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**INNOVATIVE TECHNIQUES FOR SPORT TURF  
MANAGING: AGRONOMIC AND PHYSIOLOGICAL  
IMPLICATIONS**

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# **Innovative techniques for sport turf managing: agronomic and physiological implications**

by

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## **ABSTRACT**

The turfgrass-industry is a sector of great economical and scientific interest. Nowadays the sport turf and in particular the golf courses are considered the highest engineered grass ecosystems. An esthetic assessment and a perfect playability require preparation procedures and maintaining techniques that determine a substantial departure from a natural soil.

The growing substrate of the putting greens is considered a key factor for a healthy turf ecosystem. For this reason on the market there is a wide range of products based on natural stimulants aimed to increase the growth of roots, to promote the interaction in the rhizosphere and finally to raise quality and performance of the turfgrass. Actually detailed study on the effects of growth promoting bacteria and biostimulants on a professional sport turf are very limited.

This thesis aims to study the effectiveness of different microorganisms and biostimulants in order to improve the knowledge relative to the relationship between the beneficial microflora and root apparatus of sport turfs.

The research project was divided in three principal steps:

- 1) Initially, different commercial products based on biostimulants and microorganisms mixes were tested on a *Lolium perenne* L. essence grown in a controlled environment. Effective microorganisms (EM), plant growth promoting rhizobacteria (PGPR) and phytohormones solution were inoculated within a close hydroponic system utilizing sterilized sand as growing medium. The principal evaluated parameters were the habitus and the color of the plants, the biomass production and the length of leaves and roots. In addition were studied the capacity of colonization of microorganisms within root tissues and rhizosphere.
- 2) In the second step, on the basis of acquired knowledges, were developed two different biostimulant solutions based on effective microorganisms, mycorrhizae and humic acids. This test was conducted both on an *Agrostis stolonifera* putting green at the Modena Golf Club and within a growth chamber on a *Lolium perenne* L. essence. In this second trial the effects of the different treatments were evaluated analyzing morphological, physiological and agronomical parameters. In addition were studied the variation of the soil layers stratigraphy and the capacity of inoculants to colonize soil and root tissues. For the whole durate of

the trial all chemicals applications, usually utilized on a golf course's putting green during the spring and summer seasons, were suspended in order to assess the effectiveness of the inoculants for nutrition and control of pests.

- 3) In the last step of this experimental thesis, different microorganism mixes and biostimulants were tested on an experimental putting green in the Turf Research Center (TRC) (Virginia Tech, United States) in a real managing situation. The effects of different treatments were studied maintaining all chemicals and mechanicals managements scheduled during a sport season.

The growth chamber results showed how EM, PGPR and biostimulants may enhance the health of the turfgrass and promote the growth of the root apparatus. In addition the EM microorganisms that colonized the root tissues and the rhizosphere were efficiently isolated and characterized. PGPR confirmed their attitude to improve the elongation of leaves and roots while EM reduced the growth of both the apparatus. Physiologic characteristics such as leaves' colorations and dry/fresh ratio were positively influenced by microorganism's mixes.

The putting green trial conducted at the Modena Golf Club showed a high attitude of the mix composed by microorganisms and biostimulants to determine a general improvement of the physiological condition. In particular evapotranspiration, chlorophyll content and coloration of the turf surface were positively influenced. In addition the thatch was partially degraded by the microbial metabolism and the mineralized substances determined an indirect fertilization shown by an increased leaves production. No beneficial effects on the pests' preventions were detected.

The putting green trial conducted at TRC confirmed some results obtained in the previously research. As in the previous study, roots were stimulated by microorganisms and biostimulants. In addition were confirmed the positive effects on the color and quality of the turf surface utilizing both visual assessment and instrumental NDVI evaluation. Physiological determination such as proline content, transpiration and photosynthetic efficiency have further confirmed the positive effect determined by biostimulants and microorganisms mixes.

# **DEDICATION**

**To my wife, Laura**

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# 1. PREFACE

The turf represents an essential element for the constitution of the green spaces such as urban landscaping, recreational areas and as surface of sport facilities. Frequently the essences that constitute the turfgrass are posed in sub-optimal or in unnatural conditions without considering their real physiological necessity (McCoy, 1998). The main purpose of a sport turf is to ensure a range of features and aesthetics in order to optimize the playability of the game. Indeed, a sport turf has to guarantee aspects correlated at the sporting fruition such as the safety of the athletes, a correct rebounding and rolling of the ball but also different aspects regarding the visual appearance such as the greenness, uniformity, density and everything related a good quality of the green surface (Beard, 1973).

The sport turf is a poor biotic system composed by different mixes of grass posed on a relatively simple growing substrate. The soil is principally constituted by sand and coarse layers to ensure a properly drainage for each weather condition and a correct response to mechanical solicitations. This unnatural top-soil composition, associated to periodical chemical treatments is the principal cause of the deviation from an optimal natural situation for the grass essences.

In order to improve the growing conditions of the turfgrass there are a wide selection of biostimulants that are specific for the maintaining and recovering of the sport turfs (Karnok, 2000). These products are principally composed by organic substances such as sea weed extracts (SWE), humic acids (HA) or aminoacids. In addition others biostimulants based on plant growth promoting rhizobacteria (PGPR) aim to promote an *in-situ* production of phytohormone, antioxidants and an increased nutrient up-take.

The effective microorganisms (EM) belong to a heterogenic group of bacteria with several beneficial characteristics, similar but not traceable to PGPR (Javaid, 2009). This group includes bacteria able to promote the solubilize mineral nutrient otherwise inaccessible for the plants (De Werra et al., 2009), to promote the production of different phytohormone such as IAA, ethylene, cytokines and gibberellins (Lee et al., 2004), to parasite soil's pathogens and to accelerate the decomposition of lignin in the soil (Javaid et al., 2008; Raajimakers et al., 2009). Whereas effects of biostimulants were studied since the last decades making them convincing products in the turfgrass industry, the effective microorganisms for sport turf solution are not explored and their effectiveness is still largely unknown.

The main objective of this thesis was to improve the physiological condition of a sport turf, throughout the inoculum of effective microorganisms and biostimulants. In particular were tested different solutions in order to improve the growing of roots, the quality of the turf surface and the *in-situ* production of antioxidants and phytohormons. In addition were evaluated the effectiveness of the treatments considering different agronomical and physiological parameters such as evapotranspiration, content of photosynthetic pigments, variation in the rhizosphere composition and re-circulating of the nutrients.

An expected result of this research is to develop new microbial solutions specific for sport turf on the basis of the knowledge acquired and integrate these mixes in a management program.

## 2. INTRODUCTION

### 2.1 THE SPORT TURF

A sort turf is a high engineered grassy surface whose purpose is to allow the performance of the agonistic activities. The construction of a turf following the normative standards is a fundamental requisite for the enjoyment, the safety and playability at every level in which sport can be practiced. For these reasons, in recent decades research and scientific experimentation on this field of study has detached the sport turf from the concept of natural lawn in order to accommodate playing needs, functional and aesthetic requirements of the various outdoor sports. Nowadays the research on the sport turf sector receives high scientific and economic interest because constitutes the playing surface of the most spread outdoor sports and, as completing part of a large sector of entertainment, includes innovation of agronomic practices, studies of substrates, varietal selection, maintenance technologies, fertilizer and all the sciences related to interaction-athletes grassland. The list of sports played on a turf surface is long and variegated and includes disciplines with a great audience such as soccer, football, baseball and golf. A distinction between the various sports that use sport turf can be made in terms of wear and tear, depending the activities that take place above and the type quality, color and mowing-height that has to be maintained during the sport season. Another distinction can be made on the base of the use. Indeed some sports need a short-season playability and others need almost the entire year. A consequence of the durate of the sport-season will imply the choice of the essences utilized such as annual or perennial.

All athletic fields are subjected to intense traffic, represented by trampling of athletes, crossing of vehicles for maintenance and a multitude of environmental factor affect continuously on the condition of the turf as weather events, soil moisture (Beard, 1973). The sport turf, regardless the discipline, are widely different from a natural lawn and as consequence managing techniques are necessary to maintain stable this condition that is not stable and not self-sustaining in a natural condition.



**Figure 1:** Auburn hills golf club (Virginia)

### 2.1.1 THE GOLF COURSE

The golf belongs to a minority of sports that have not a standardized playing field. Any field in the world is different in its characteristics even if some elements can be found everywhere. The only constraint in a golf course is relative at the number of holes that has to be 9 or 18 and the total length that has to oscillate between a maximum of 6400 m and a minimum of 3700 m. An 18-hole golf course in the United States is generally constituted by a 0,8 – 1,2 hectares of putting green surface, 0,6 – 1,2 hectares of teeing area and 10 – 20 hectares of fairway surface (Table 1).

**Table 1:** Comparative turf use by area for representative 18-holes golf course

Turf Use	Area (hectares)	Area (%)
Rough-water-woodland	52,6	72,2
Fairways	16,2	22,2
Building-parking lots	2,1	2,9
Putting greens	1,01	1,4
Tees	0,93	1,3
Total area	72,9	100,0

Golf course paths must be designed in function of the landscape in order to ensure a degree of heterogeneity and showmanship during the course of the game. The golf clubs are usually divided into several areas: the playing field, the club house, which typically offers on-site dining, services and area of practice (practice or driving range).

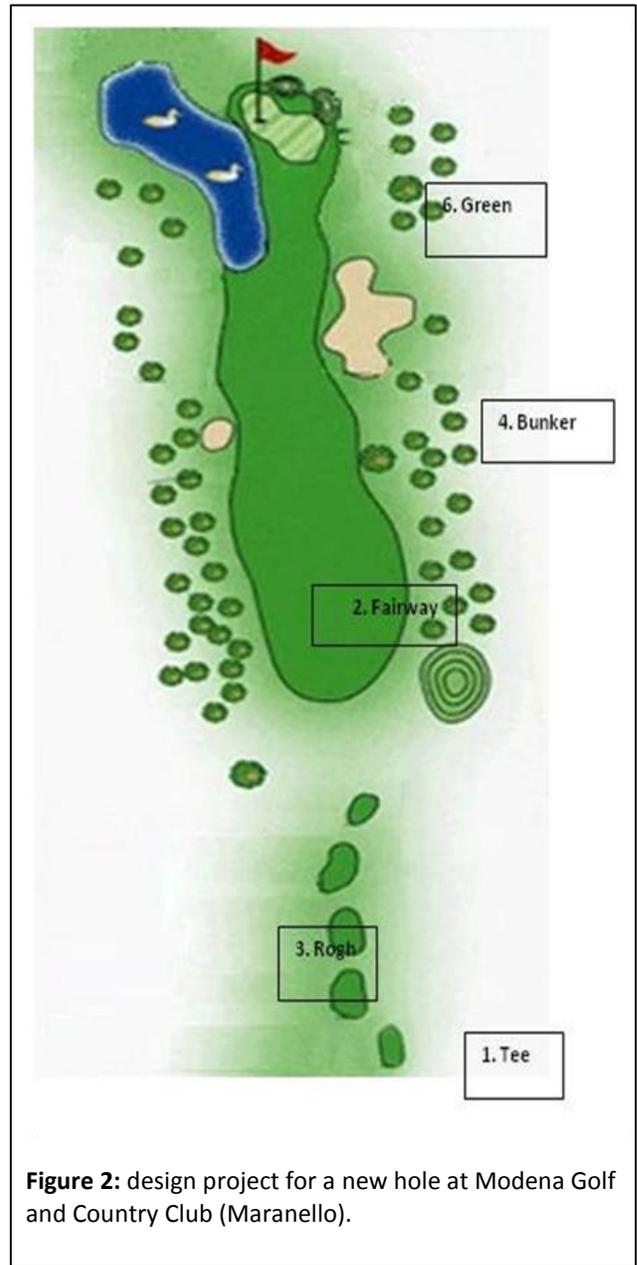
The area of the golf course is divided into different elements (Figure 2), which will be briefly described below:

- **Tees** or starting area is the area in which is played the first stroke of each hole, said tee shot. It is a flat area of several hundred square meters carefully shaved (1 to 1.5 cm) and dry, to have maximum stability and on which the golfer prepares the shot resting the ball on a tack, about 5 cm in wood or plastic, called tee. In the longer exist tees, generally four tee maker marked with different colors, according to the status (amateur or professional) and gender (male or female) to which they are dedicated.
- **Fairway** or paths is the course between the tees and the greens. It has cross-mown grass (1.5 - 2.5 cm) which shall have good density, uniformity and flexibility so as to facilitate the ball game. These areas have an extension that can go from 90 to 550 meters, while the width varies between 25 and 55 meters.

- **Rough** or uncultivated area is formed by tall grass, almost uncultivated, located outside of the fairways. This area constitutes a penalty for scoring incorrectly the previous shot.
- **Bunker** or sand trap is pits of varying sizes, filled with sand, with the function of making the game more difficult and challenging.
- **Water hazard** is a made up streams, river, pool, pond, lake and sea. It can be natural or artificial and it is built to make more difficult the approach to the hole.

- **Green** or putting green or pitch of arrival is the area of arrival of the game (Figure 3). This surface of the putting green is finely shaved (20 to 48 mm height) to allow a perfect rolling. This surface must achieve the maximum uniformity, homogeneity, strength and elasticity.

The size of this area is proportional to the length of the approach shot and on average remains around 600 m<sup>2</sup>. The green have slopes more or less pronounced, both to drain the most of the water (since the drainage must be perfect), both to make the putt of greatest difficulty. The position of the hole (Ø 11 cm) is not fixed, but is continuously moved (1 per month) for two principal reasons: to make the playability putting green always different and in order to prevent excessive damage from foot traffic in the same area .



**Figure 2:** design project for a new hole at Modena Golf and Country Club (Maranello).



**Figure 3:** Putting green at the end of the hole n° 3 of the Modena Golf & Country Club

## 2.1.2 GRASS ESSENCES OF A GOLF COURSE

### Grass essences of a tee

Tees are principally sown with *Agrostis stolonifera*, *Cynodon* spp., *Zoysia* spp., or by a mix of *L.perenne*, *F. rubra* and *P. pratensis* (Panella, 2000). The choice of seed depends by the latitude, the composition of the soil and by the intensity of playing. Generally tees are mowed 3-4 time per week and may be occasionally replaced with over-seeding or deposition of sods due to frequent shots that remove portion of grass.

### Grass essences of a tee

Fairway's grass essences are represented *Cynodon* spp., *Agrostis* spp., *L. perenne*, *Zoysis* spp., *Paspalum vaginatum* or by a mix of *L.perenne*, *F. rubra* and *P. pratensis* (Panella 2000). Due to the high managing cost of a *Agrostis* only fairway, it is preferred the choice of a mix composed by *Poa pratensis*, *Festuca rubra* and *Agrostis* spp. Generally the fairways are mowed 3-4 time per week.

### Grass essences of a putting green

The grassy essence used in most of putting greens is the *Agrostis stolonifera* (Figure 4). It is a perennial cool-season C3 turfgrass belonging to the family of Poaceae and Genus *Agrostis*, a large genus of plant that counts more than 100 species. *Agrostis stolonifera* is characterized by a typical prostrate habitus, with a strongly and vigorous stolons which developing on the surface of the soil, allowing the initiation of new roots and new culms at each node. These characteristics give the possibility to obtain a low mowing height, between 2 – 4 mm, and a dense mat to cover uniformly the whole surface of the putting greens. In the sport fields this variety is the principal grass specie of the golf putting in Europe and United States, although in the south climate during

summer with high daytime temperature and warm nighttime temperatures could bring to adverse conditions. For this reason in the south regions putting green is principally utilized a warm –season C4 turfgrass the hybrid of Bermuda grass [*Cynodon dactylon* L. Pers. X *Cynodon transvaalensis* Burt Davy].



**Figure 4:** *Agrostis stolonifera* L. grown for a growth chamber trial at Virginia Tech (Virginia) and in nature.

In nature, the chorological element of *Agrostis stolonifera* is circumboreal and can be found in on edges of ponds and marshes, banks and uncultivated wet from a height of 0 m to more than 2000 meters over the sea level. *Agrostis stolonifera* L. has a height that can reach 20 – 50 cm, creeping culms with rooted nodes and epigeal stolons. The leaves have blade wide 4-6 mm and with 10-12 nerves.

