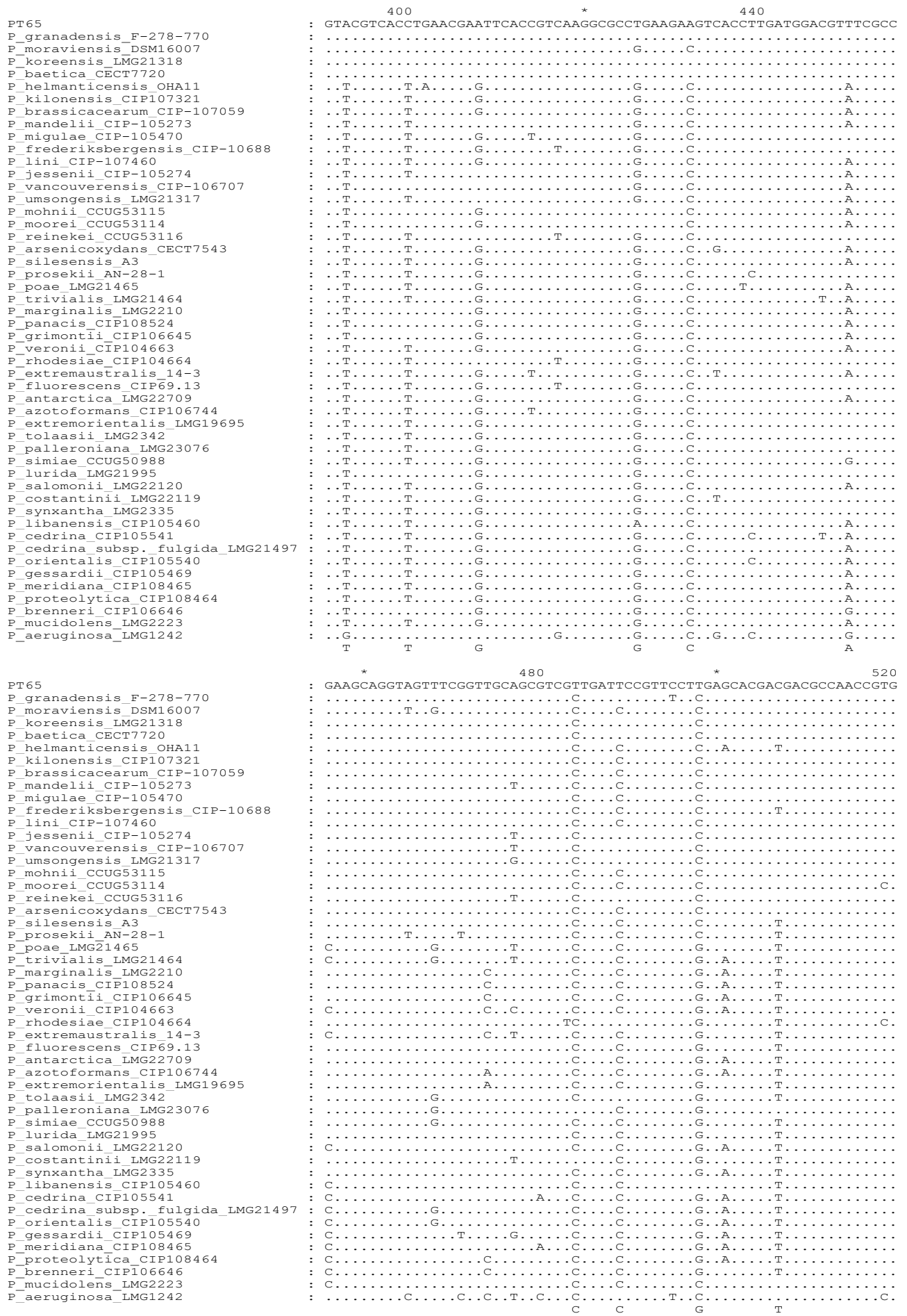
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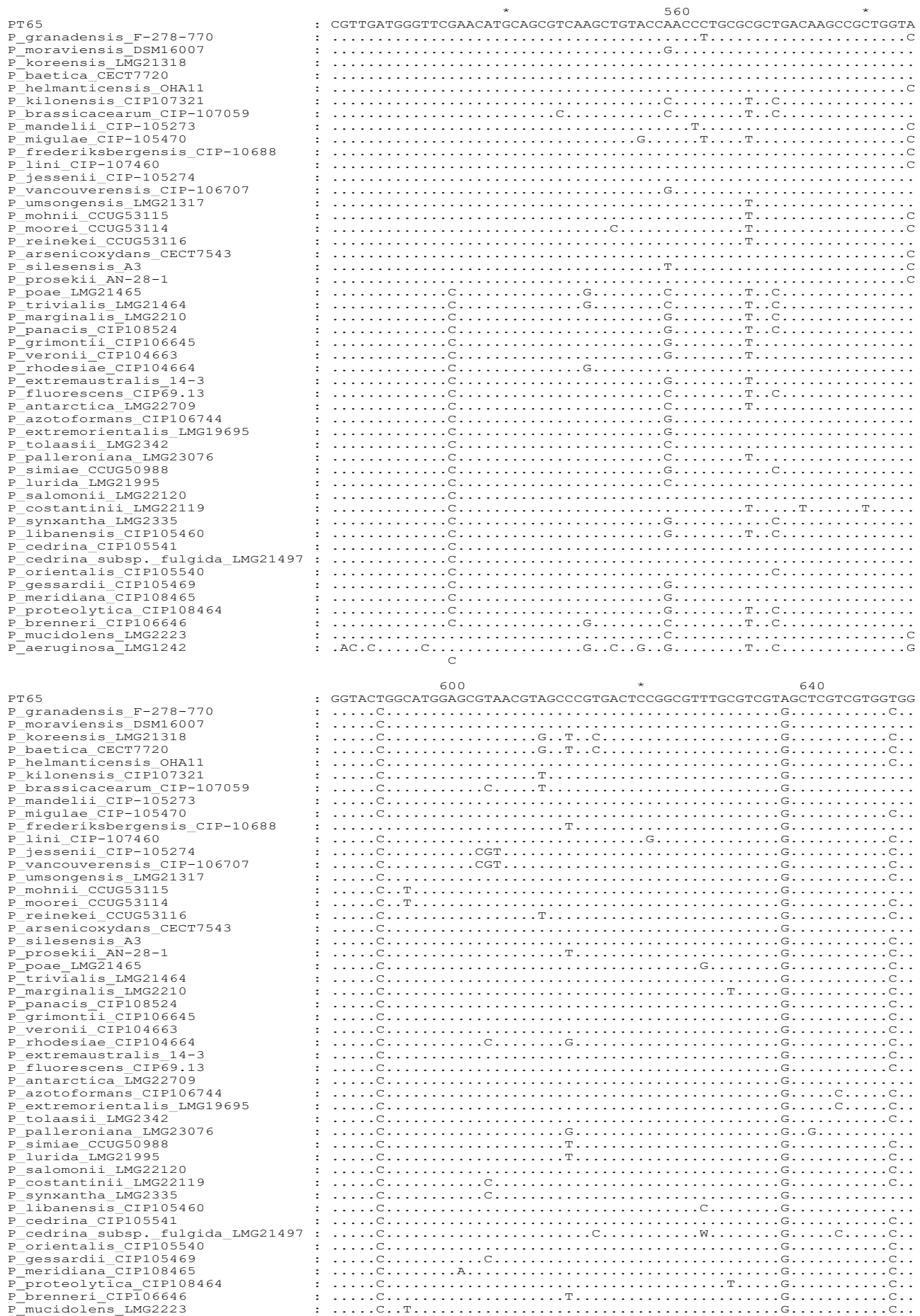
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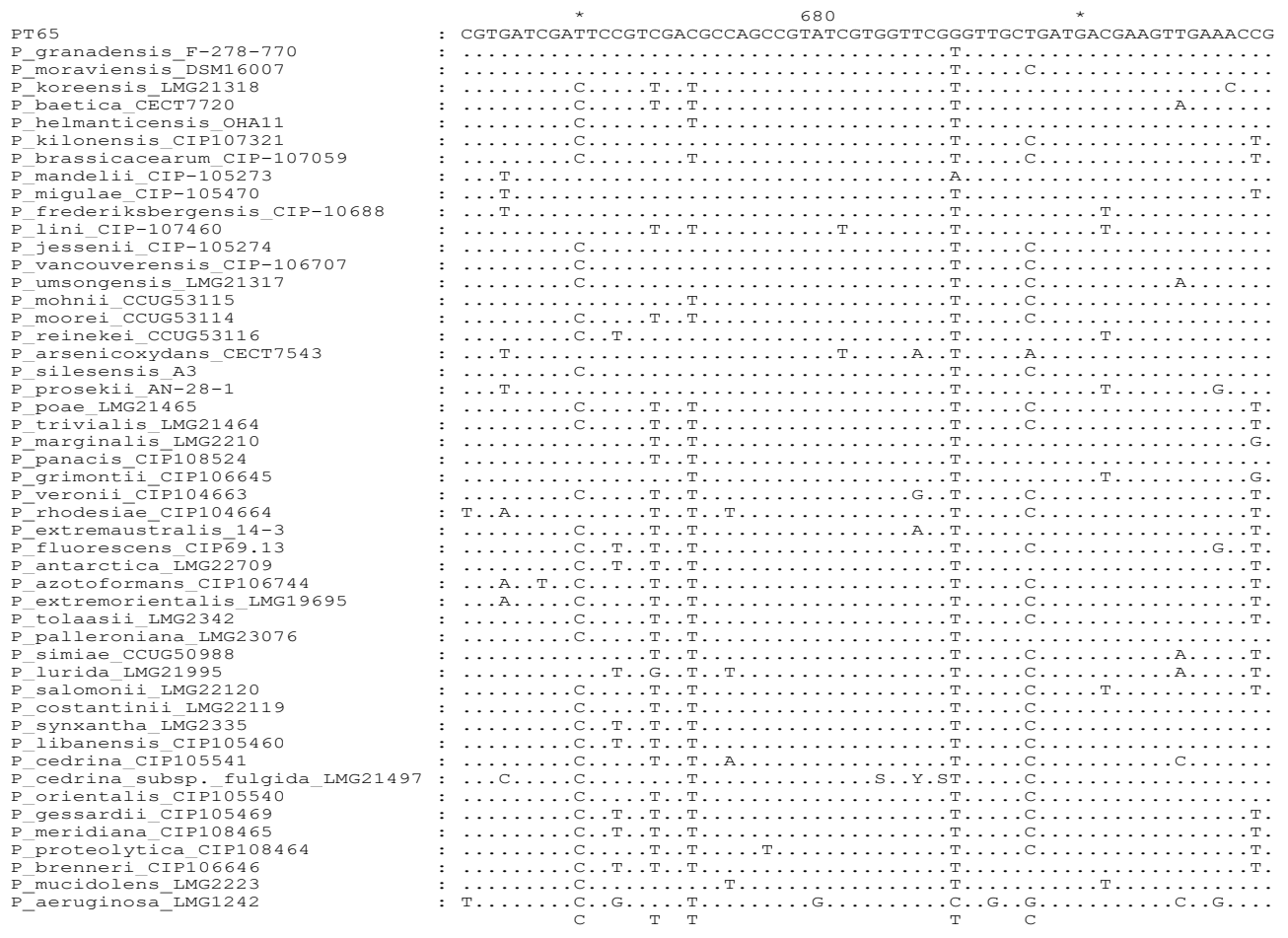
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P_koreensis_LMG21318
P_baetica_CECT7720
P_helmanticensis_OHA11
P_kilonensis_CIP107321
P_brassicacearum_CIP-107059
P_mandelii_CIP-105273
P_migulae_CIP-105470
P_frederiksbergensis_CIP-10688
P_lini_CIP-107460
P_jessenii_CIP-105274
P_vancouverensis_CIP-106707
P_umsongensis_LMG21317
P_mohnii_CCUG53115
P_moorei_CCUG53114
P_reinekei_CCUG53116
P_arsenicoxydans_CECT7543
P_silesensis_A3
P_prosekii_AN-28-1
P_poae_LMG21465
P_trivialis_LMG21464
P_marginalis_LMG2210
P_panacis_CIP108524
P_grimontii_CIP106645
P_veronii_CIP104663
P_rhodesiae_CIP104664
P_extremaustralis_14-3
P_fluorescens_CIP69.13
P_antarctica_LMG22709
P_azotoformans_CIP106744
P_extremorientalis_LMG19695
P_tolaasii_LMG2342
P_palleroniana_LMG23076
P_simiae_CCUG50988
P_lurida_LMG21995
P_salomonii_LMG22120
P_costantinii_LMG22119
P_synxantha_LMG2335
P_libanensis_CIP105460
P_cedrina_CIP105541
P_cedrina_subsp._fulgida_LMG21497
P_orientalis_CIP105540
P_gessardii_CIP105469
P_meridiana_CIP108465
P_proteolytica_CIP108464
P_brenneri_CIP106646
P_mucidolens_LMG2223
P_aeruginosa_LMG1242
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760
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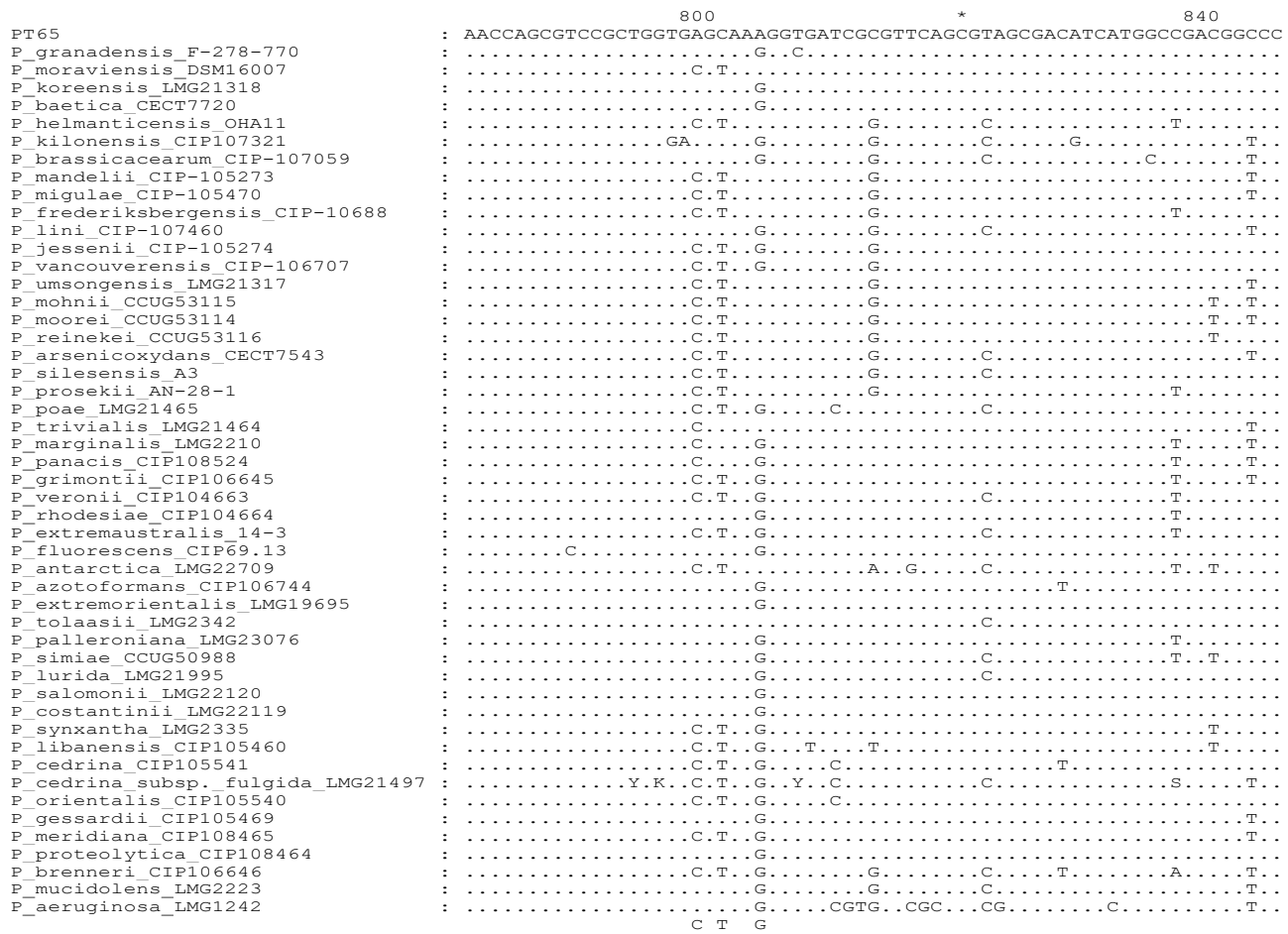
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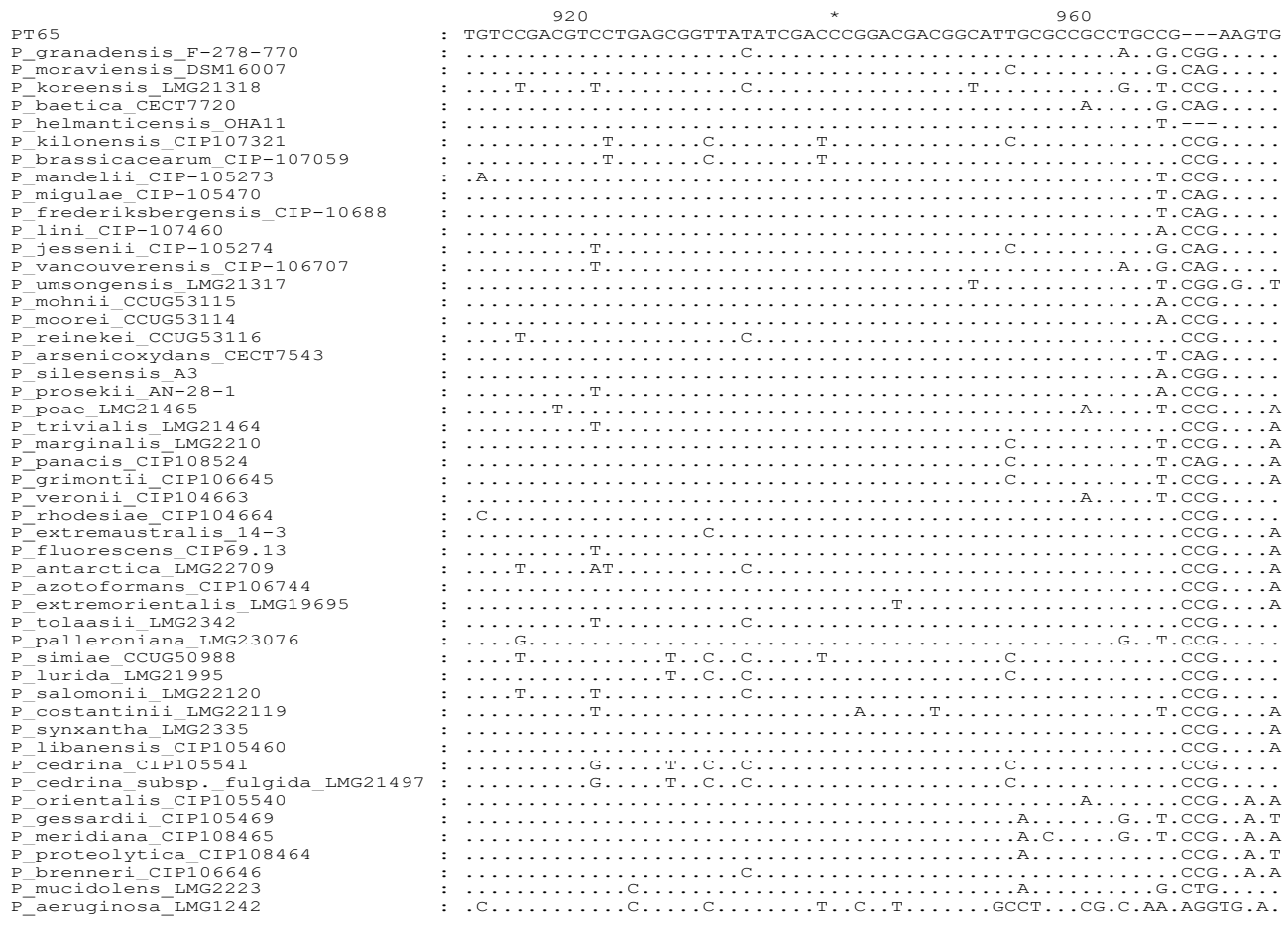
P_granadensis_F-278-770 : .....G..C.....
P_moraviensis_DSM16007 : .....C.T.....
P_koreensis_LMG21318 : .....G.....
P_baetica_CECT7720 : .....G.....
P_helmanticensis_OHA11 : .....C.T.....G.....C.....T.....
P_kilonensis_CIP107321 : .....GA.....G.....G.....C.....G.....T..