**Table 2:** Agronomical characteristics of *Agrostis stolonifera*

PARAMETERS	CHARACTERISTICS
Texture	On average - fine
Density	Excellent
Propagation	Seed, stolon, sod
Color	Light green – slightly gray
Leaf shape	2-3 mm width, tip spired, pronounced ribbing on the upper page
Soil	Highly fertile, irrigated, fine texture, well-fertilized, pH 5,5/6,5
Settlement	Low
Potential recovery	Moderate
Tolerance to wear / traffic	Low
Tolerance to hot	Moderate
Tolerance to cold	High
Tolerance to drought	Low
Tolerance to shade	Low-Moderate

Tolerance to drowing	Moderate
Tolerance to sality	Moderate
Tolerance to pathogens	Very low
Management required	Very low
Mow height	3-15 mm
Nitrogen necessity	0,35 / 0,65 kg – 100 m <sup>2</sup> each 30 gg
Water needs	Very high

### 2.1.3 THE SOIL OF A SPORT TURF

The soil is a complex mixture of gasses, liquids, minerals, organic matter and a myriad of micro- and macro-organisms. In the turf science the soil is essential for the development of the turf, because it is subjected to intense constraints dictated by the technical requirements. It's primarily functions are to stock nutritive substances, maintain a certain porosity and air fraction, allowing a proper water drainage for a physiological growth of the turf and an ideal sport fruition. The air fraction has a fundamental role in the supplying oxygen to the roots and accelerates the microbial degradation of organic residues which tend to accumulate, causing the thickening of the thatch. The microbial component in the soil plays a central role in improving the physical and chemical properties in the rhizosphere such as the density and structure of the soil, the degradation of the organic substance and the availability of nutrients (Huhta, 2007).

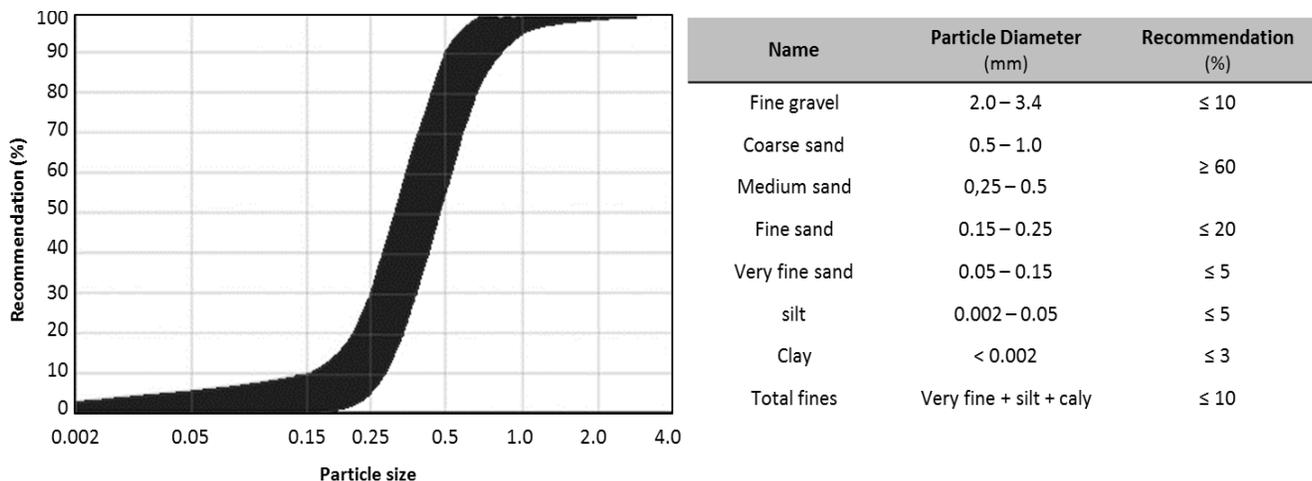
The percentage of sand, silt and clay constituting the growing medium influence the characteristics of the mechanical and physiological response of the turf, directing towards to one or another one specificity of utilization. An important feature in sports fields, that must ensure a proper rebound of the game items such as balls and in some contact sports the fall down of the athletes, is the response to mechanical compression. The ground must be able to withstand intense actions of exploitation without losing its mechanical characteristics.

### 2.1.4 THE SOIL OF A PUTTING GREEN

USGA, in the past 30 years has evolved the guide lines about the composition of putting green soils, in order to bring to a surface always playable, according to the highest aesthetic standards and discern the response to the mechanical stresses of the game (USGA Green Section Staff, 1993).

The USGA guide lines for the construction of putting green growing medium, in the past 30 years has evolved into artificially constructed soils, built from a predetermined mixture of 80 – 90 % sand and 10 – 20 % organic matter (Figure 5). The USGA specifications indicate a soil stratigraphy composed by a topsoil mixture with a minimum thickness of 30 cm, lying over a 2.5 – 5 cm of a coarse sand layer, which is above a 10 cm layer of

washed pea gravel with a particle size of 0.5 – 0.75 cm, which covers other 10 cm tile drain imbedded in the subgrade of native soil.



**Figure 5:** Recommended standard granulometric curve for growth medium of a golf's putting green. USGA

The sand component should be contained at least 92%, with particles size suggested by USGA standard comprised between 1.0 to 0.10 mm, and the remaining fraction composed by less than 5% silt, and less than 3% clay. Clay content is always maintained in a minimum percentage, above all because clay is highly implicated in the reducing of cation exchange capacity (CEC) and in the increasing of water retention (Bigelow et al., 2004). Currently the putting greens of golf courses, including those present at the Modena Golf & Country Club, are basically constructed according to the USGA directives, which in most cases provide sufficient water retention of the substrate and a good penetration of the roots grass, reducing problems due to compaction.

### The organic matter

The organic matter content in putting green topsoil should be around 10 – 20%. The organic matter, even if present in small percentage, has a great importance both for chemical and for physical characteristics of the soil. Indeed it promotes the growth and development of turf essences, is a reserve and propagation of soil microorganisms, and has a significant importance in water retentions and movements in the soils and the availability of nutrients. Regarding the chemical properties, the organic matter acts as a reserve of nitrogen, sulfur and a part of phosphorus and promotes the absorption of other nutrients for the increase of the cation exchange capacity (CEC). In regard as physical characteristics it improves the soil structure, increasing the cohesion soil particles and increasing the water absorption capacity. In a sports field, the addition of organic matter can occur naturally by the deposition of foliar residues mown and the replacement of the roots, or by

artificial application with seasonal treatments in the topsoil mixture utilizing peat moss, reed sedge peat or other well-degraded organic matter (USGGA Green Section Staff, 1993)

### **The C:N ratio**

The Carbon Nitrogen ratio (C/N ratio) provides useful information about the kind of organic matter present in the soil and the degree of mineralization of the nitrogen. It is directly correlated with the activity of microorganisms that feed by N and expel CO<sub>2</sub>. If the ratio is less than 20 there is an excess of nitrogen compared to the necessity of a good humification. This excess is released during demolition of organic matter by microorganisms and will become part of the mineral component of the soil. If the ratio is high (more than 30-35) the soil contains a little amount of nitrogen, which will be fully utilized and as consequence immobilized by the metabolisms of the microorganisms. For values between 20 and 30-35, there is a situation of equilibrium and the nitrogen present in the residues is totally used in the process of humification. Therefore, there is no release or immobilization. When C:N ratio arrives at values around 10, it means that the process of humification is finished.

## **2.1.5 ROOTS AND THATCH OF A SPORT TURF**

### **Roots**

The ability of the plants to absorb water and nutrients from the growing medium is related to their ability to develop a root system. Roots are the primary apparatus for the absorption of all mineral elements necessary for the plant growth and their development and extension in the soil is strongly influenced by the patches of nutrients as nitrogen and phosphorus in the soil (Glinski and Lipiec, 1990; Durieux et al., 1994).

The importance of the construction of a efficient root apparatus is supported by the 33% of global annual net primary production, used for finer root production. In addition, more than 50% of daily photosynthetic production may be utilized in the growth and maintenance of a root apparatus (Lambers, 1987).

High mowing frequency at 2-3mm height of creeping bentgrass (*Agrostis palustris* H.) is one of the principal reasons of the low growth of the root system in the golf course putting greens (Jordan et al. 2003; Shepard, 2000). A deeper root system with extensive root branching is considered an important characteristic improve resistance at drought stress favoring the absorption of water (Russel, 1977) and a lower attitude to resists at environmental stress induced by a shallow rooted turf is largely reported (Jordan et al. 2003).

This roots concentration in the upper portions of soil profile does not allow a prolonged capacity to supply water and as consequence frequent precipitation or irrigation are necessary to avoid drought stress.

## Studies concerning roots in the sport turf industry

In turfgrass industry the root development and as consequence the plant health is a primary topic. Each year new growth promoters are formulated to ensure a more efficient radical development, and this concerns especially the varieties used in the putting green areas. The possibility to develop and use plant varieties with an improved root growth and a higher drought-resistance has gradually reduced the water consumption but the argument remains still of great interest. Nowadays the sport turf industry is developing some fertilizers containing precursors to natural phytohormones, and biostimulants such as microorganisms, sea weed extracts and humic acids. Many researches describe the *Bacillus* strains capacity to produce phytohormone (e.g. indole-3-ethanol) in a non-toxic form. This kind of interaction between plant-microorganisms responds more effectively to the metabolic needs and development of the plant in different growth phase, being metabolites synthesized directly in the rhizosphere.

## Thatch

The term thatch is referred to a layer of plant material, dead or alive, not decomposed that positioned between the aerial parts of the plant, immediately below the turf surface and resting on the layer of growing medium underneath (Figure 6).



**Figure 6:** Thatch layer highlighted between two yellow lines. Picture taken at Modena Golf & Country Club.

This layer adversely affects the health and metabolism of the turf, because it goes to alter the gas-water exchange between the atmosphere and soil. Additionally, it facilitates the spread of fungal diseases (eg. *Sclerotinia homoeocarpa* and *Rhizoctonia* spp.) and parasites due to the formation of an optimum microclimate for the development of pathogens; being the felt layer practically hermetic, is also reduced the effectiveness of fungicides and plant protection products administered for the control of these diseases. Another feature of the felt, is that favors the loss of anchorage of the turf, as, retaining water inside it facilitates a surface development

of the radical, thus leading to a carpet less stable and more prone to deterioration. Finally, the formation of shallow root system makes the turf more susceptible to damage from extreme temperatures and/or drought.

The main causes of formation of thatch are:

- the excessive levels of nitrogen fertilization that determine a greater vigor of the epigeal part which, once cut, will go to increase the layer of thatch;
- Reducing condition and acid pH are condition that may reduce the activity of soil microorganisms and, therefore, the decomposition of the undecomposed organic matter;
- Infrequent cuts and excessive cutting heights, together with abandonment in the field of grass clippings after cutting.



**Figure 7:** Scheme of a mechanical aerification and an aerification performed at TRC (Turf Research Center, Virginia Tech).

To contain / decrease the felt layer, can be made different operational choices. With the preventive method, pretty common, you take all those agronomic practices that promote the decomposition of organic matter, such as topdressing, pH correction by liming, unpack the soil, the containment of carbon / nitrogen ratio of 25-30: 1. For curative method, however, means the mechanical removal of the felt through the practice of aeration (Figure 7) (or verticutting), or, alternatively, may be used another technique such as coring.

### 2.1.6 QUALITY OF GOLF COURSE'S TURF

The quality of turf is essential to optimize the playability and to allow at the player to use the lowest amount of shots during the competition. A correct maintenance plays a key role to eliminate as much as possible the negative factors that interfere with the success of the match. The essential qualities of a good golf turfgrass are:

- **Uniformity** - the playing field should be as uniform as possible, especially on the green's surface in order to have a proper sliding of the ball, but just as important on the tees to provide a stable base to the player during the swing.
- **Density** - it is expressed as the number of plants per unit area. The density of the mat provides a perfect support during rolling. This characteristic is achieved by regular maintenance, such as a higher frequency of cutting heights with the use of optimal grassy essences used and proper management of fertilizer and irrigation.
- **Weaving** - it is dependent on the width of the leaf blade. This feature influences the rolling of the ball, which is optimal when the texture of a green is medium-fine. This quality can be obtained by the choice of adequate turf essence, the height of the mow and from the practice of topdressing.
- **Smoothness** - this parameter is a quality index for a field that does not have damage, obstructions or depressions which may affect the run of the ball. This is a characteristic has to be preserved by weed, pests diseases and other fungal or pathongens.

#### Quality and color of the turfgrass

The aesthetic parameters such as the quality and the color of the green surface are the most important in the maintaining of a golf course, especially for the evaluation of a golf course's putting green.

The color parameter considers the mean coloration of the green surface and acquire a high value when leave have greener/darker coloration. The quality takes in consideration a range of factors affecting the visual appearance of the grassy surface.

Turf quality of a putting green often decline during summer when golf putting greens are subjected to an high human traffic and seasonal stresses such as high temperatures and intense solar radiation (Lucas, 1995; Carrow, 1996).

#### Visual assessment

The evaluation of visual parameters such as quality and color is routinely used to assess aesthetic characteristic of turfgrass cultivars. Quality and color rating is based on a relative evaluation between the same turfgrass specie within the same season. Indeed quality and color rating may change significantly depending on the seasons or agronomic practices that are carried out. The visual assessment of the turf surface, as most as aesthetic rating

collected on turf grass, is based on a 1-9 rating scale as reported in the guide lines of National Turfgrass Evaluation Program (NTEP), an approach that is accepted and used since many years. The NTEP guide line is a necessary support for researchers and practitioners to evaluate turfgrass health and to take management decisions.

The visual evaluation traditionally has always been a subjective method that requires good personal practices and sometime may happen that data can be quite variable and difficult to reproduce due to different evaluation techniques and personal interpretation (Trenholm et al. 1999). This kind of valuation is very useful for golf course superintendents, sports turf managers, sod growers, lawn care service operators and ground managers in order to study which adjustments, or seed varieties utilize in the different seasons.

Rating systems are commonly employed to evaluate phenotypic variation in colors, density and uniformity of turfgrass stands in year. Therefore, quality and color are key components for water and nutrient status often utilized also in growth chamber or greenhouse trials (Xu and Huang, 2000).

The rating of turfgrass quality is evaluated 9 for the perfect and ideal turf surface and 1 for the poorest quality aspect. A rating of 6 is generally considered acceptable but for putting greens quality standard would be better do not fall below of 7. Quality ratings are not based on color alone, but on a combination of color, density, uniformity, texture, and disease or environmental stress. The rating of the color represents a relative evaluation of the greenness of the turfgrass surface and is inherent to the genotype color variation affected by different treatments and soil or environmental conditions that act on the experimental plots.

This kind of measure, subjected to criticism for time consuming and subjective characteristics. In the past several techniques were used to measure objectively the color such as reflectance measurements (Birth and McVey, 1968), Chlorophyll and amino acid analysis (Nelson and Sosulski, 1984) and comparison with standardized colors (Beard, 1973) but all these method have the advantage to be relatively expensive and to be time consuming.

### **Instrumental assessment**

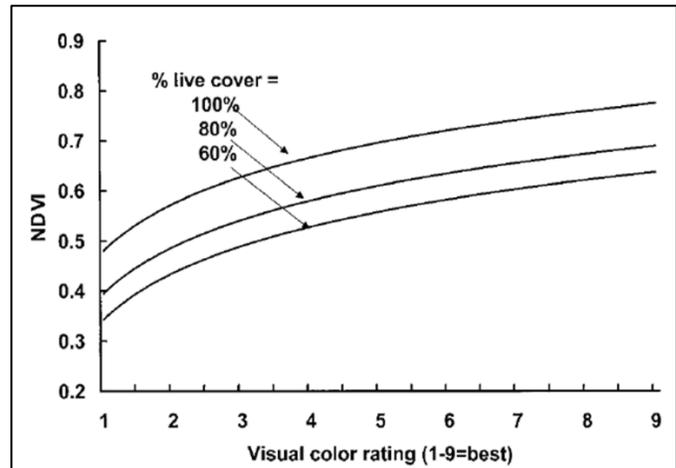
Nowadays specialized instruments for the turfgrass color analysis allow to slide out from a subjective rating and lead to an objective approach to make color and health evaluation and make management decisions.