P_brassicacearum_CIP-107059 : .....G.....G.....C.....C.....T..
P_mandelii_CIP-105273 : .....C.T.....G.....T..
P_migulae_CIP-105470 : .....C.T.....G.....T..
P_frederiksbergensis_CIP-10688 : .....C.T.....G.....T.....
P_lini_CIP-107460 : .....G.....G.....C.....T..
P_jessenii_CIP-105274 : .....C.T..G.....G.....
P_vancouverensis_CIP-106707 : .....C.T..G.....G.....
P_umsongensis_LMG21317 : .....C.T.....G.....T..
P_mohnii_CCUG53115 : .....C.T.....G.....T..T..
P_moorei_CCUG53114 : .....C.T.....G.....T..T..
P_reinekei_CCUG53116 : .....C.T.....G.....T.....
P_arsenicoxydans_CECT7543 : .....C.T.....G.....C.....T..
P_silesensis_A3 : .....C.T.....G.....C.....
P_prosekii_AN-28-1 : .....C.T.....G.....T.....
P_poae_LMG21465 : .....C.T..G.....C.....C.....
P_trivialis_LMG21464 : .....C.....T..
P_marginalis_LMG2210 : .....C....G.....T.....T..
P_panacis_CIP108524 : .....C....G.....T.....T..
P_grimontii_CIP106645 : .....C.T..G.....T.....T..
P_veronii_CIP104663 : .....C.T..G.....C.....T.....
P_rhodesiae_CIP104664 : .....G.....T.....
P_extremaustralis_14-3 : .....C.T..G.....C.....T.....
P_fluorescens_CIP69.13 : .....C.....G.....
P_antarctica_LMG22709 : .....C.T.....A..G.....C.....T..T.....
P_azotoformans_CIP106744 : .....G.....T.....
P_extremorientalis_LMG19695 : .....G.....
P_tolaasii_LMG2342 : .....C.....
P_palleroniana_LMG23076 : .....G.....T.....
P_simiae_CCUG50988 : .....G.....C.....T..T.....
P_lurida_LMG21995 : .....G.....C.....
P_salomonii_LMG22120 : .....G.....
P_costantinii_LMG22119 : .....G.....
P_synxantha_LMG2335 : .....C.T..G.....T.....
P_libanensis_CIP105460 : .....C.T..G...T....T.....T.....
P_cedrina_CIP105541 : .....C.T..G.....C.....T.....
P_cedrina_subsp._fulgida_LMG21497 : .....Y.K..C.T..G..Y..C.....C.....S.....T..
P_orientalis_CIP105540 : .....C.T..G.....C.....
P_gessardii_CIP105469 : .....G.....T..
P_meridiana_CIP108465 : .....C.T..G.....T..
P_proteolytica_CIP108464 : .....G.....
P_brenneri_CIP106646 : .....C.T..G.....G.....C.....T.....A.....T..
P_mucidolens_LMG2223 : .....G.....G.....C.....T..
P_aeruginosa_LMG1242 : .....G.....CGTG..CGC...CG.....C.....T..
C T G
* 880 *
PT65 : GTCCACCGACACGGTTGACCACATTCTCTCCGAGTACACCCGCGTCACCACCGAAGGTGGTCGCC
P_granadensis_F-278-770 : .....G..G.....
P_moraviensis_DSM16007 : .....T
P_koreensis_LMG21318 : .....C.....T.....G.....
P_baetica_CECT7720 : .....T.....A.....C.....T.....
P_helmanticensis_OHA11 : .....T.....
P_kilonensis_CIP107321 : .....T.....T.....G....
P_brassicacearum_CIP-107059 : .....T..T.....T.....C....
P_mandelii_CIP-105273 : .....T.....T.....T.....C....
P_migulae_CIP-105470 : .....T.....T.....T.....C....
P_frederiksbergensis_CIP-10688 : .....T.....A.....T.....C....
P_lini_CIP-107460 : .....T.....T.....T.....C....
P_jessenii_CIP-105274 : .....T..T.....
P_vancouverensis_CIP-106707 : .....T..T.....A.....
P_umsongensis_LMG21317 : .....T..T.....A.....T.....T.....C....
P_mohnii_CCUG53115 : .....T.....T..T.....T.....
P_moorei_CCUG53114 : .....T.....T.....T.....
P_reinekei_CCUG53116 : .....T..C.....T.....G.....
P_arsenicoxydans_CECT7543 : .....T.....T.....A.....T.....C....
P_silesensis_A3 : .....T.....A.....T.....C....
P_prosekii_AN-28-1 : .....G.....A.....T.....
P_poae_LMG21465 : .....T.....C....
P_trivialis_LMG21464 : .....T.....C....
P_marginalis_LMG2210 : .....T.....T.....C....
P_panacis_CIP108524 : .....T.....T.....C....
P_grimontii_CIP106645 : .....T.....T.....C....