New instruments that utilize the spectral proprieties of the plants, give the possibility to analyze the color proprieties of the turf surface with a no time consuming method.

Remote sensing technologies that utilize the visible infrared (VIS) and near infrared (NIR) light, measure with optical sensors the irradiance reflected from turfgrass over which they travel (Bell et al., 2002).



**Figure 8:** Vehicle-mounted optical sensing utilized at Virginia Tech. Crop Circle ACS-470.



**Figure 9:** relationship between the NDVI revealed with a vehicle-mounted optical sensing and 1-9 rating by visual assessment (Bell et al. 2002) in *Agrostis stolonifera*.

The Crop Circle ACS-470 (Figure 8) is an active crop canopy sensor that provides NDVI vegetation index. The Crop Circle is not limited by ambient lighting conditions because this instrument remove the effect given by the differences of light by time of the day and weather condition removing the effects of ambient radiation, processing only the radiance emitted by the integrated source.

Optical sensors detect the returning irradiance emitted by the instrument and reflected by turf surface at red radiation (600-700 nm wavelength) and at the near infrared radiation (700-800 nm wl). VIS reflected by plants is very low if compared with a soil surface because efficiently absorbed by photosynthetic tissues of leaves (Knipling, 1970, Tucker, 1979). NIR radiation is poorly absorbed by photosynthetic tissues and more highly reflected (Daughtry et al., 1992). The relationship established between these two different wavelengths, collected by the surface of leaf plant arterial, is the basis for a relatively simple calculation necessary to obtain the normalized difference vegetation index (NDVI). The NDVI algorithm is measured as follows:

$$NDVI = (NIR - VIS) / (NIR + VIS)$$

Normalized difference vegetation index was widely utilized to determine chlorophyll content (Howell, 1999; Zhao et al., 2003), turf injury and quality (Trenholm et al., 1999; Bell et al., 2000). In addition NDVI parameters was studied for predictive models relating to plant biomass, plant nitrogen content, stress severity, drought stress and nitrogen deficiency. Bell et al. (2001) with a vehicle-mounted optical sensing, reported a strong correlation between the visual color assessment and the NDVI instrumental approach (Figure 9). That study was conducted on tall fescue and creeping bentgrass that shown respectively a coefficient of determination of 0.8 and 0.5 respectively, a good result considering the heterogeneity of the visual prediction model on which the comparison was based on.

## 2.1.7 PATHOLOGIES AFFECTING A GOLF COURSE

Creeping bentgrass (*Agrostis stolonifera*) has gained the endorsement of superintends and athletes for its characteristic of density and quality of the turf surface. The interest for this specie is specie was increased in United States and Europe in the last two decades (White, 1996). However this specie is a cool season grass and suffers warm and drought condition that may occur in the hot and dry summer in the south of United States and Europe. Summer conditions often reduce the quality of color and quality of the grassy surface and in addition these are the precursor of diseases onset (e.g. fungal pathologies) and common physiological stresses (Lawlor, 1995). Environmental stressful condition such as water deficit, high irradiation and high temperature increase the oxidative stress that can cause loss of vigor to the plant tissue, cellular damage and a reduction of shoot growth. Less tolerance of summer condition in creeping bentgrass is strongly correlated with a shallow root system and an excess of shoot growth determined by a fertilizer and irrigation over applying to reduce the loss of aesthetic quality (Hull, 1992). An interruption of electron transport along the photosynthetic process, determined by an increase of the stress induced on the plant as solar irradiation and high temperature, causes an increase in reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, hydroxyl anions and singlet oxygen (Scandalios, 1997; Smirnoff, 1995). The removal of ROS can be implemented by superoxide dismutase, an efficient enzyme for the removal of superoxide anions (Bowler et al., 1992). Has been shown that seaweed extract (*Ascophyllum nodosum*) abbreviated as SWE and humic acids (HA) have an radical scavenging activity (Zhang,1997).

### Dollar spot

Dollar sport is a serious creeping bentgrass disease of golf course caused by *Sclerotinia homoeocarpa*, a fungal pathogen member of Sclerotiniaceae family. To measure its global spread, in 1992, the golf course industry in the United States spent around \$ 56.5 million on fungicides trying to control the dollar spot fungus (Smile et al, 1993). The fungus incites foliar blight leaf tissues with the mycelium forming round yellow-light brow patches with a 1-5 diameter size (Figure 10).



**Figure 2:** dollar spot pathology affecting a putting green at the TRC (Virginia Tech).

The grass species mainly affected are *Agrostis stolonifera*, *Poa annua*, *Poa pratensis*, *Lolium perenne* and *Cynodon dactylon* (Couch, 1985; Smile et al., 2007). Dollar spot proliferation occurs in a period between spring and autumn, when temperature range from 18 and 33°C during and nights are humid and around 10°C. The difference of temperature from night and day and the dew formation on leaves determine the propagation of the disease (Smile et al., 2007). Control of dollar spot can be performed with agronomic practices, through a less frequent irrigation, avoiding irrigating at night and removing the dew from the leaves in the early hours of the day. Being the occurrence of dollar spot strongly correlated with moisture in the soil, removal of felt or other treatments implement to increase evapotranspiration can guarantee a lower potential for occurrence of the disease. The golf courses and especially the putting greens always require an aesthetic quality close to excellence, so at the first manifestations of this disease is necessary to act with chemicals to eradicate the presence of dollar spot. Many kind of fungicide are labeled to control dollar spot such as including benzimidazoles, carboxamides, nitriles, dicarboxamides, and demethylation inhibitors. During the last decade certain strains of dollar spot showed the earliest forms of tolerance against chemical compounds, it is good practice to rotate them frequently to prevent this from happening (Smile et al., 2007). Alternative method to prevent the occurrence of the dollar spot, have been studied in 90s with poor results. The utilization of effective microorganisms as antagonistic microorganisms for a biological control of soil disease was reported in many works by Lin (1991) and Higa (1994). Kremer in 1999 reported a 4% inhibition of the *S. homoeocarpa* growth in plots pre-treated with effective microorganisms.

### **Brown patch**

Brown patch is a common pathology affecting the golf courses caused by the fungal parasite *Rhizoctonia solani*. It is a fungal disease that occurs during early summer and autumn, indeed its propagation rises for temperature between 22-28°C and high air humidity. With temperatures over 30°C its propagations it suffers a drastic downturn. This disease can occurs in the sports fields maintained with a low mow height that have a high concentration of nitrogen in the soil and show case of waterlogging (Figure 11). *R. solani* rises throughout round or irregular patches (2 cm – 2 m diameter), and grass affected by this disease presents a light brown coloration.



**Figure 3:** On the left, *Rhizoctonia solani* affecting a fairway. On the right, *R. solani* hyphae (Wikimedia Commons).

Control of *R. solani* can be performed with agronomic practices, through balanced fertilizations of nitrogen, good levels of phosphates and potassium in order to increase the plant's defenses, decrease the humidity present in the top-soil and on the leaves especially in the shadow areas and a correct managing of the thatch. The active substances utilized for the chemical control are the Tolclofosmethyl, iprodione and the pyraclostrobin.

Some previously works showed an effectiveness of some microorganisms in the control of this pathology. For example *R. solani* inhibitions was showed with bacteria of the rhizosphere such as *B. subtilis* and *B. lentimorbus*. These bacteria resulted effective for the in vitro control of growth of *R. solani* isolate (Montealegre et al., 2003)

### **Pythium blight**

Pythium is a fungal pathology that rises in golf courses during summer and early autumn (Figure 12). The infection occurs between 29 – 35 °C. All microterms and some macroterm (especially *Cynodon* sp.) are the essences affected by this pathology in the sport turfs. Badly ventilated sport field that have soil with a high humidity are more subjected. At first stages the disease presents with circular spots that may vary from 2-3 to about 15 cm diameter. The plants affected appear dark, brown, and greasy and may be covered by a mass of white cottony mycelium. The insurgence of pythium can be reduced by a properly agronomical maintenance such as balanced ferti-irrigation, a reduction of the waterlogging, the removal of thatch in excess and increasing the air exposition. Some previously research demonstrated the effectiveness of bio-control in reduction of pythium insurgence. Indeed as Loper (1988) showed the capacity of *Pseudmonas fluorescens* to reduce the colonization and the pre-emergence caused by *Pythium* sp.



**Figure 4:** Pythium disease at the Auburn hills golf club (Virginia)

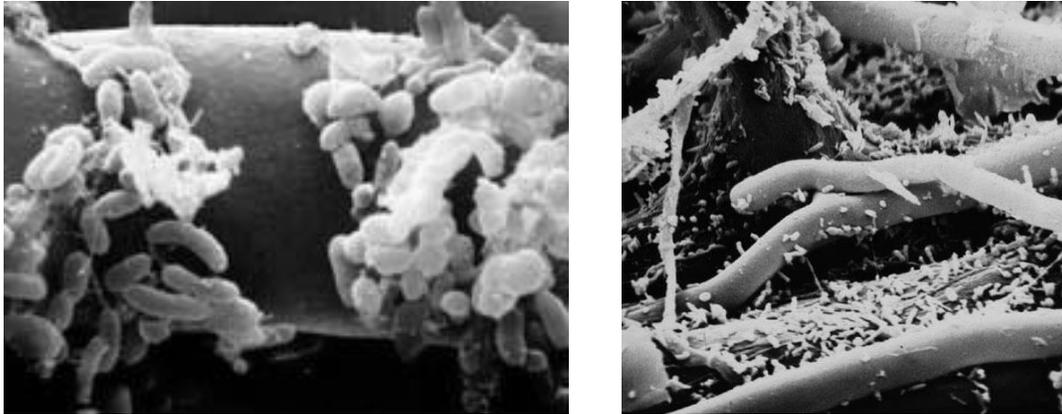
## 2.2 MICROORGANISMS OF THE SOIL

The soil microorganisms play a central role in many edaphic processes and establish complex interactions between them, other organisms and plants (Figure 13).

Although the study of the micro fauna in agriculture is a topic of great interest since many years, in the sport fields it is finding interest in commercial and scientific area only recently. Indeed the micro-fauna stands out as a determinant for carrying out biotic soil interaction; which explains why the quality of the soil depends primarily on its microbiological activity (De Luca, Picione, 2009). It also represent the most abundant biotic component in soil and is estimated that in a gram of soil there are hundreds of millions of fungi and bacteria, of which only the 5% in know (Uphoff et al., 2006).

Bacteria and fungi have a key role principally in the rhizosphere where they are primarily related to the maintenance of soil functions. These microorganisms are in fact involved in many processes such as the formation of soil structure, decomposition of organic matter, removal of toxic substances and recycling of the elements. They are also involved in the control of diseases by impoverishment of the soil due to high management practices and changes in vegetation (Garbeva et al., 2004).

The main groups of organisms constituent rhizosphere are bacteria and archaea, fungi, actinomycetes and algae. Bacteria and archaea are most abundant (up to  $10^9$  UFC – Unit Forming Colony per gram of soil), actinomycetes and fungi count respectively  $10^6$ - $10^7$  UFC/g soil, while algae count  $10^5$  UFC/g soil (Paul and Clark, 1996).



**Figure 13:** On the left are represented bacteria on a root ([www.indiana.edu](http://www.indiana.edu)). On the right , phungal iphae in the soil ([www.soils.usda.gov](http://www.soils.usda.gov)).

### 2.2.1 MICROORGANISMS IN SPORT TURF INDUSTRY

In the management of sports fields, the use of microorganisms is still a not totally explored area of study but of growing worldwide interest. Natural and undisturbed soil contains high micro-organism population but USGA based sand soil, the commonly utilized standard for the construction of golf turfgrass, do not allow a rich population of microorganisms (Nunan, 2003). In order to obviate this deficiency managers and green keeper utilize a variety of artificial method and commercial products as biostimulants and ammendands.

Several published works has confirmed the positive effects given by microbial inoculation associated with turf grasses, especially in golf industry where there is the concern that these kind of turf system are not sustainable, due to intensive management (kind of soil, fumigation, pesticide,) that reduce significantly the microbial population.

Depending on the mode of action and effects, these products can be used as bio-fertilizers plant strengtheners, phytostimulators and biopesticides (Berg, 2009). The principal positive aspects given by bio-fertilizers are the improved acquisition of nutrients and in-situ hormonal stimulation.

Golf courses' putting greens need a management similar to cropping systems for intensive applications of pesticides, fertilizer and irrigation. Newly putting greens undergo a soil pretreatment that consist in fumigation with methyl bromide before sowing. This application allows destructing weed seeds, parasitic nematodes and common Bermuda grass (*Cynodon dactylon*) but reduces tragically the microbial population (Elliot and Des Jardin, 2001). Although, successive studies demonstrated how after fumigation the population of microorganisms is able to rebound (Elliot et al. 2004)

A new concept of turfgrass is required not only to avoid chemical treatment abuses and all correlated problematic, but also to ensure an healthy turf surface more resistant to abiotic stress as drought and biotic as

fungal pathologies to reduce the cost management that that nowadays are a crucial step in the management of sports fields.

### **2.2.2 PLANT GROWTH PROMOTING RHYZOBACTERIA (PGPR)**

The term PRPG (Plant growth promoting rhyzobacteria) was coined in 1978 by Koepller and Schroth to indicate a group of rhyzobacteria able to promote significantly the growth of plants. Podile and Kashore in 2007 defined rhyzobacteria able to colonize aggressively the rhizosphere of plants but only a part of them (2-5%) is also able to promote the plant growth (Antoun and Koepller, 2001).

Different kinds of bacteria belong to PGPR, in particular *Bacillus* and *Pseudomonas* genre are the predominant (Podile and Kishore, 2007; Singh et al., 2011).

These microorganisms are able to promote directly the growth of plants establishing symbiotic associations with the root system, and releasing growth regulators or plant hormones such as auxins, cytokinins, gibberellins, ascorbic acid and ethylene (Arshad and Frankenberger, 1998).

PGPR exert their action on the main growth parameters, on the health and on the productivity of the plants in general as a result of multiple mechanisms, closely dependent on the species of rhyzobacteria involved (Singh *et al.*, 2011). PGPR main functions may be divided in six groups:

- Phytohormone stimulations;
- Solubilization of inorganic phosphates;
- production of siderophores that chelate iron making it unavailable to pathogens (Glick et al., 1995)
- Volatility of the compounds that affect the regulation signals;
- Control of the harmful microorganisms;
- Competition for space, nutrients and induction of systemic resistance against a broad spectrum of pathogens.

### **2.2.3 EFFECTIVE MICROORGANISMS**

EM (Effective Microorganisms) is another microorganism's group with several beneficial characteristics, similar but not traceable to PGRP (Javaid, 2009). The effects of effective microorganisms have been shown for the first time in the 60s by Professor Teruo Higa (University of the Ryukyus, Okinawa, Japan). Higa discovered the capacity to grow in the same solution aerobic and facultative anaerobic microorganisms, without compromising the mutual growth or induce degenerative processes. EMs are able to improve growing conditions of plants (Gaggia et al., 2013) stimulating the photosynthetic process, producing bioactive substances such as

phytohormone and vitamins (Desoky I.M. et al., 2001); controlling soil disease and accelerating the decomposition of lignin in soil (Javaid et al., 2008).

### **EM – growth promotion in plants**

Many studies report the capacity of bacteria to produce phytohormone precursor or stimulate the production through metabolic cross-talk in the plant (Lee et al., 2004). Plant hormones are naturally synthesized by the plants but the interaction with different kinds of soil bacteria stimulates the production that may take place also directly in microorganisms as exudates. The phytohormone of which has been reported the production upon interaction with microorganisms are indole-3-acetic acid (IAA) (Lee et al., 2004), ethylene (Pierik et al., 2006), cytokinins and gibberellins. Some bacteria pertaining to *Azospirillum* and *Pseudomonas* sp. showed the ability to acidify the growing medium and as consequence to solubilize mineral nutrient such as organic phosphate otherwise inaccessible to the plant (De Werra et al., 2009).

### **EM – Beneficial effects**

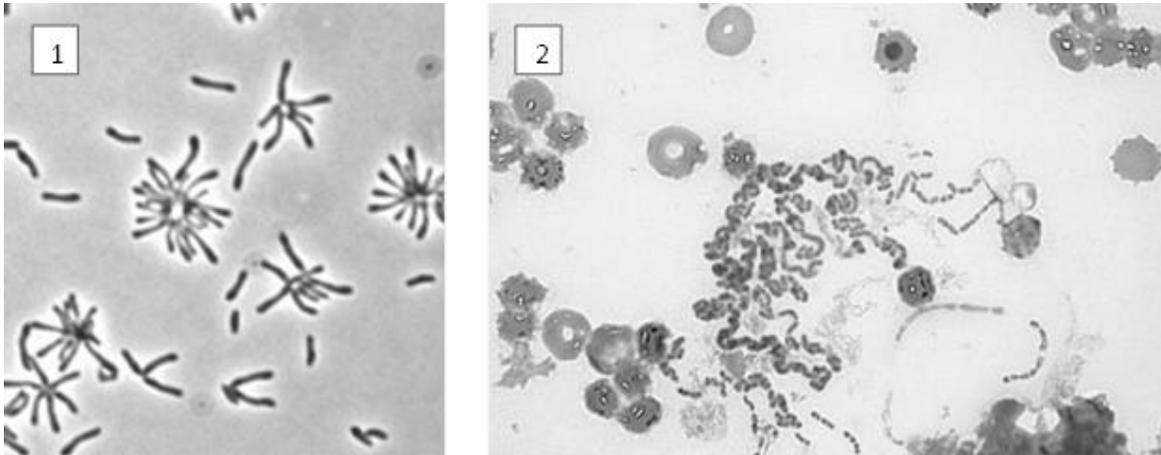
Effective microorganisms' effect may derive both from a direct interaction host plant and also indirectly due to their antagonistic activity against plant pathogens (Berg, 2009). Has been reported that EMs' allopathic effects derives from secondary metabolites as antibiotics, antifungal, antiviral, insecticidal and immune-suppressant agents (Ryan et al., 2008). In addition, inoculated EMs may parasite bacterial pathogens through production of extracellular cell wall-degrading enzymes such as chitinases and B-1,2-glucanase (Raajmakers et al., 2009).

EMs applied with a pre-treatment in creeping bentgrass plots, shown a 4% inhibition effect of the occurrence of the dollar spot (Kremer, 1999). Other turfgrass disturbs that may result from fungal, bacterial, and viral attack has been demonstrated as can be reduced through inoculation with bacterial / fungal entophytes (Sturz et al., 1996; Kerry et al., 2000; Berg et al., 2006).

### **EM – Main microbial groups**

Effective microorganisms include more than 80 microbial species but the main microbial groups are photosynthetic bacteria (*Rhodospseudomonas* and *Rhodobacter* spp.), lactobacilli (*Lactobacillus* spp. and *Streptococcus* spp.) yeast (*Saccharomyces* spp.) and actinomycetes (*Streptomyces* spp.).

## Photosynthetic bacteria



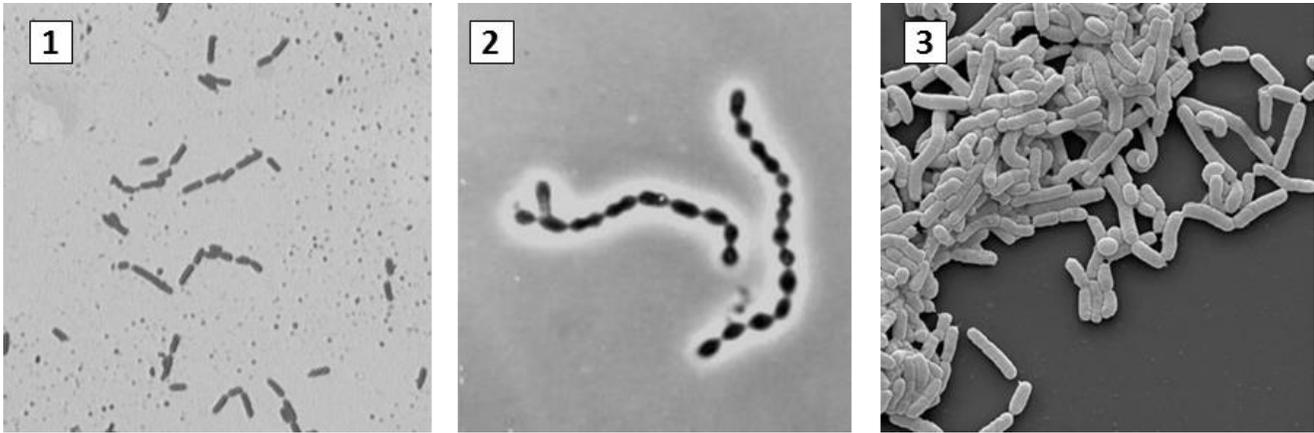
**Figure 14:** Two photosynthetic bacteria belonging to EMs' group: *Rhodospirillum rubrum* (1), *Rhodospirillum rubrum* sp(2).

The photosynthetic bacteria (also known as phototrophic bacteria) utilize solar energy to metabolize the organic and inorganic substances (Figure 14). This phototrophic potential is useful particularly in the environmental field, because they are able to effectively decompose the organic materials. Photosynthetic bacteria includes microorganisms belonging to different phyla constitute a very diversified group: some of these proteobacteria belonging to the class of  $\alpha$ -proteobacteria. Due to the heterogeneity within proteobacteria, these are divided into 5 classes: alpha ( $\alpha$ ), beta ( $\beta$ ), gamma ( $\gamma$ ), delta ( $\delta$ ) and epsilon ( $\epsilon$ ). Within alpha proteobacteria belong phototrophic bacteria, capable to fix atmospheric nitrogen in symbiosis with plants.

In addition the phototrophic bacteria are involved in various metabolic systems, and play an important role in the nitrogen cycle and the carbon cycle, allowing a coexistence with others microorganisms.

## Lactic acid bacteria

Lactobacilli (Figure 15) are a large and heterogeneous group of microorganisms that are characterized by their fermentative metabolism: lactobacilli by the fermentation of the carbohydrates produce lactic acid (Hussain et al., 2002). Lactic acid bacteria are heterotrophic and chemiorganotrophic microorganisms adapted to live in complex substrates, and require important growth factors such as amino acids, nucleotides and vitamins and carbohydrates as source of energy.

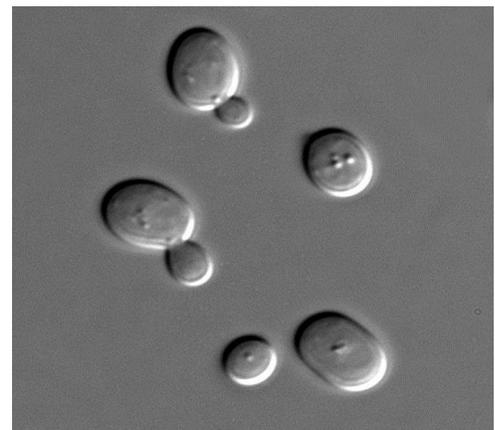


**Figure 15:** Three lactobacilli belonging EMS': *Lactobacillus plantarum* (1), *Lactococcus lactis* (2) and *Lactobacillus casei* (3).

Lactic acid bacteria improve mineral availability for plants in the rhizosphere through the production of enzymes and organic acids (Lopez et al., 2001; Kinjio and Higa 1991) and control the diffusion of different pathogens by the productions of several compounds. Indeed some in vitro studies showed inhibition effects against pathogenic fungi (Wang et al., 2011; Wang et al., 2012). Their effectiveness in improving the stability of silage with the production of metabolites that inhibited moulds and fungi propagation has been documented for different species of *Lactobacillus*, including some sp. identified in this study such as *L. buchneri*, *L. parafarraginis* and *L. diolivorans*. Moreover was verified their ability to promote the decomposition of the organic substance promoting the fermentation and the decomposition of recalcitrant compounds such as lignin and cellulose, thereby limiting undesirable effects related to the presence in the soil of undecomposed material (Gao et al., 2008; Valerio et al., 2008).

### Yeasts

Yeasts (Figure 16) are eukaryotic microorganisms, unicellular, belonging to the group of the Fungi. Yeasts are devoid of mycelial apparatus, although some species can form a pseudo-mycelium that makes them look like micro-molds. Yeasts are known as promoters of fermentation processes and produce many biologically active agents such as amino acids and polysaccharides. Many studies conducted on the effects of yeasts on plant have shown increase in the growth of vegetation and roots, resistance to drought, in containing the spread of fungal diseases such as *Sclerotinia homeocarpa*, and in reducing stress caused by the strong maintenance of lawns intensive (Kremer et al. , 2000).



**Figure 16:** *Sacharomyces cerevisiae* cells under DIC microscopy (Wikimedia Commons)

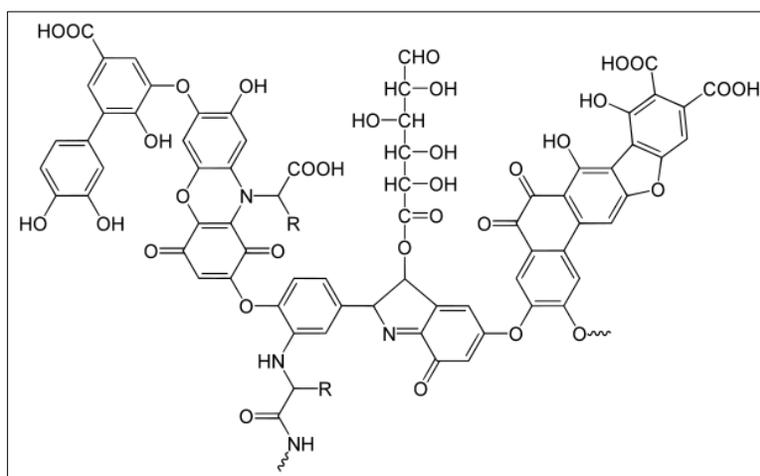
Regarding the rhizosphere, the main species studied for their effectiveness are *Sporobolomyces roseus* (Perondi et al., 1996), *Rhodotorula* spp. (Abd El - Hafez and Shehata, 2001), *valid Candida*, *Rhodotorula glutinis* and *Trichosporon asahii* (El - Tarabily, 2004). These studies have attested yeasts' ability to promote plant growth, and for this reason they were defined growth plant - promoting yeasts, PGPY. The studies of Abo-Elyousr and Mohamed (2009) demonstrated the possibility of using the species *Saccharomyces cerevisiae*, *Candida sake* and *Pichia membranifaciens* for the biological control agent of Fusarium in maize.

## 2.2.4 NATURAL AMMENDANDS FOR SPORT FIELDS

In the last decades the sport turf industry introduced a wide range of natural treatments which, over the years, have gained a considerable importance especially in the high quality sport field such as golf courses. In addition to the PGPR, in most evaluable turfgrass products could be content substances such as humic compounds, mycorrhizae, and sea weed extract which are described below.

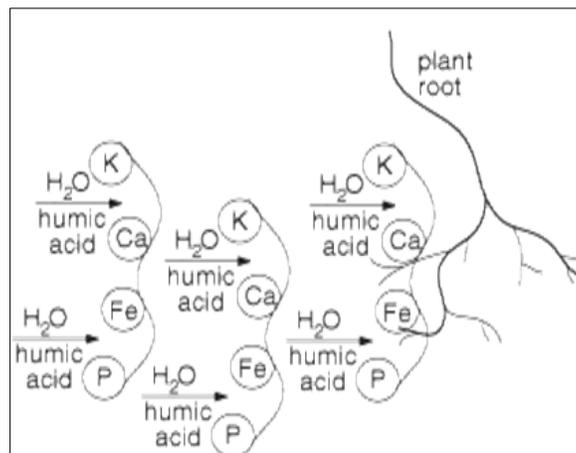
### Humic acids

Humic acids are natural acidic polymers component of humic substance of the soil humus (Figure 17). Humic substances can be divided in humin, fulvic acid, and humic acid in function of the degree of solubility depending by the pH (Stevenson, 1994). Humic acid consist of a hydrophobic framework of aromatic rings linked by flexible carbon chains, with alcohol, amide, amine, carboxylic, carbonyl phenol and quinone functional groups (Davies and Ghabbour, 1998).



**Figure 175:** Example of a typical humic acid, having a variety of components including quinone, phenol, catechol and sugar moieties (Stevenson, 1994)

Organic structure of humic acid is naturally oxidized giving it a negative charge (Figure 18). Positive ions (cations), attracted to broken bonds at the site of the oxidation, create sites for micronutrients and microflora. The actions relatively at the cations allow to the plant to absorb more nutrients holds by HA, improving the transference of these absorbed elements at the plant's circulation system. The capacity of the roots to absorb nutrients would be maintained until the root's negative charged remains greater of the humic acid's negative charge. It is hypothesized that the mechanism of transport from soil to plant's tissues is given by the capacity of the micronutrients carried (Humic Acids) to move into close proximity of the root system, while the capacity of the plants to absorb water will complete the final gap.



**Figure 18:** scheme of the action of humic acids to bring micro-nutrients to plant's root

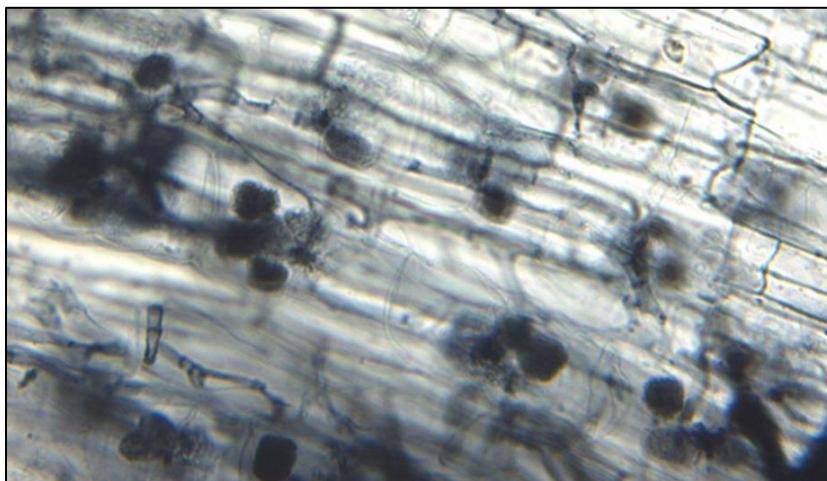
In order to study the effects of humic acids, several studies have been carried out on crop species. In the last decade a fervent interest grew around a possible application in the sport turf sector. Studies conducted in controlled environment have given excellent result regarding the effects of humic acids, such as the increasing of the root biomass and length (Zhang et al., 2003; Liu, 1998; Cooper, 1998). Studies conducted in growth chamber on *Agrostis stolonifera* L., showed positive effects on the photosynthesis rate in plants treated with humic acid (Liu, 1998; Zhang, 2004). In contrast with these good responses, experiments conducted in the open field condition have not yet confirmed the same positive effects (Kaminski, 2004). Humic acids present in many commercial turf products claims to improve turfgrass performance under environmental stress when used in conjunction with a standard fertilization program (Sachs, 1996).

The reasons for results that have been previously obtained in controlled environment trials compared at the field condition experiments could be attribute to a multitude of causes. First of all field trials are not enough accurate to show small effect determined by single component due to the heterogeneity of the field condition. In addition commercial products based on humic acids may contain other substances which could compromise the result both in grow chamber and in field trial.

## Mycorrhizae

Mycorrhizal fungi are obligate biotrophs soil organisms that develop a symbiotic association with root systems called mycorrhiza (Figure 19), a mutualistic relationship that establishes a network which allows plant to utilize mineral nutrients from the soil that the root system would not be able to access otherwise (Marschner et al., 1994).