P_veronii_CIP104663 : .....T.....C....
P_rhodesiae_CIP104664 : .....T..G.....C....
P_extremaustralis_14-3 : .....T.....C....
P_fluorescens_CIP69.13 : .....T.....
P_antarctica_LMG22709 : .....A.....
P_azotoformans_CIP106744 : .....T.....A.....C....
P_extremorientalis_LMG19695 : .....
P_tolaasii_LMG2342 : .....A.....G....
P_palleroniana_LMG23076 : .....A.....T....G.....C....
P_simiae_CCUG50988 : .....T.....C....
P_lurida_LMG21995 : .....T.....T.....
P_salomonii_LMG22120 : .....T.....C....
P_costantinii_LMG22119 : .....T.....C....
P_synxantha_LMG2335 : .....T.....C....
P_libanensis_CIP105460 : .....C....
P_cedrina_CIP105541 : .....A.....C.....
P_cedrina_subsp._fulgida_LMG21497 : .....C.....C....
P_orientalis_CIP105540 : .....T.....T.....C....
P_gessardii_CIP105469 : T.....T.....G.....C....
P_meridiana_CIP108465 : .....T.....T.....G.....T..T..G.....G.....C....
P_proteolytica_CIP108464 : .....T.....T.....C....
P_brenneri_CIP106646 : .....T.....T.....C....
P_mucidolens_LMG2223 : .....T.....A.....T.....
P_aeruginosa_LMG1242 : .....G...AG...C..GG....C....AT...A..GT.G.....C.....
C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960
PT65
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P_helmanticensis_OHA11
P_kilonensis_CIP107321
P_brassicacearum_CIP-107059
P_mandelii_CIP-105273
P_migulae_CIP-105470
P_frederiksbergensis_CIP-10688
P_lini_CIP-107460
P_jessenii_CIP-105274
P_vancouverensis_CIP-106707
P_umsongensis_LMG21317
P_mohnii_CCUG53115
P_moorei_CCUG53114
P_reinekei_CCUG53116
P_arsenicoxydans_CECT7543
P_silesensis_A3
P_prosekii_AN-28-1
P_poae_LMG21465
P_trivialis_LMG21464
P_marginalis_LMG2210
P_panacis_CIP108524
P_grimontii_CIP106645
P_veronii_CIP104663
P_rhodesiae_CIP104664
P_extremaustralis_14-3
P_fluorescens_CIP69.13
P_antarctica_LMG22709
P_azotoformans_CIP106744
P_extremorientalis_LMG19695
P_tolaasii_LMG2342
P_palleroniana_LMG23076
P_simiae_CCUG50988
P_lurida_LMG21995
P_salomonii_LMG22120
P_costantinii_LMG22119
P_synxantha_LMG2335
P_libanensis_CIP105460
P_cedrina_CIP105541
P_cedrina_subsp._fulgida_LMG21497
P_orientalis_CIP105540
P_gessardii_CIP105469
P_meridiana_CIP108465
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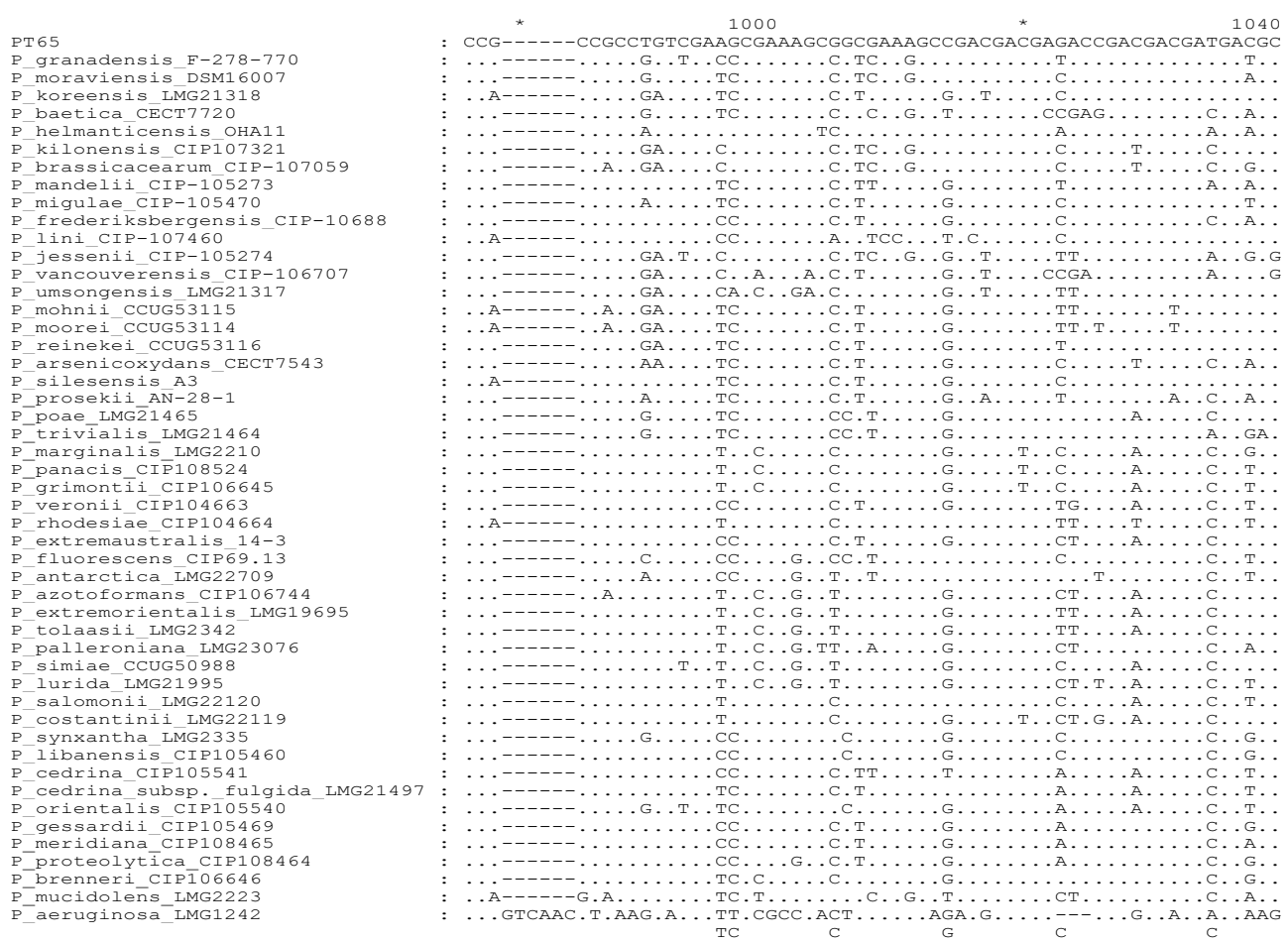
P_brenneri_CIP106646
P_mucidolens_LMG2223
P_aeruginosa_LMG1242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1000
*
1040
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P_koreensis_LMG21318
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P_kilonensis_CIP107321