Mycorrhizal fungi are represented by two principal groups: ectomycorrhizal and endomycorrhizal. Ectomycorrhizae mainly colonize woody Angiosperms and Gymnosperms, in which Basidiomycetes Ascomycetes and Zygomycetes develop intercellular hyphae from a mycelian sheath covering the surface of short lateral roots. Endomycorrhizas colonize plant with an intraradical growth and an intracellular proliferation which are formed by Basidiomycetes in the Orchidaceae, Ascomycetes in the Ericales and Zygomycetes (arbuscular mycorrhiza) in most other terrestrial plant taxa (Brundrett, 1991; Harley and Smith, 1983). The most important group of endomycorrhiza is represented by Arbuscular mycorrhizal fungi (AM-fungi or AMF), that is compatible with more than 80% of extant plant families. AM-fungi are fundamental for many ecological aspects as the capacity to transfer nutrients from plants exchanging with photosynthetically fixed carbon. The abundantly nutrients uptake and supply resulting from the mutualistic association between mycorrhiza and plants has been widely documented for many ions as P,  $\text{NH}_4^+$ , K, Ca,  $\text{SO}_4^{2-}$ , Cu and Zn (Johansen et al., 1992; Tobar et al., 1994). Some studies demonstrated that arbuscular mycorrhizal fungi, associated with host plants in agricultural or natural soil in different climate condition, increase the capacity to uptake amino acid (Näsholm et al., 1998; Hawkins H.J., et al. 2000) and transfer complex organic nitrogen, otherwise unavailable for uptake by the majority of plants (Swift et al., 1979).



**Figure 69:** Arbuscular mycorrhiza seen under microscope. MS Turnel, University of Manitoba, Plant Science Department.

In addition has been reported an improved photosynthesis and an higher plant growth, probably as consequence of an enhanced phytohormone cytokinin level (Drüge and Schonbeck, 1993). Some beneficial bioprotective roles against adverse environmental condition were evaluated as an increased resistance by heavy metal pollutants and a certain number of common soilborne pathogens (Benthlenfalvay and Linderman, 1992; Fernando W.G.D and Linderman R.G. 1996).

The results of studies on the application of mycorrhizae in sport turf have shown a marked effect on the limitations of the growth of weeds such as *Poa annua* ( Ganges A. , L. Whitfield , 2004) , and in a larger area for the exchange of nutrients for both symbiotic organisms (Amaranthus M. 2001) .

### **Sea weed extract**

Seaweed (*Ascophyllum nodosum* Jol.) extracts (SWE) is novel variety of organic material that is utilized in various products that promise protecting turfgrass against oxidative stresses. SWE derives from *Ascophyllum nodosum*, a common brown algae belonging to the Fucaceae family, which can be found in the northern Atlantic Ocean.

The first researches on the effects of SWE are dating back to the early 90s, when Schmidt and his collaborators studied the effects that *A. nodosum* has on leaf growth rate and senescence, nutrient uptake, root mass and photochemical activity (Goatley and Schimidt, 1990; Zhang 1997).

SWE contains different compounds such as amino acids and micronutrients (Fike et al., 2001). In addition was reported that SWE has a hormonal activity equivalent to 50 mg/L kinetin. Furthermore, Crouch and Van Staden (1993) utilizing GC-MS techniques have quantified auxin and cytokinins in SWE. Also some betaine forms were found in SWE by Blunden et al (1986).

Nowadays SWE are widely used in various biostimulant product formulations. This compound has been reported to contain phytohormones and osmoprotectants such as cytokinins, auxins, polyamines and betaines. In addition, some researches shown that SWEs have the capacity to improve the resistance to environmental stresses such as drought (Zhang, 1997) and salinity (Nabati et al., 1994). Successive researches have demonstrated that the capacity to resist at the environmental factors could be attributed at an increasing of the anti-oxidant contents (Zhang and Schmidt, 1997; Zhang and Schmidt 1999).

## 2.3 ENVIRONMENTAL IMPACT OF A GOLF COURSE

A well designed golf course, that respects the principles of environmental sustainability, has a potentially significant role in environmental reconstruction/protection and become an integral part of the land use planning. For these reasons, the European Golf Association in 1994, has instituted a label unit, the European Golf Association - Ecology, aims to bring at the public understanding the role that golf can play in the environmental preservation.

There are several legislations that regulate the construction and management of a golf course. In the case that the golf course was built on a industrial or agricultural land there was a change in land destination use. Otherwise urban zones such as green areas for sport and recreational activity include the possibility to build a golf course. Furthermore, regulations for environmental protection in a territory on may include other constraints as environmental, forestry, hydro-geological and faunal limitations.

Green keepers, stakeholders and athletes have the interest to work and play golf in a healthy and self-sustaining as possible area. A significant amount of research has been done to quantify the potential leaching and run-off of nutrients and pesticides from golf courses (Kenna and Snow, 2000). Innovations in the last years regard reduction of water consumption with more efficiently agronomical practices and drought resistant grass varieties, utilization of new generation fertilizer, fuel consumption and noise implicated with machineries utilization that may interfere with the wildlife, and less use of plant protection products with the introduction of Integrated Pest Management.

These practices are all related with an undirected well maintenance of the territory and a good relationship with local community. The benefits that a golf course leads to the environment can be summarized as follows:

- **Conservation of biodiversity.** Maintenance of native flora and fauna thanks to the ban on hunting and habitat conservation within the property of the golf club may have the function of " protected area", defending the species from the pressure human activities (Hammond and Hudson, 2007; Beard, 1994);
- **Preservation of the environment and cultural heritage.** Within the golf courses, especially in the oldest, can be preserved pieces of agro-ecosystems, monuments and historical artifacts.
- **Environmental education.** Managers and green keepers have as their objective to hold high the standard of knowledge of correct principles of environmental management and the willingness to apply them in this way can become a new kind of professionals for the environmentally sound management of the populated areas.
- **Decrease of the damage threshold in the noise.** The grass is able to absorb and refract the sound waves by reducing the noise of 20-30% (Rasmussen, 1981). This characteristic could bring range benefits to human health in case of recreational area or golf courses close to large city or very busy streets.

- **Daily temperature mitigation.** Turf is able to reduce thermal peaks thanks to the absorption of heat during the day and a slow release of the heat, previously absorbed, during the night (Beard, 1994). It has been studied that a grassy moderates about 40% of heat from solar radiation, there was a differential of 10-15 °C between the surface of the golf course and a sidewalk.
- **Absorption of air pollutants and reduction of atmospheric dust.** Turf surface and trees are able to absorb pollutants such as nitrogen oxide, sulfur dioxide and carbon monoxide, ozone and particulate matter, produced by the gaseous emissions in residential or manufacturing areas (Nowak et al., 2004). Turfs are also able to capture the atmospheric dust that settles on the plate as condensation and precipitation.
- **Production of oxygen.** Through the process of photosynthesis, trees and grass essences present in the golf course release into the atmosphere a considerable amount of oxygen. 1 m<sup>2</sup> of turf surface produces about 5.4 kg of oxygen per day. In addition the production of oxygen relative to a tree, is around 0.31 kg of oxygen per day.
- **Erosion control.** Recently the soil erosion is a common occurrence in hilly and mountain areas, and it is amplified by the increasing of heavy rainfall. An area such as a golf course that has a high density vegetation cover, offer a greater degree of protection respect to the erosion.
- **Environmental regeneration.** A golf course represent an environmental revaluation if built in an area previously occupied by industries for exemple the Modena Golf & Country Club.

Environmental concerns regarding the use of land for golf courses have grown over the past decades. The issues include the amount of water needed for irrigation, use of pesticides and chemical fertilizers for maintenance, as well as the dismantling of wetlands and other important areas of environmental protection during the construction of the golf club. Following are summarized some environmental adverse impacts derived by the presence of a golf course:

- **Impact on water resources.** Water consumption is the most significant environmental impact in the management of a golf course. Nowadays this is a topic of great interest especially for the water emergency almost all regions of the world. The United Nations estimates that, worldwide, golf courses consume about 9.5 billion liters of water per day. On the basis of estimates compiled by the European Golf Association, the average water consumption for a 18-hole course, is about 1,500-2,000 cubic meters of water per day; this corresponds at the consumption of a 8,000 people village or at the water needed for the production of 2 tons of wheat.

“During drought period and in the cases of scarcity of resources water [...] must be ensured, after human consumption, the priority of the agricultural use" (art.28 Italian national Law n° 36 of 5 January 1994). A recreational use contradicts the principle of sustainable use of natural resources advocated by Agenda

21 (Rio De Janeiro in 1992), is even less tolerated by the community. To minimize this inconvenience many golf courses are irrigated with non-potable water and rainwater collected in reservoirs constructed on the site.

- **Impact on soil and groundwater:** The negative impact that a golf course has on soil and groundwater can be attributed at the fact that the turf, has to guarantee impeccable condition of uniformity and smoothness especially in the areas of greens and tees. In order to obtain this quality, the green surface is treated with chemicals such as fungicides, pesticides and herbicides, with quantity and frequency higher than those used in a normal agricultural cultivation. The Journal of Pesticides Reform estimates that in the United States in a standard golf course will employ 750 pounds per year.

## **Influence of microorganisms and bio-regulators on a *Lolium perenne* L. essence grown in a controlled environmental system**

### **3.1 SECTION OBJECTIVES**

The main purpose of the first experiment was to study the effects that, various growth promoter utilized in different agronomical contexts, have on perennial ryegrass (*Lolium perenne* L.). During this experiment different products were chosen among the commercial solutions, including the original mixture of effective bacteria EM-RO®. These products were tested with a hydroponic system inside a growth chamber where light, temperature and photoperiod were controlled, utilizing a growth medium composed by a stratigraphy of sand, pumice and vermiculite 8:1:1.

The effects that the microbial mixes induced on the ryegrass plants were studied considering different physiological and morphological parameters. Furthermore microbiological analysis were conducted in order to evaluate endophytic colonization of roots by EM-1 (EM-RO®) bacterial mix.

## 3.2 MATERIALS AND METHODS

### 3.2.1 SEED AND PLANT GROWTH CONDITION

The plant used in this preliminary study was Perennial ryegrass (*Lolium perenne* L.) provided by Fratelli Ingegneroli (Milan, Italy). *Lolium perenne* is a pilot specie commonly utilized for the preliminary cases of growth chamber studies (Schweinsberg-Mickan and Müller, 2009; Ervin, 2007). Inside the growth chamber was assembled a close hydroponic system composed by four separate lines of irrigation (Figure 20). Each irrigation line was assembled with a 40 L water tank, a submersible electro pump (TOP 2 – LA) to flow solution up to drip irrigation stick placed in each pot. The water regime was controlled by a timer that supplies irrigation solution for 5 minutes twice per hour at 0,1 L/min per pot.

Pots utilized for the sowing were 10 cm diameter and 12 cm height. Pots were partially closed on the bottom with a plastic filter 1 mm thick, and then filled with 2 cm pumice layer and 12cm of sand, and lastly 0,5 cm of vermiculite (Figure 21). The growing medium was sterilized in autoclave before the preparation of the pots. *Lolium* seeds were sowed with a density of 0,7 g/pot (50 Kg/ha) and placed at depth of 1 cm.



**Figure 20:** Growth chamber and close hydroponic system. . Trays and water tank are wrapped with aluminum in order to prevent the entry of light.

**Table 3:** Ingredients of Hoagland solutions.

Hoagland components	g/L of solution
2M KNO <sub>3</sub>	202g/L
2M Ca(NO <sub>3</sub> ) <sub>2</sub> x 4H <sub>2</sub> O	236g/0.5L
Ferro chelato	15g/L
2M MgSO <sub>4</sub> x 7H <sub>2</sub> O	493g/L
1M NH <sub>4</sub> NO <sub>3</sub>	80g/L
H <sub>3</sub> BO <sub>3</sub>	2.86g/L
MnCl <sub>2</sub> x 4H <sub>2</sub> O	1.81g/L
ZnSO <sub>4</sub> x 7H <sub>2</sub> O	0.22g/L
CuSO <sub>4</sub>	0.051g/L
H <sub>3</sub> MoO <sub>4</sub> x H <sub>2</sub> O	0.09g/L
1M KH <sub>2</sub> PO <sub>4</sub>	136g/L

To prevent arising and propagation of algae that may inadvertently be found in some water tank and pots, all areas exposed to light of hydroponic circles, were wrapped with aluminum. This precaution has prevented the application of growth inhibitors for algae that could interfere with the microbial metabolism in the hydroponic solution.

The nutrient solution was prepared according to Hoagland and Arnon method(Hoagland,1950) and diluted 1:1 (v/v) with distilled water (Table 3). To prevent an ionic accumulation in this close hydroponic solution, especially on the bottom of the tank, electro conductivity was checked every week.

Plant water / nutrients uptake was weekly compensated with fresh nutrient solution refilled directly in the tanks. Growth chamber settings were set up 24°C and 70% relative humidity (RH) during the day, and 20°C and 50% RH in night conditions. Light was supplemented with an artificial illumination at 550  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ . Photoperiod was set at 16 hours of light and 8 hour of dark condition in accord with Dudeck (1986).

During the experiment, plants were mow 4 cm above the soil level once a week, for 60 days. The experiment plan was a completely randomized design, consisting of eight pots for each treatment placed in a random position on a shelf in the growth chamber.



**Figure 21:** Perennial ryegrass 6 days after the germination.

### 3.2.2 TREATMENTS

Different commercial products, composed by microorganisms, phytohormones solution and nutrients were tested in this preliminary study (Table 4).

**Table 4:** Table with the different treatments

N° THESIS	TREATMENT	PRODUCER
1°	Control (Only Water) + Hoagland	-
2°	Embio Bacteria's / Green Gold (F/NF)	Embio
3°	Embio Filtered Bacteria's + Hoagland (F/NF)	Embio
4°	Agrisystem Phytohormones + Hoagland	Fast-Speed Top, Agrisystem
7°	EM-1 (1:500) F/NF	EM-RO
8°	EM-1 (1:500) + Hoagland (F/NF)	EM-RO

**Green Gold** (Embio, Brunico, Bolzano, Italy) is a base microbial commercial solution marked to integrate in different contexts, including a lack of effective microorganisms in the soil.

**Agrisystem's phytohormone solution (HRM treatment)** (Fast-Speed Top, Agrisystem, Lamezia Terme, Italy) is a commercial product containing gibberellic acid (GA3) (1.65 g/l) and naphthalene acetic acid (NAA) (3.30 g/l). Before use, the stock solution was diluted 1:2000 (v/v) in distilled water.

**EM-1** (EM-RO<sup>®</sup>, Okinawa, Japan) is a commercial product containing many effective microorganisms in particular lactic acid bacteria, yeast and phototrophic bacteria.

Before using the microbial products (Green Gold and EM-1) an activation step was required and it consisted in the addition of 5% (v/v) microbial products and 5% (v/v) molasses in water. Activation was carried out inside a plastic container with a specific resistance that maintains a fixed temperature of 35° ± 1°C for five days (Figure 22).



**Figure 22:** EM-1 activation.

**Pre-Sowing treatments** All treatments, including the control, were tested with and without a pre-sowing imbibition of the seed (priming). For each thesis, imbibition was performed dipping seeds in treatment solution at the same concentration used for the following 5 weeks experiment. Priming condition was set at 20°C, an optimum temperature for *Lolium* sp. (Copeland, 1978) and in a in dark condition for 6 hours. After hydration, seeds were washed with distilled water to remove the solution from the surface and then dried in hoven at 22°C for 6 hours (T.K Danneberg et al. 1992). Seeds with and without a pre-sowing imbibition were sowed in pots simultaneously.

**Filtered and Not-Filtered treatments**

In order to study the effect of microbial solutions without microbial metabolites, each microbial treatments (Green Gold and EM-1) were filtered through a 0.20 µm nylon filter (Millipore). Filtered solutions were diluted at the same concentration of not filtered treatments (1:500 v/v) in distilled water.

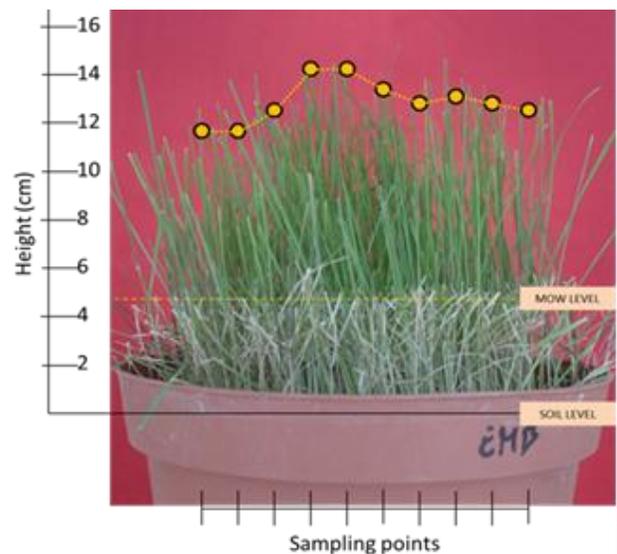
**3.2.3 DETERMINATIONS DURING THE EXPERIMENT**

The first mowing was done 10 days after germination, when all plants reached a height of 8 cm. For the whole experiment duration, plants were mowed weekly at 4 cm height.

Before the weekly mowing, the parametric analyzes focused on the shoots heights (steam and leaves together), colors of the leaves (RGB and HIS scale), tear resistance, electro conductivity and pH of the water solution inside the tanks. After the weekly mowing, leaves were collected to measure the fresh and dry weight. Other parameter as total shoot length, total shoot fresh and dry weight were determined as sum of all measurements.

**Leaves growth and biomass**

The analysis of leaves growth in height was conducted before the weekly mowing. A picture for each pot were taken with a Canon Reflex EOS 350D placed on a tripod with an adjustable height in horizontal position relative to the plane of the leaves. With an image analysis process (Figure 23), the heights of the leaves were determined in 10 points transversally along the diameter of the pot. The height of leaves for each pot was measured as average of the 10 transversally points. After the mowing, clipped leaves were collected in paper bags to be weighed in



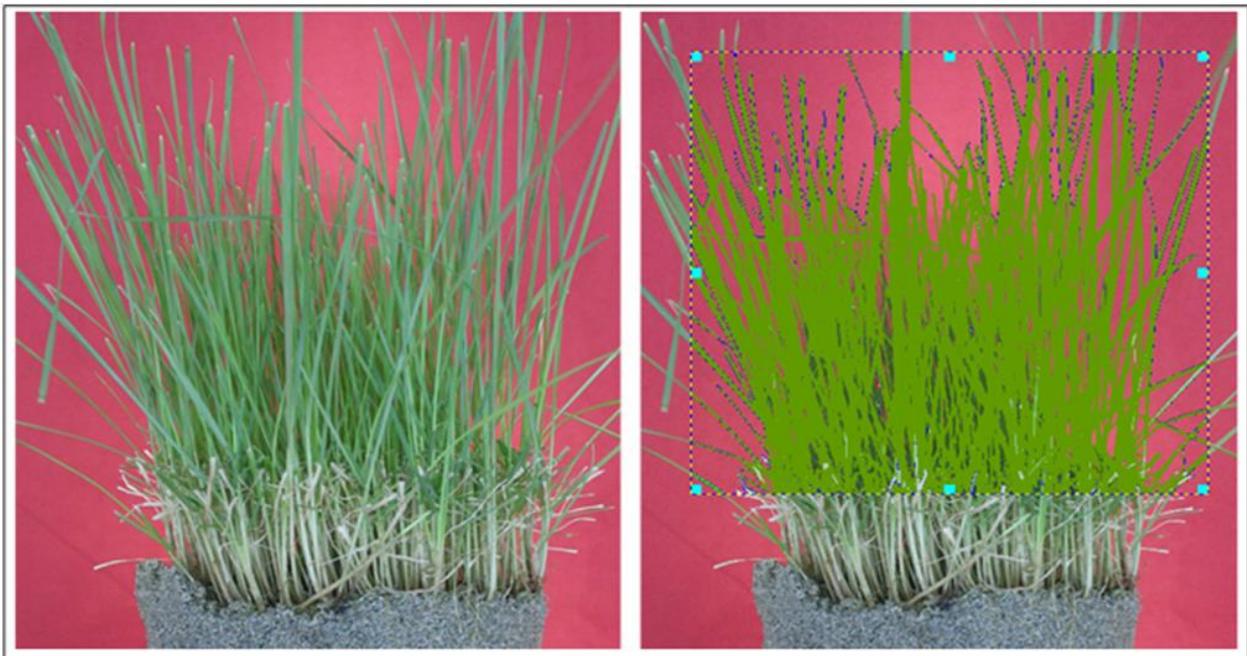
**Figure 23:** *Lolium perenne* within ligh-box.

laboratory. Fresh weight was measured immediately after the mowing, instead dry weight was determined

### Tear resistance

Before each weekly mowing, tear shoot resistance was tested utilizing a mechanical dynamometer (PCE Instrument, Lucca, Italy). The method used consists in measuring the force required to tearing out of the aerial apparatus. The clamp placed at the base of the dynamometer, composed by small not sharp teeth, was attached to the base of the stem of the ryegrass plant. A red marker, placed inside the dynamometer, pointed out the maximum force utilized at the moment of the tear out of the stem from the soil.16 repetition were sampled for each plot.

### Image analysis



**Figure 24:** Perennial ryegrass within the light-box during the color evaluation process.

The image analysis was carried out considering both the RGB color space. The pictures necessary to the color analysis were taken previously at each mow. Pictures were taken in RAW quality by a Canon Reflex EOS 350D and then elaborated with APS ASSESS 2.0 image software. The iris opening and the exposure of the camera were set respectively to f/6,3 and 1/100 seconds.

Plants were placed inside a light-box, assembled specifically for this experiment, in order to take pictures in homogeneous lighting conditions throughout all the duration of the trial. Light-box was equipped with a red

background (mean values R-200, G-105, B-115) to maximize the contrast with the green of the leaves (Figure 24). Two different groups of cool-white LED provided lighting inside the lightbox: the front LED-string provided to a frontal lighting and the lateral illumination deleted the shadows from the background to homogenize the image.

### 3.2.4 FINAL DETERMINATIONS

#### Root growth and biomass

60 days after sowing, plants were removed from pots and with a camera Canon EOS 350D were taken pictures from each plant within the lightbox.

After this picture sampling, 50 grams of soil, containing rhizosphere and roots from each pot, were collected for future analysis of microbial flora. After that, roots were washed lightly in a tray with distilled water. Washed plants were extended on a flat surface and then measured in length.

Leaves and washed roots were separated cutting the Lolium at the base of the stem. Roots and leaves were dried completely utilizing an absorbent paper and then measured the fresh weights were determined. After 5 days at 50°C in a hoven respective dry weight were measured.

#### Mycorrhizal analysis

Mycorrhizal presence within root tissues was analyzed from 2 grams of roots for each sample. According with techniques utilized by Brundrett et al. (1984), roots of Lolium were washed lightly but accurately in distilled water and then transferred in a solution 10% (w/v) KOH in a 50 ml in an autoclave-resistant jar for tissues discoloration. Roots were completely submerged by KOH solution to remove cell contents and cell wall pigments, a widely used protocol for viewing internal features in plant tissues (Gardner, 1988). Different root thickness may affect the success of this discoloration process but in order use a standardized method, it was always utilized one hour heating at 90°C in a hoven. Samples were removed from the hoven and lightly washed again with deionized water. Roots were moved in a staining solution of 0.03% (w/v) CBE (Chlorazol Black E, Sigma-Aldrich) in lactoglycerol (1:1:1: lactic acid, glycerol and water) and soaked within an autoclave-resistant jars. Samples were



**Figure 25:** MEIJI microscope utilized for the acquisition of the mycorrhizal

stained 1 hour at 90° in CBE. Roots were washed lightly and abundantly with deionized water in order to remove all the CBE solution from the surface of the root tissues. Before to observe VA mycorrhizal colonization, roots were plunged in a 50% glycerol solution and were randomly arranged in a petri plastic dish (90mm diameter, Sterlin®) marked on the bottom with a grid line (split 1cm squarest) that act as a device for the observation of the mycorrhizal hyphae inside the roots (Giovannetti & Mosse 1980; Brundret et al., 1996). Samples were observed with microscope in dark field phase contrast (MEIJI, Japan) with a 400X zoom (Figure 25). Images were successively elaborated with the software ImageJ 1.31 (open source Java-written) in order to increase the image contrast and improve the analyzing of the samples. The presence of fungal hyphae within the root tissues was expressed as the percentage of squares with hyphae / total square of the petri plastic dish.

### **3.2.5 MICROBIAL ANALYSIS**

#### **Microbial analysis of the EM-1® mix**

##### **Microbial quantification**

The original mix of bacteria known as effective microorganisms, EM-1®, was used to determine the bacteria concentration before and after the activation step. Thereby 10 mL of both EM's solutions (activated and not-activated) were suspended in 90 mL of sterile water. Throughout successive dilutions, and after incubation, the number of colony forming units/ml (cfu/ml) of Lactobacilli, aerobic mesophilic bacteria and yeasts were counted. This analysis was replicated three times to have a more accurate response. Specific growth medium were used for each genera of bacteria. Lactobacilli were inoculated in de Man, Rogosa and Sharpe Broth (MRS, Merck, Darmstadt, Germany) containing 0.2% (w/v) sorbic acid (Sigma–Aldrich, Milan, Italy) and 0.1% (w/v) cycloheximide (Sigma–Aldrich) to inhibit growth of yeasts, and incubated anaerobically for 2 – 3 days at 30°C. Aerobic mesophilic bacteria were inoculated in Nutrient Agar (NA, Merck) containing 0.1% (w/v) cycloheximide and incubated for 3 days at 30°C. Yeasts were enumerated by inoculation on Sabouraud Dextrose Agar (SDA, Merck) containing 0.1% (w/v) chloramphenicol (Sigma– Aldrich) after 3–5 day incubation at 25°C. 20-30 colonies from each growing medium were selected, purified and stored at -80°C for successive phenotypic identification.

## **Phenotypic identification**

The colonies of microorganisms were examined by the stereomicroscope in order to identify the morphological characteristics and then group them by different isolates. These isolates were characterized phenotypically by Gram stain's test, catalase test, oxidase test and by the profile of carbohydrate fermentation (API® Test).

### **API® Test**

The performance of carbohydrate metabolism was tested with API 50 CH kit (Biomérieux) for *Lactobacillus* identification in 48 hours. API 20C-AUX kit was used for yeast identification after 48-72 hour growth of cells in Sabouraud Dextrose Broth (SDB, Merck).

### **PCR – genre identification**

The isolates of EM solution were analyzed for genotypic characterization, after a previous extraction of genomic DNA. The extraction was performed utilizing a commercial kit, the Wizard® Genomic DNA Purification Kit (Promega, Madison, USA), which allows the extraction from a pure culture. The bacterial solution were initially grown overnight. Subsequently the bacterial cells were extracted from 2 mL of culture by centrifugation at 6000 rpm for 10 minutes, and subjected to a pre-treatment to promote cell lysis by the addition of 120 µL of lysozyme (20 mg/mL) and 5 µL of mutanolisina (50 U/ µL). The pellet, consisting of bacterial cells, was suspended, transferred to 1,5 mL eppendorf and incubated for two hours at  $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ . Then samples were centrifuged 2 minutes at 18000 rpm and the supernatant was removed.

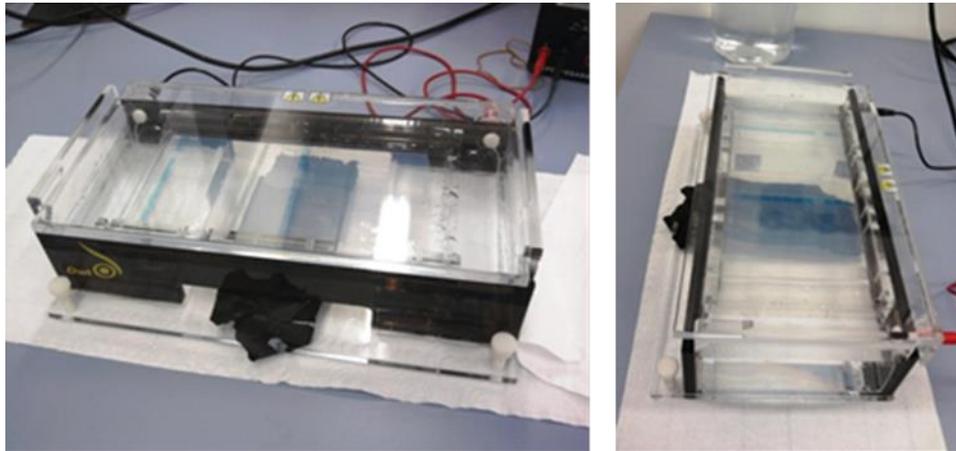
The bacterial cells were resuspended in 600 µL of Nuclei Lysis Solution, then they were incubated at  $80^{\circ}\text{C} \pm 1^{\circ}\text{C}$  for 5 minutes and cool down to room temperature. Successively, 3 µL of RNase Solution and the samples were agitated and then incubated at  $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$  for 40 minutes and then the samples were cool down to room temperature. Subsequently 200 µL of Protein Precipitation Solution were added. Samples were incubated on ice for 5 minutes, and then centrifuged for 4 minutes at 18000 rpm to recover the supernatant containing the extracted DNA. To carried out the precipitation of DNA, the supernatant was transferred into new eppendorf, in which was previously dispensed 600 µL of isopropanol and stirred reversing, by centrifugation for 2 minutes at 18000 rpm and removal of the supernatant was recovered DNA. Then proceed to the washing with the addition of 600 µL of 70% ethanol at room temperature, and the final precipitation by centrifugation 2 minutes at 18000 rpm. The supernatant was removed and DNA was dried overnight in a sterile hood. Subsequently DNA was suspended in 100 µL of DNA Rehydration Solution and incubated at  $4^{\circ}\text{C}$  overnight. The storage takes place at  $-20^{\circ}\text{C}$ .

The quantification and the purity of DNA were determined with a TECAN Infinite 200, at the laboratories of Agricultural Chemistry of DiSTA. An acceptable purity level of DNA was 1.8 – 2.0. Genus-specific PCR was performed according to Walter et al. (2001) to confirm the affiliation to the genus *Lactobacillus*.

Molecular biology-based grouping of lactobacilli and aerobic isolates was performed by ERIC-PCR. The amplification reaction was performed utilizing the primers ERIC-I (*enterobacterial repetitive intergenic consensus*) and ERI-II (Ventura and Zink, 2002):

- ERIC I: 5' – ATG TAA GCT CCT GGG GAT TCA C – 3'
- ERIC II: 5' – AAG TAAG TGA C TG GGG TGA GCG – 3'

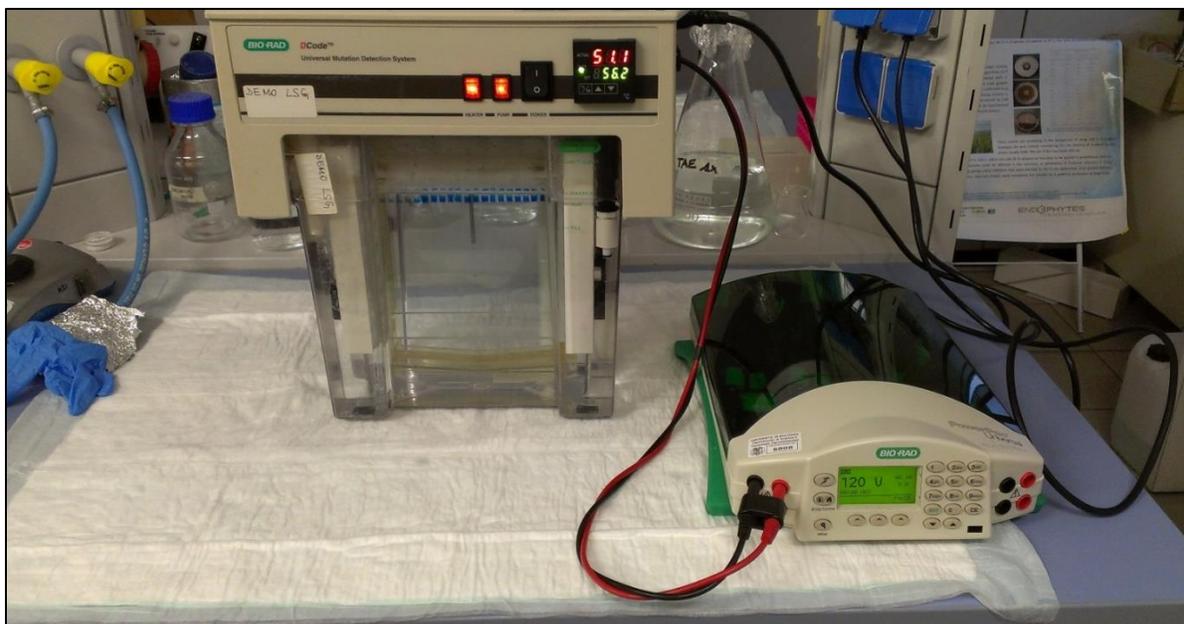
The amplification was performed in a 20  $\mu$ L solution composed by 2  $\mu$ L DNA (previously diluted at 12ng/ $\mu$ L concentration) and Master Mix composed by 10  $\mu$ L Hot Start Taq Plus Master Mix, 1,5  $\mu$ L MgCl<sub>2</sub>, 1  $\mu$ L ERIC-I, 1  $\mu$ L ERIC-II, 4,6  $\mu$ L H<sub>2</sub>O and 2  $\mu$ L DNA. The samples were separated by electrophoresis with a 2% w/v of agarose gel at 50 V for a durate of 4 hours (Figure 26). Samples were stained with ethidium bromide and visualized with the gel documentation system Gel Doc™ XR (Bio-Rad, Hercules, CA, USA).