P_brassicacearum_CIP-107059
P_mandelii_CIP-105273
P_migulae_CIP-105470
P_frederiksbergensis_CIP-10688
P_lini_CIP-107460
P_jessenii_CIP-105274
P_vancouverensis_CIP-106707
P_umsongensis_LMG21317
P_mohnii_CCUG53115
P_moorei_CCUG53114
P_reinekei_CCUG53116
P_arsenicoxydans_CECT7543
P_silesensis_A3
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P_extremorientalis_LMG19695
P_tolaasii_LMG2342
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P_mucidolens_LMG2223
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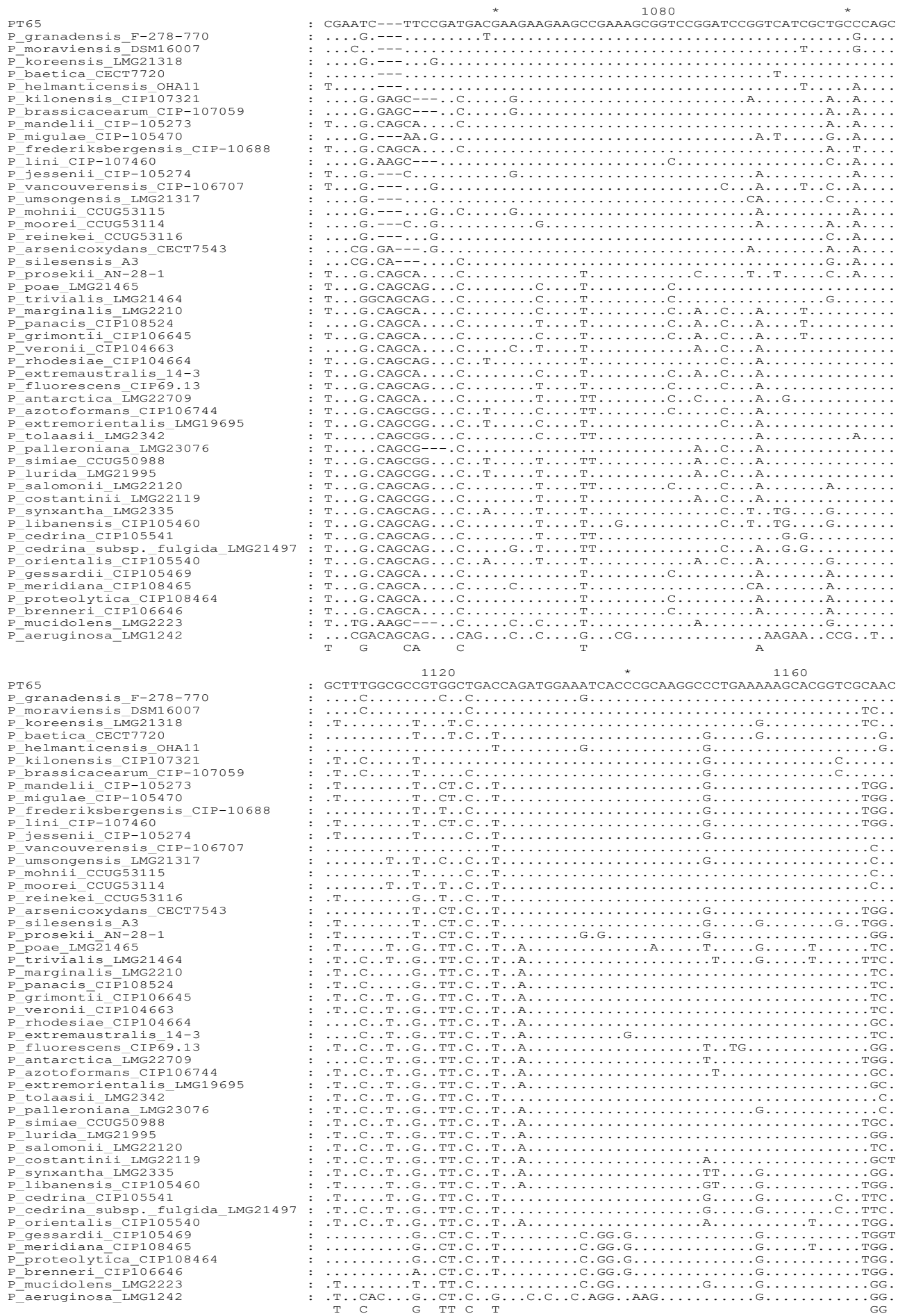
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1120
1160
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P_brassicacearum_CIP-107059
P_mandelii_CIP-105273
P_migulae_CIP-105470
P_frederiksbergensis_CIP-10688
P_lini_CIP-107460
P_jessenii_CIP-105274
P_vancouverensis_CIP-106707
P_umsongensis_LMG21317
P_mohnii_CCUG53115
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P_reinekei_CCUG53116
P_arsenicoxydans_CECT7543
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P_azotoformans_CIP106744
P_extremorientalis_LMG19695
P_tolaasii_LMG2342
P_palleroniana_LMG23076
P_simiae_CCUG50988
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P_salomonii_LMG22120
P_costantinii_LMG22119
P_synxantha_LMG2335
P_libanensis_CIP105460
P_cedrina_CIP105541
P_cedrina_subsp._fulgida_LMG21497
P_orientalis_CIP105540
P_gessardii_CIP105469
P_meridiana_CIP108465
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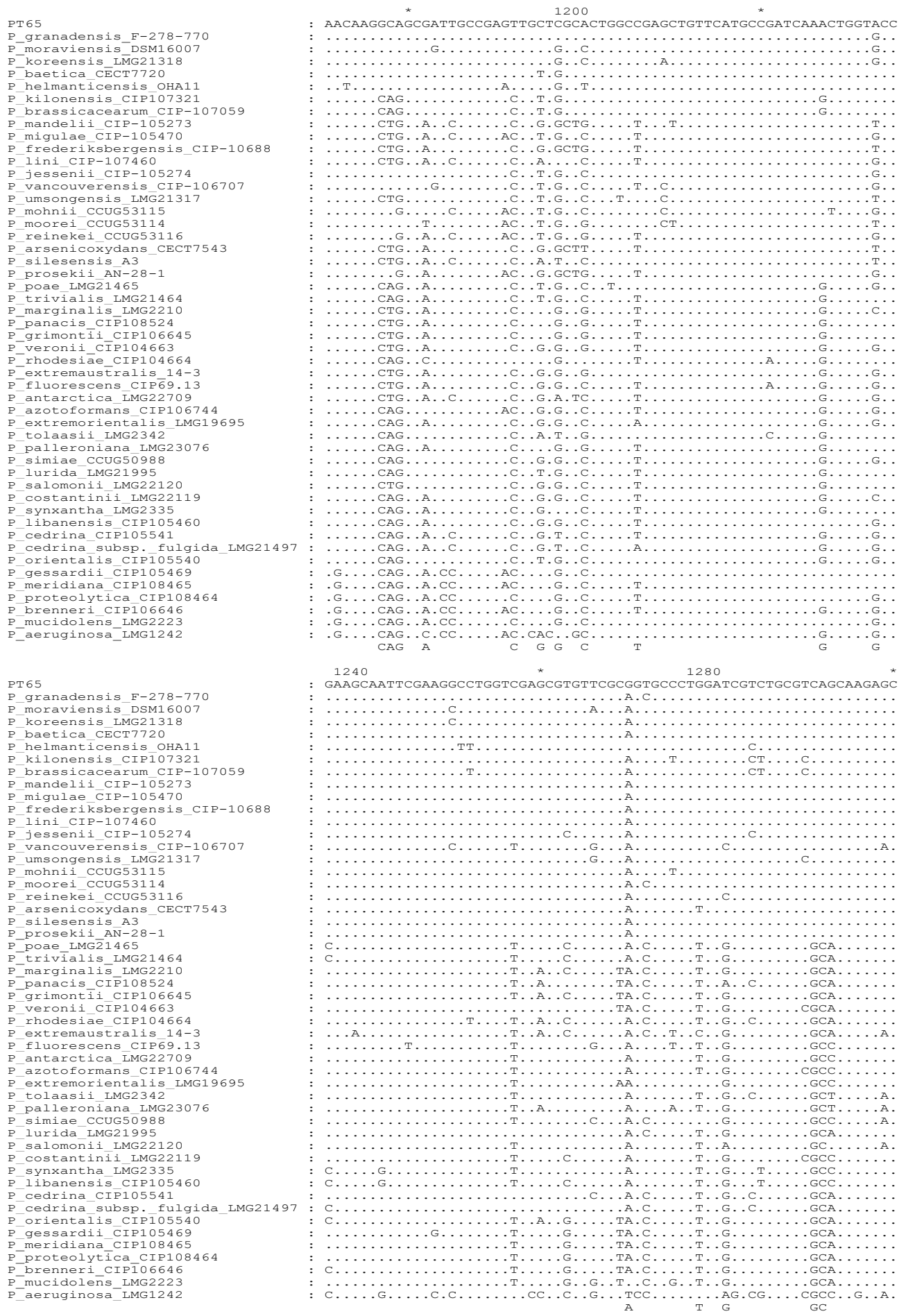
P_brenneri_CIP106646
P_mucidolens_LMG2223
P_aeruginosa_LMG1242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P_helmanticensis_OHA11
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P_brassicacearum_CIP-107059
P_mandelii_CIP-105273
P_migulae_CIP-105470
P_frederiksbergensis_CIP-10688
P_lini_CIP-107460
P_jessenii_CIP-105274
P_vancouverensis_CIP-106707
P_umsongensis_LMG21317
P_mohnii_CCUG53115
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P_reinekei_CCUG53116
P_arsenicoxydans_CECT7543
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P_prosekii_AN-28-1
P_poae_LMG21465
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P_rhodesiae_CIP104664
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P_libanensis_CIP105460
P_cedrina_CIP105541
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P_orientalis_CIP105540
P_gessardii_CIP105469
P_meridiana_CIP108465
P_proteolytica_CIP108464
P_brenneri_CIP106646
P_mucidolens_LMG2223
P_aeruginosa_LMG1242
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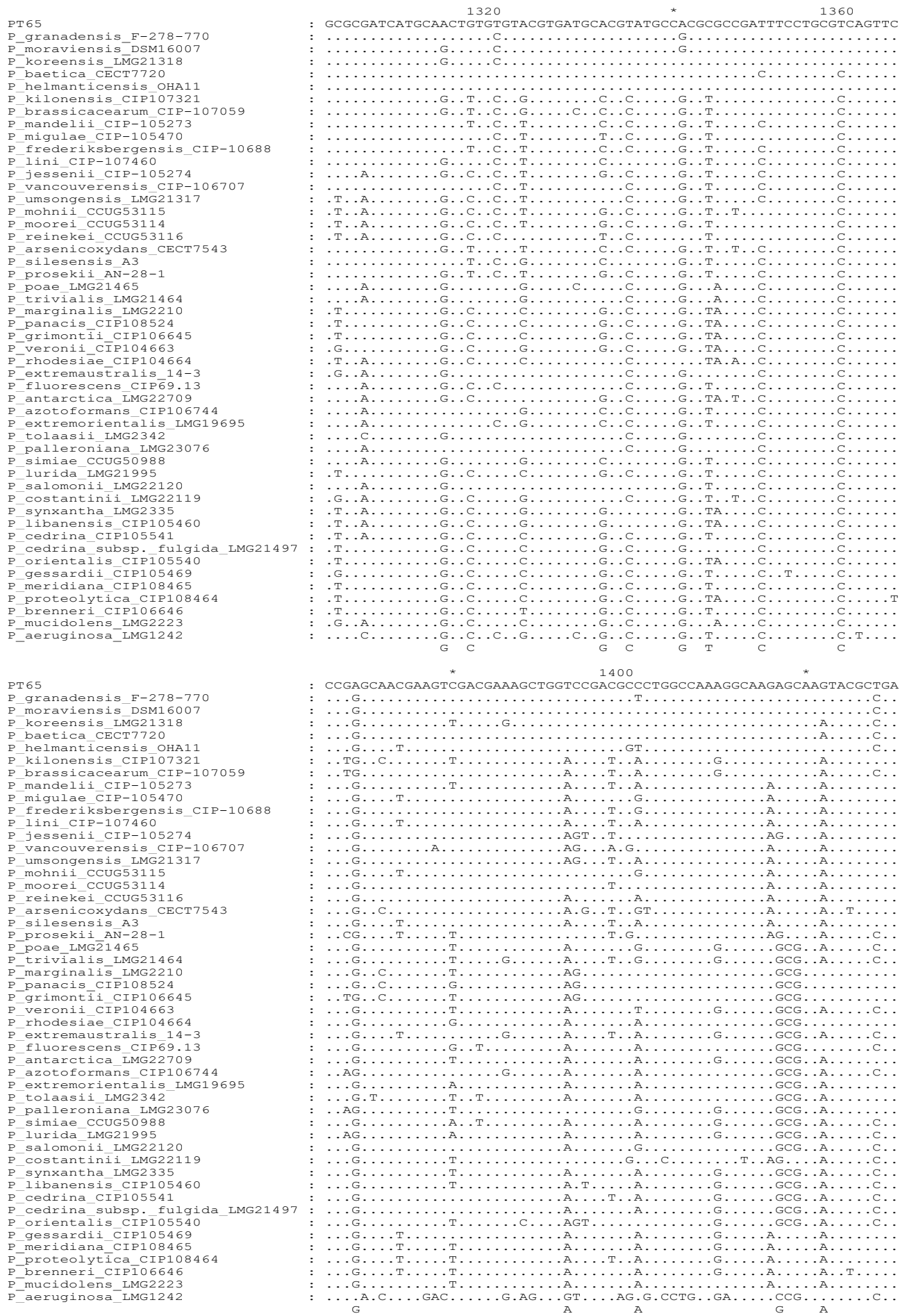
1280
GAAGCAATTCGAAGGCCTGGTCGAGCGTGTTCGCGGTGCCCTGGATCGTCTGCGTCAGCAAGAGC


1320 * 1360
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P_koreensis_LMG21318
P_baetica_CECT7720
P_helmanticensis_OHA11
P_kilonensis_CIP107321
P_brassicacearum_CIP-107059
P_mandelii_CIP-105273
P_migulae_CIP-105470
P_frederiksbergensis_CIP-10688
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P_vancouverensis_CIP-106707
P_umsongensis_LMG21317
P_mohnii_CCUG53115
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P_reinekei_CCUG53116
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P_rhodesiae_CIP104664
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P_gessardii_CIP105469
P_meridiana_CIP108465
P_proteolytica_CIP108464
P_brenneri_CIP106646
P_mucidolens_LMG2223
P_aeruginosa_LMG1242
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P_koreensis_LMG21318
P_baetica_CECT7720
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P_brassicacearum_CIP-107059
P_mandelii_CIP-105273
P_migulae_CIP-105470
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P_jessenii_CIP-105274
P_vancouverensis_CIP-106707
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P_mohnii_CCUG53115
P_moorei_CCUG53114
P_reinekei_CCUG53116
P_arsenicoxydans_CECT7543
P_silesensis_A3
P_prosekii_AN-28-1
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P_trivialis_LMG21464
P_marginalis_LMG2210
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P_grimontii_CIP106645
P_veronii_CIP104663
P_rhodesiae_CIP104664
P_extremaustralis_14-3
P_fluorescens_CIP69.13
P_antarctica_LMG22709
P_azotoformans_CIP106744
P_extremorientalis_LMG19695
P_tolaasii_LMG2342
P_palleroniana_LMG23076
P_simiae_CCUG50988
P_lurida_LMG21995
P_salomonii_LMG22120
P_costantinii_LMG22119
P_synxantha_LMG2335
P_libanensis_CIP105460
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P_orientalis_CIP105540
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P_meridiana_CIP108465
P_proteolytica_CIP108464
P_brenneri_CIP106646
P_mucidolens_LMG2223
P_aeruginosa_LMG1242

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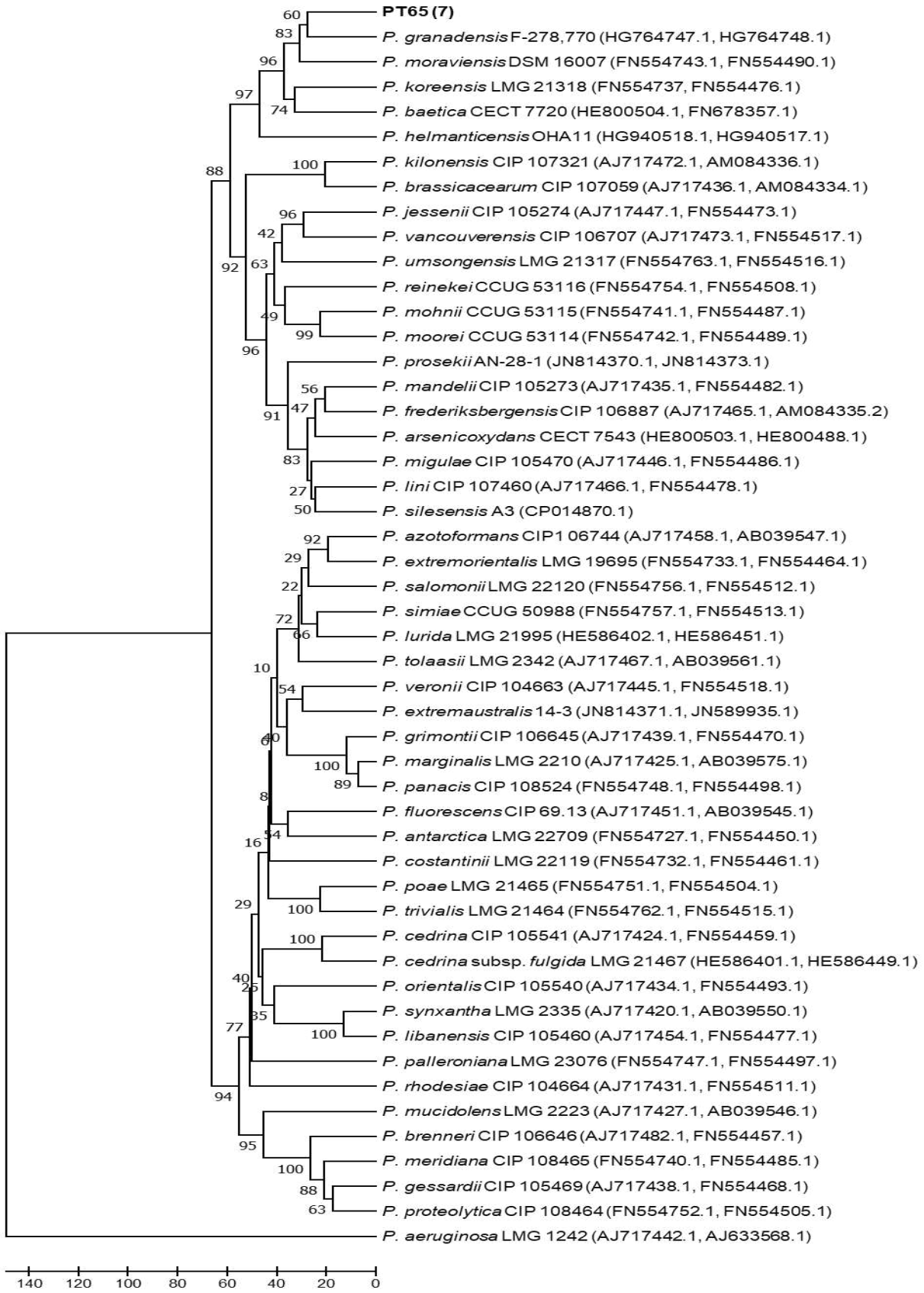
                                          1440                  *                1480
PT65                                    : AGCCATCGGTCGCGTGCAGCCGGACATCATTCGTTGCCAGCAGAAACTGATCGCGCTGGAAACCG
P_granadensis_F-278-770                 : ..............A.............................G...................