**Figure 26:** samples stained with ethidium bromide within the electrophoresis chamber

An Image Lab software (Bio-Rad) was used to elaborate the images taken from the extraction and a binary matrix has been constructed. In order to obtain the phylogenetic trees, based on neighbor-joining method (Tamura et al., 2011), was utilized softer Mega 5.1.





**Figure 27:** The DCode system for DGGE analysis (BIO-RAD).

All the analysis in PCR-DGGE were carried out in 50  $\mu$ l volume containing 1.5 U AmpliTaq Gold DNA polymerase (Applied Biosystem), 5  $\mu$ l of 10xPCR Gold Buffer (Applied Biosystem), 200  $\mu$ M of each deoxynucleotide triphosphate (Fermentas GmbH, St. Leon-Rot, Germany), 1.50 mM MgCl<sub>2</sub> (Fermentas), 0.45  $\mu$ M of each primer (MWG), 2.5% (w/v) bovine serum albumin (Fermentas), 4  $\mu$ l DNA template and sterile MilliQ water.

The PCR with universal primers was performed under the following thermo-cycling program: 5 min initial denaturation at 95°C; 35 cycles of 95°C for 30 s, 55°C for 60 s, 72°C for 40 s, and by a final elongation step of 72°C for 7 min. In accordance with Walter et al. (2001), the PCR with the primers Lac1f and Lac2r-GC followed the manufacturer's instructions. 2 mL of samples were utilized to estimate the size and the amount of the PCR product by 1.5% agarose gel (w/v) electrophoresis and ethidium bromide staining.

The DGGE analysis was basically performed as first described by Muyzer et al. (1993), using a DCode System apparatus (Bio-Rad). Polyacrylamide gels [7% (w/v) acrylamide:bisacrylamide (37.5:1) (Bio-Rad)] in 1 x Tris-Acetate-EDTA (TAE) buffer were prepared using a Bio-Rad Gradient Delivery System (Model 475, Bio-Rad), using solutions containing 35–55% denaturant [100% denaturant corresponds to 7 M urea (Sigma-Aldrich) and 40% (v/v) formamide (Sigma-Aldrich)]. The electrophoresis was run at 55 V for 16 hours at 60°C. Gels were stained in a solution of 1x SYBR-Green (Sigma-Aldrich) in 1x TAE for 20 min and their images captured in UV transillumination with Gel Doc™ XR apparatus (Bio-Rad).

Selected dominant bands were cut from the gel with a sterile scalpel and DNA was eluted by incubating the gel fragments for 16 hours in 50 µl of sterile deionized water at 4°C. 2 µl of the solution were then used as template to re-amplify the band fragments using the same primers without the GC-clamp and the same PCR conditions.

### **Sequence analysis of 16S rDNA of pure cultures and DGGE bands**

On the basis of phenotypic and genotypic investigations, representative isolates (lactobacilli and aerobic bacteria) were selected and the 16S rDNA amplification performed with universal primers 27f and 1492r (Lane D.J., 1991). The amplified 16S rDNA were then purified (PCR clean-up; Macherey-Nagel GmbH & Co. KG, Germany) and sequenced (Eurofins MWG Operon, Ebersberg, Germany). Concerning PCR-DGGE bands, the obtained amplicons were sequenced with primer 907r and Lac1f. Sequence chromatograms were edited and analyzed using the software programs Finch TV version 1.4.0 (Geospiza Inc., Seattle, WA, USA) and obtained sequences were subjected to taxon classification using RDP classifier, an available tool at the RDP-II website (<http://rdp.cme.msu.edu/classifier/classifier.jsp>). Moreover, SeqMatch search was used to find the closest match for each 16S rRNA fragment ([http://rdp.cme.msu.edu/seqmatch/seqmatch\\_intro.jsp](http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp)) (Cole et al., 2009).

## 3.3 RESULTS

### 3.3.1 MORPHOLOGICAL AND PHYSIOLOGICAL DETERMINATIONS

#### Growth of the leaves

Perennial ryegrass (*Lolium perenne* L.) clods were extracted from respective pots at the end of the 60 days experimental period (Figure 28). Preliminary observation of the perennial ryegrass plants showed visual differences determined by microbial treatments (Embio and EM-1) and phytohormones solution. Plants treated with Embio and phytohormonic solution appeared more vigorous if compared to the control. EM-1 mixes determined an habitus more compact with leaves and roots shorter than the control and other treatments.



**Figure 28:** *Lolium perenne* L. habitus after the 60 days experimental period. From left to right are shown *Lolium* clods thesis: 1-Control, 2-Embio NF (Embio not filtered), 3-Embio F (Embio filtered), 4-EM-1 NF (EM-1 not filtered), 5-EM-1 F (EM-1 filtered), 6-HRM (Green Gold hormone solution).

**Table 5:**

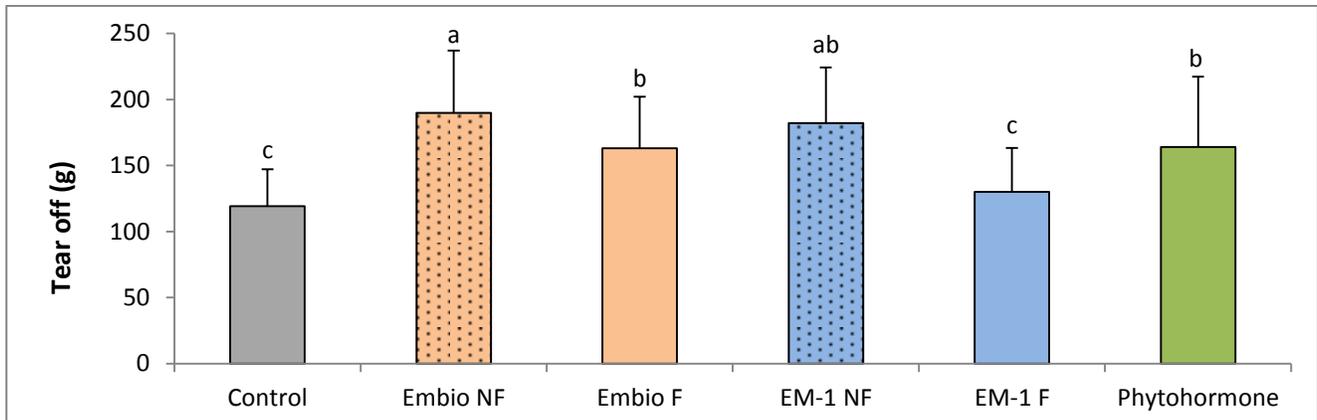
Effects of different treatments on leaf apparatus. Leaf analysis on Leaf length at 60 DAT (LL), Leaf Fresh Weight at 60DAT (LFW), Leaf Dry Weight/Leaf Fresh Weight percentage ratio (LDW/LFW) and Leaf Dry Weight/ Leaf Length (LDW/LL).

Leaf analysis	Control	Embio NF	Embio F	EM-1 NF	EM-1 F	HRM
<b>LL (cm)</b>	7,04 ± 0,58 b	6,32 ± 0,82 c	7,95 ± 0,61 a	4,68 ± 0,24 d	4,43 ± 0,64 d	7,94 ± 0,66 a
<b>LFW (g)</b>	4,05 ± 0,54 c	6,33 ± 1,28 b	6,11 ± 1,10 b	3,35 ± 0,20 c	3,69 ± 0,67 c	9,86 ± 1,62 a
<b>LDW/LFW (%)</b>	18,84 ± 1,92 a	13,47 ± 0,73 b	13,82 ± 0,69 b	20,54 ± 1,45 a	20,62 ± 4,13 a	12,64 ± 1,78 b
<b>LDW/LL (mg/cm)</b>	1,08 ± 0,19 c	1,39 ± 0,27 ab	1,07 ± 0,18 c	1,48 ± 0,16 ab	1,70 ± 0,28 a	1,56 ± 0,17 ab

Not filtered and filtered Embio determined opposed effects on the LL values, inducing respectively a reduction of 10% and a growth of 12% respect to the control. The EM-1 microbial mix determined similar effects for filter and not filtered conditions inducing a reduction of foliar length of 37% and 34% compared to the control. Green Golf hormone solution promoted the length of the leaves similarly to filtered Embio, showing significant difference respect to the control. Leaf fresh weight (LFW) was increased by microbial mixes without statistical differences between filtered and not filtered conditions within the same product. Hormones solution determined the higher foliar biomass production at the end of the experimental period, increasing the foliar yield of 143% compared to the control. The dry-fresh weight ratio (LDW/LFW) showed a significant and similar effect of Embio NF, Embio F and HRM solution in the diminution of the dry biomass content with a reduction of 28%, 27% and 31% respectively compared to the control. The leaf dry biomass per unit of foliar height (LDW/LL) showed similar effects for EM-1 microbial mixes and HRM solution, whereas Embio microbial mixes had different results. EM-1 preparation determined a 56% improving of LDW/LFW ratio compared to the control. However, Embio and EM-1 not filtered and Hormone solution increased the biomass yield per cm leaf if compared to the control.

### **Tear resistance**

The tear test was conducted on the Lolium plants at the end of the experimental period. The graph below (Graph 1) shows the values obtained by tearing the leaves at the base of the collar with a mechanical dynamometer. Embio NF and EM-1 NF determined the higher values, with values improved by 59% and 53% respectively if compared to the control. Embio filtered mix and phytormone solution showed similar significant differences respect to the control improving the tear resistance by 37% - 38% compared to the control. EM-1 filtered mix did not determined tear off effects.



**Graph 1:** Tear off resistance of *Lolium perenne* L. Analysis were conducted at the end of the experimental trial (60 DAT) in a growth chamber at DiSTA.

### Image analysis

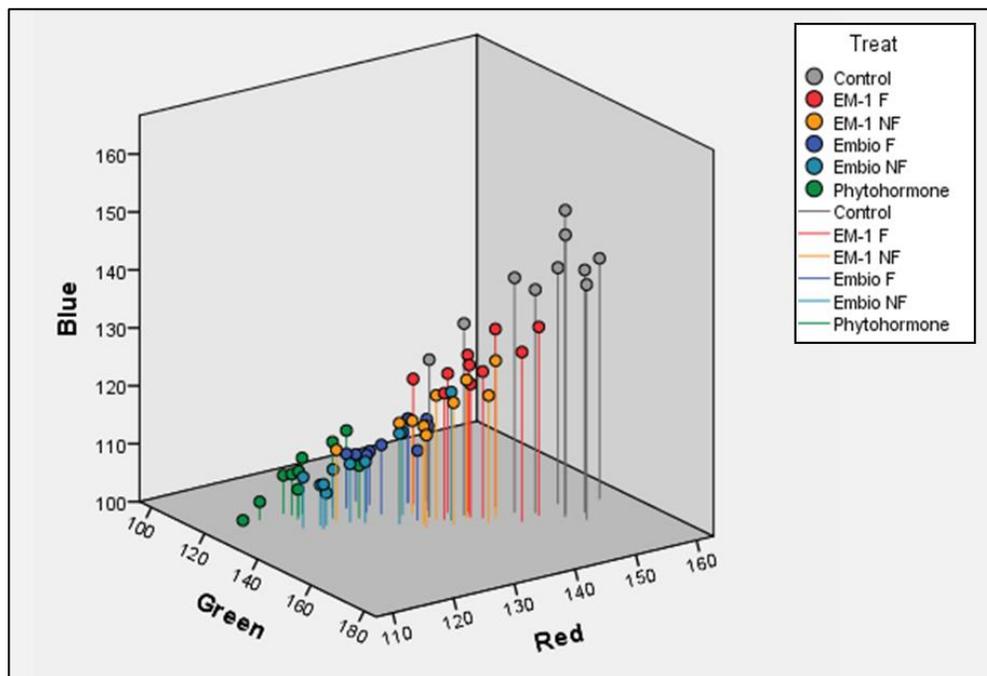
The image analysis was conducted measuring individually the values of the three color channels in RGB space. The pictures were captured within a light-box in order to homogenize the lighting conditions of the environment (Figure 29) and were afterward analyzed with Assess software (for a complete description of the picturing process and analysis refer to the relative Materials and Method section).



**Figure 29:** Leaves of a *Lolium perenne* L. pot. Pictures were isolated from the red screen in the background and analyzed only from the 4 cm mowing height. Leaves section utilized for the color analysis were processed in Assess image analysis software.

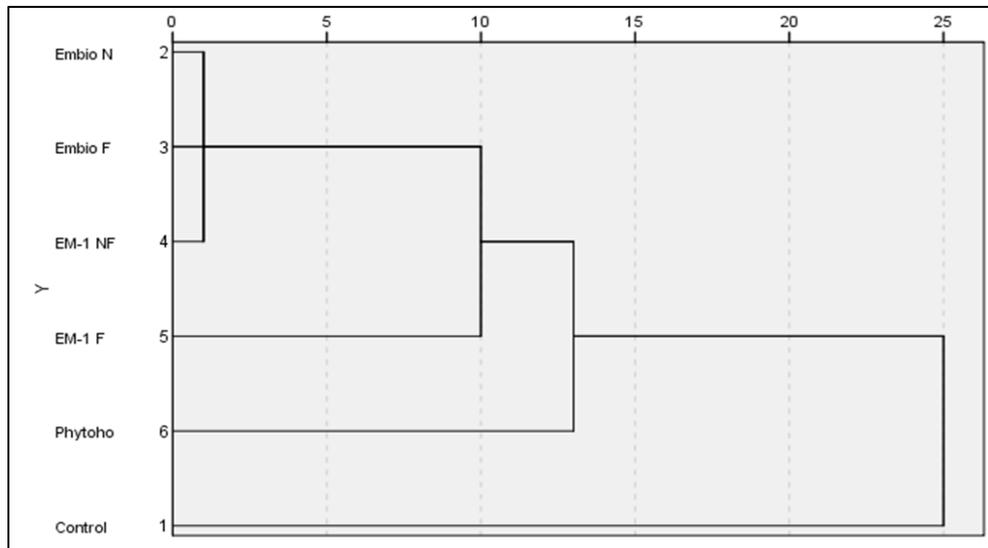
The leaves' color analysis shows an intensification of the color due to the tested product. Ryegrass treated with Control thesis (water and Hoagland solution) is placed at higher values for Red, Green and Blue, therefore the grassy surface showed the brightest coloration. Between the different tested products the phytohormone thesis seemed to have darkened the color of the leaves.