P_moraviensis_DSM16007                  : ................................C.....................................
P_koreensis_LMG21318                    : ......T........A.............C.....................T.............
P_baetica_CECT7720                      : .........C..T..T........T.....C..............G....CG.............
P_helmanticensis_OHA11                  : ..............T.....................................................
P_kilonensis_CIP107321                  : ......T......C....A.........................G....C...............
P_brassicacearum_CIP-107059             : ......T.CC...C....A.........G..............G..C.GT........GG...
P_mandelii_CIP-105273                   : ......T......C....A.......................A..G....C.......C......
P_migulae_CIP-105470                    : ......T......C..............T..............G....C...............
P_frederiksbergensis_CIP-10688          : ......T......C....A........................G..A.C.......C......
P_lini_CIP-107460                       : ......T......C....A........................G....C...............
P_jessenii_CIP-105274                   : ......T.....TC.............C.............G....C...............
P_vancouverensis_CIP-106707             : ......T......C.............C...........A..G....C...A...........
P_umsongensis_LMG21317                  : ........................T.....................G....C...............
P_mohnii_CCUG53115                      : ............C.................C........A..G....C...............
P_moorei_CCUG53114                      : ......T......C....A........................A..G....C...............
P_reinekei_CCUG53116                    : ......T......C.............................G....C.........G....
P_arsenicoxydans_CECT7543               : ......T......C....A........................G....AA..............
P_silesensis_A3                         : ......T......C..............................G....C.......C......
P_prosekii_AN-28-1                      : ...G..T......C....A.........C.G.............G.............C......
P_poae_LMG21465                         : ......T......C.......C..T.....C..C.........G....C......T..G....
P_trivialis_LMG21464                    : ......T......C.......C..T.....C............G....C......T..G....
P_marginalis_LMG2210                    : ......T......C..............C..............G....C......T..G....
P_panacis_CIP108524                     : ......T..C...C..............C..............G....C...A..C..G....
P_grimontii_CIP106645                   : ......T......C..............C..............G....C...A..T..G....
P_veronii_CIP104663                     : ......T......C.......C..T..................G....C...A..TC.G....
P_rhodesiae_CIP104664                   : ............C....A............C............G....G......C..GG...
P_extremaustralis_14-3                  : ............C..............................C..G....C......T..G....
P_fluorescens_CIP69.13                  : ......T......C..............C.............G....C......TC......
P_antarctica_LMG22709                   : ......T......C...........T.....C............G....C...CT..C.G....
P_azotoformans_CIP106744                : ......T......C..............C.............G....CT.....T.......
P_extremorientalis_LMG19695             : ............C..............C.............G....CT.....T..G....
P_tolaasii_LMG2342                      : ......T......C..............C..C..........G....C......T..G....
P_palleroniana_LMG23076                 : ......T..........A..........C...........G....C......T..G....
P_simiae_CCUG50988                      : ......T......C....A.........C............G....C......T..G....
P_lurida_LMG21995                       : ......T......C.......C.......C..C.......A..G....C......T..G....
P_salomonii_LMG22120                    : ......T......C..............C............G....C......T..G....
P_costantinii_LMG22119                  : ......T......C....A........C............A..G........A...C......
P_synxantha_LMG2335                     : ............C..............................G.........T..C.G....
P_libanensis_CIP105460                  : ............C..............................G.........T..C.G....
P_cedrina_CIP105541                     : ............C............................A..G............C.G....
P_cedrina_subsp._fulgida_LMG21497       : ......T......C..............................A..G............C.G....
P_orientalis_CIP105540                  : ......T......C..............C..................G...A.....GG.T.
P_gessardii_CIP105469                   : ......T......T.C...G.C......G..............G....CT..AT..C.G....
P_meridiana_CIP108465                   : ......T......T.C...G.C......G..............G....CT..A...C.G....
P_proteolytica_CIP108464                : ......T......T.C...G.C......G..............G....CT..AT..C.G....
P_brenneri_CIP106646                    : ......T......T.C...G.C......G..............G....CT..AT..C.G....
P_mucidolens_LMG2223                    : ......T......T.C...G.C......G..............G....CA..AT..C.G....
P_aeruginosa_LMG1242                    : G.....T.AG...C...GCGAC......C.G..CAA.........G...GCG..C.......G..
                                                T       C                                G    C          G

                                           *
PT65                                    : AAACCGGT
P_granadensis_F-278-770                 : ........
P_moraviensis_DSM16007                  : .G......
P_koreensis_LMG21318                    : .G.....C
P_baetica_CECT7720                      : ........
P_helmanticensis_OHA11                  : .G......
P_kilonensis_CIP107321                  : ........
P_brassicacearum_CIP-107059             : .G..T..C
P_mandelii_CIP-105273                   : ........
P_migulae_CIP-105470                    : ........
P_frederiksbergensis_CIP-10688          : ........
P_lini_CIP-107460                       : ........
P_jessenii_CIP-105274                   : ........
P_vancouverensis_CIP-106707             : ........
P_umsongensis_LMG21317                  : ........
P_mohnii_CCUG53115                      : ........
P_moorei_CCUG53114                      : ........
P_reinekei_CCUG53116                    : ........
P_arsenicoxydans_CECT7543               : ........
P_silesensis_A3                         : ........
P_prosekii_AN-28-1                      : ........
P_poae_LMG21465                         : .G......
P_trivialis_LMG21464                    : .G......
P_marginalis_LMG2210                    : ........
P_panacis_CIP108524                     : ........
P_grimontii_CIP106645                   : ........
P_veronii_CIP104663                     : ........
P_rhodesiae_CIP104664                   : ........
P_extremaustralis_14-3                  : .......C
P_fluorescens_CIP69.13                  : ........
P_antarctica_LMG22709                   : ....T...
P_azotoformans_CIP106744                : .G.....C
P_extremorientalis_LMG19695             : ........
P_tolaasii_LMG2342                      : ........
P_palleroniana_LMG23076                 : .......C
P_simiae_CCUG50988                      : ........
P_lurida_LMG21995                       : .G......
P_salomonii_LMG22120                    : ........
P_costantinii_LMG22119                  : .G......
P_synxantha_LMG2335                     : ........
P_libanensis_CIP105460                  : ........
P_cedrina_CIP105541                     : .......C
P_cedrina_subsp._fulgida_LMG21497       : .......C
P_orientalis_CIP105540                  : ....A..C
P_gessardii_CIP105469                   : ........
P_meridiana_CIP108465                   : ........
P_proteolytica_CIP108464                : ........
P_brenneri_CIP106646                    : ........
P_mucidolens_LMG2223                    : .......C
P_aeruginosa_LMG1242                    : .GGT..AG
```

Annex E – Phylogenetic tree obtained from the alignment of the concatenated sequence for the *rpoB* and *rpoD* genes shown in Annex D, with the "UPGMA" statistical method, "N° of differences" substitution model and "Complete Deletion" mode (No. of bootstrap replications: 1000).



Annex F - References

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