To assess the statistical significance of the differences highlighted in graph 2, a MANOVA test has been conducted: The red, green and blue color values measured from different pots (130 sampling points for each pot) have been used as dependent variables, while the treatments / control as fixed factors. The test reported a significant difference between the factors ( $F(15, 2131) = 64,043, p < 0,005$ ), and it was therefore followed by a Tukey's post-hoc analysis. The post-hoc test showed that the Control was significantly different from all the treatments in the whole color space, while EMBIOF and Phytohormone, instead, resulted not significantly different from each other in the whole color space. EM1F and EM1NF showed non-significant difference in both red and green, but a significant difference in the blue. For a detailed report of the test results refer to Table X in the Appendix.



**Graph 2:** 3D scatter plot of the color space in RGB mode. Three axes indicate different color variables X:Green, Y: Red, Z: Blue

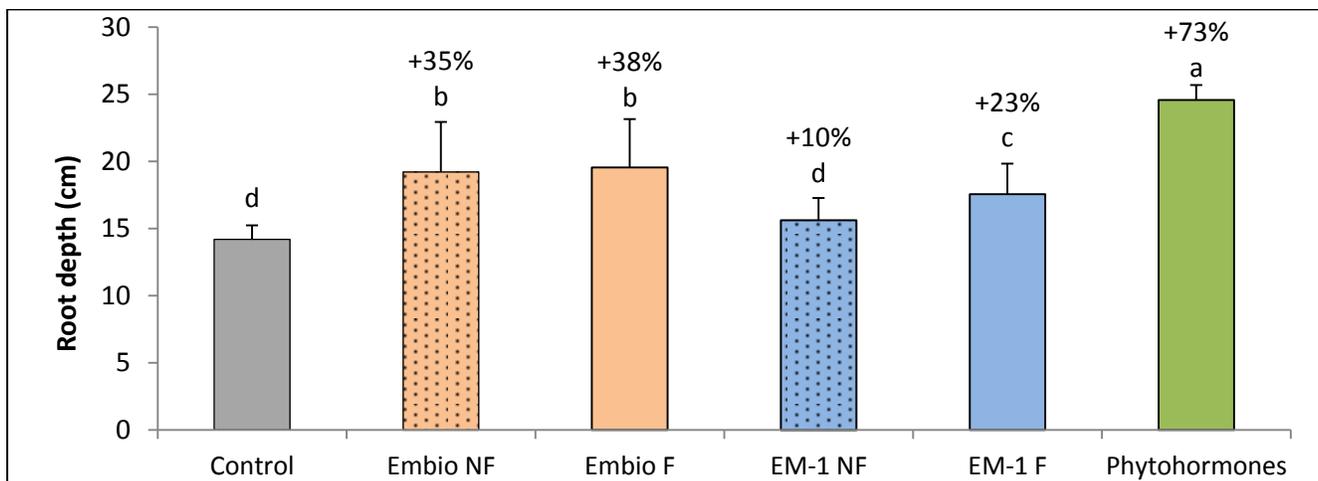
A cluster analysis (squared distance, single linkage) has been conducted in order to obtain a graphic representation of the differences suggested by MANOVA test. The following plot (Fig X) shows a dendrogram of growth chamber trial where the different treatments / control were clustered on the basis of the RGB variables.



**Graph 3:** Dendrogram representing the differences by mean color obtained for different treatments within growth chamber.

The treatments Embio NF, Embio F and EM-1 NF form a well distinct cluster, while the Control is a clearly separate entity with a much longer clustering distance. EM-1 and Phytohormone treatments are clustered independently and separated comparing to the control.

### Growth of roots

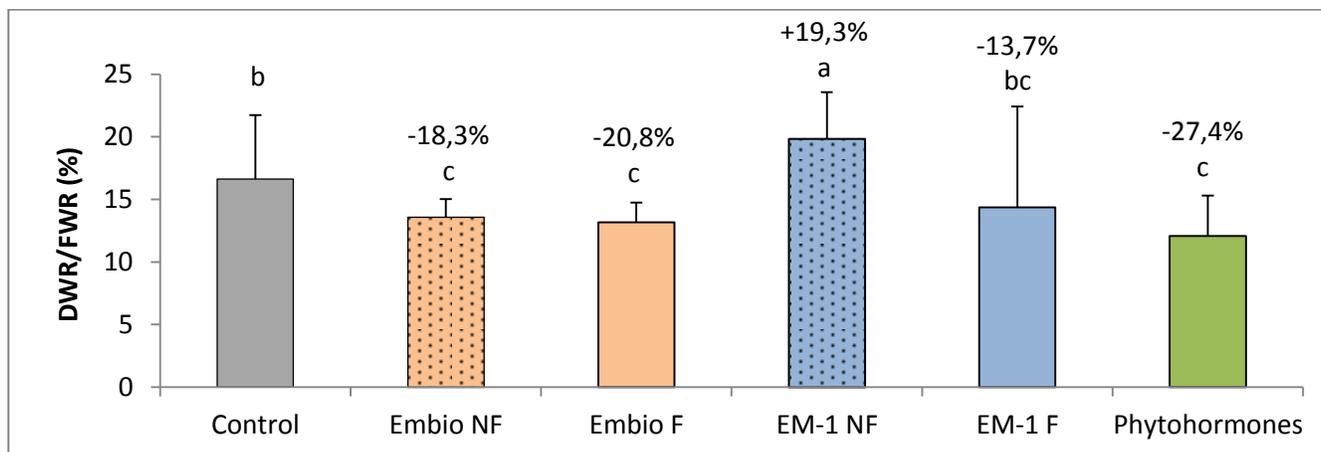


**Graph 4:** Root growth values at 60 DAT.

The roots of the perennial ryegrass showed the highest growth with the application of phytohormone treatment, with an increasing of 73% if compared to the control. Embio bacterial mix induced a growth of 35% - 38% (not filtered and filtered respectively) comparing to the control. A statistical difference was not detected between the

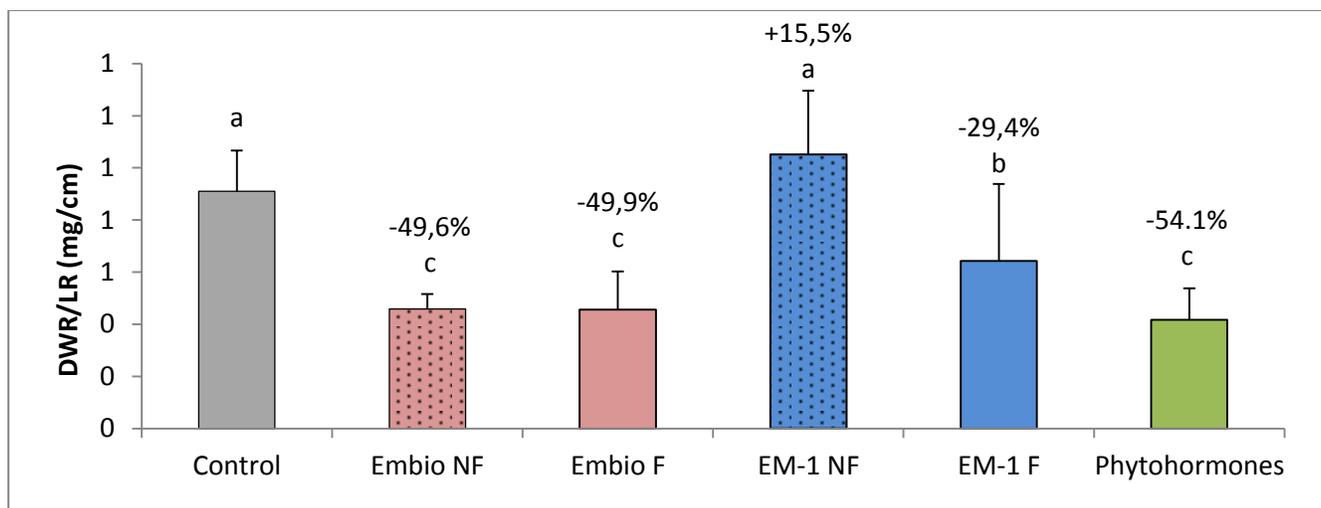
two Embio treatments. EM-1 showed a root development less pronounced than Embio treatments but higher compared to the control. A significant statistical difference was found between the two EM-1 treatments examined.

### Biomass of roots



**Graph 5:** Dry biomass of roots/Fresh biomass of roots percentage ratio (DWR/FWR %) at 60 DAT.

The dry / fresh biomass ratio showed different effects determined by these tested (Graph 5). Embio treatments determined a 18% - 21% decreasing compared to the control and did not show statistical differences between not filter and filtered solution. Not filtered EM-1 mix showed an increasing of 19,3% comparing to the control otherwise filtered EM-1 did not determine a significant difference respect to the control. Phytohormone solutions, similarly to Embio solutions decreased the dry / fresh ratio by 27,4% comparing to the control.

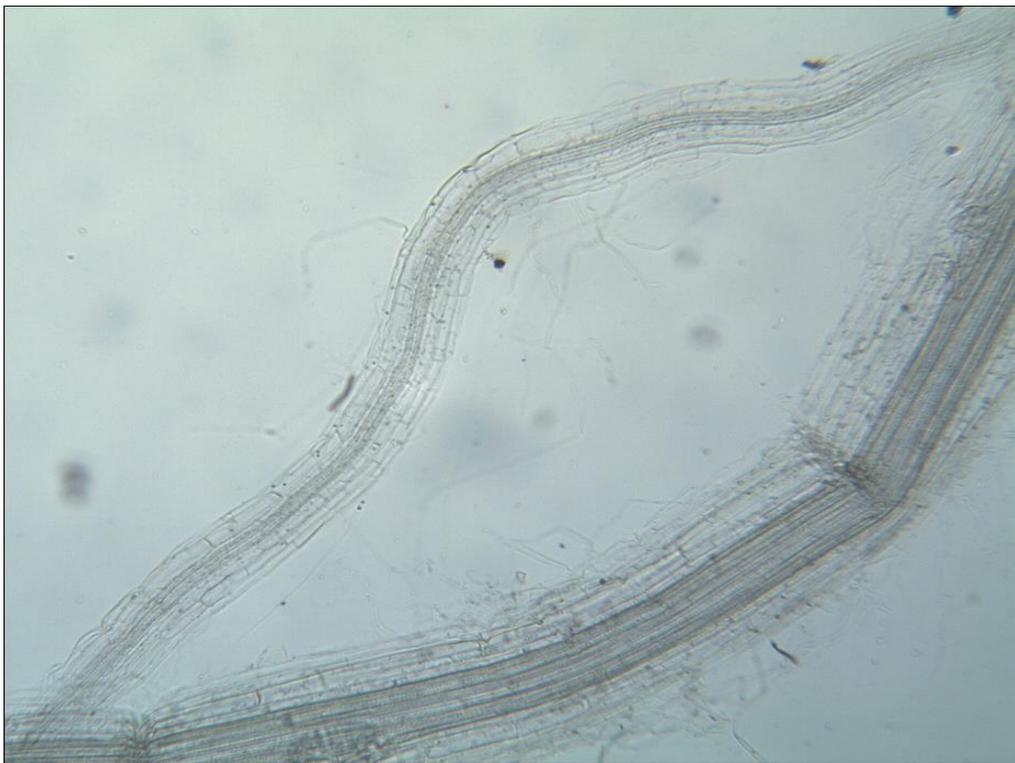


**Graph 6:** Dry weight of roots / length of roots ratio graph. The DWR / LR ratio indicated the mg of root per cm of roots.

DWR / LR ratio showed a similar effect of both Embio solutions to determine a decreasing of dry biomass for length of root. Not filtered EM-1 bacterial solution determined an increasing by 15,5% of the DWR/LR ration if compared to the control. Filtered EM-1 solution decreased the DWR/LR ratio by 29,4% with significant difference with the control thesis. Phytormones solution showed a similar effect compared to Embio theses.

### **Mycorrhizal analysis**

The presence of entophytic mycorrhizal within the root tissues was conduct utilizing Chlorazol Black E (CBE), a specific stain for fungal hyphae, as described in the concerning paragraph (M&M 1). The analysis of mycorrhizal presence in the root tissues did not reveal the presence of mycorrhizal hyphae for all treatments applied. Some death spores were found but were presumably a remnant of the sand used as growth medium before to be autoclaved. As shown in the picture below (Fig 30), following the root pigments removal through KOH treatment, root tissues was devoid of blue-stained hyphae characteristic of arbuscular mycorrhizae presence focused by CBE.



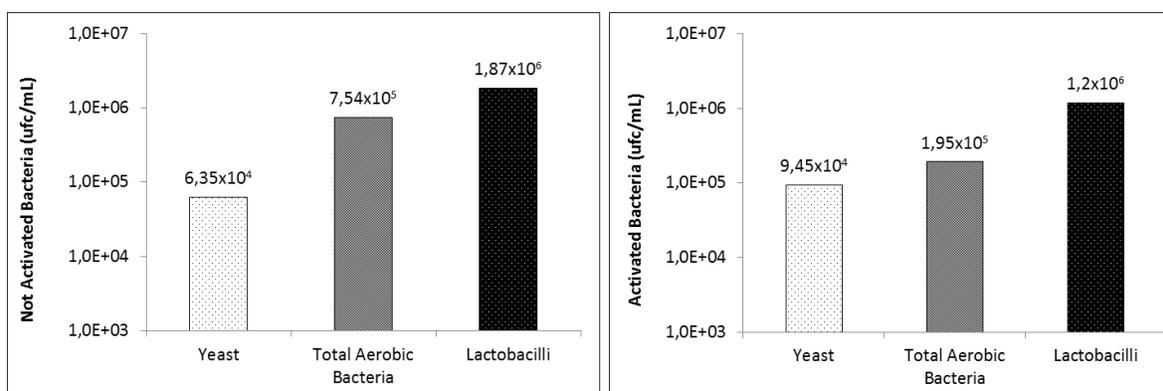
**Figure 30:** microscope captured picture of *Lolium perenne* roots.

### 3.3.2 MICROBIAL ANALYSES

#### Microbial analysis of the EM-1® mix

##### Microbial quantification

Lactobacilli, total aerobic bacteria and yeasts were counted on MRS agar, SDA and NA respectively. The bacteria counts evidenced that activation step revitalized the inoculum but did not increase the bacterial number (Graph 7). Lactobacilli were the most represented group of microorganisms, being at levels of  $10^6$  cfu/ml, while total aerobic bacteria and yeast were counted at  $10^5$  sfu/ml and  $10^5$  cfu/ml respectively.



**Graph 7:** total yeast, aerobic bacteria and lactobacilli content (UFC/mL) in the Not-Activated EM-1 and Activated EM-1.

##### Phenotypic identification

The isolated colonies were observed at the stereomicroscope and have been identified the main morphological characteristics. The morphological analysis of the isolated colonies conducted with the optical microscope allowed to certify, for all strains isolated, presumptively lactic acid bacteria, the presence of stick morphology. The bacteria isolated in TSA showed a rod-shaped and could form groups of two, three, or more individuals. The morphological observation of the isolates in Sabouraud DA confirmed their belonging to the group of yeasts.



**Figure 31:** View with a optical microscope by immersion (1000X) of a group of lactobacilli (gram stained).

## API® Test

The phenotypic identification conducted on lactobacilli and yeast by the analysis of the fermentation profiles and assimilation of different carbon sources, has allowed a preliminary identification to species level of microbial isolates. The metabolic profiles of lactobacilli and yeasts were analyzed with APIWEB® software, utilizing two different kit: API50 CHL® and API20 C AUX® for lactobacilli and yeasts respectively (Figure 32). Utilizing the API50 CHL® test, strains LA2, LA3, LA6, LA8, LA9, LA10, LA11 and LA12 were attributed to the specie *Lactobacillus brevis*, while strain LA7 was instead identified as *Lactobacillus buchneri* (Table 7).



**Figure 32:** Fermentation profile of 50 galleries API 50 CHL® obtained after 72 hours of fermentation. On the left: LA6 strain, identified as *Lactobacillus brevis* 2; on the right LA7, identified as *Lactobacillus buchneri*.

The identification of yeasts isolated was performed with the API20 C AUX® test. Yeasts identified strains were L1, L2 and L3 to L4 species as *Candida utilis* and the species as *Candida krusei* / *inconspicua* (Table 8).

STRAIN	Identification %
LA 1	nd
LA 2	<i>Lactobacillus brevis</i> 3 (94,0%)
LA 3	<i>Lactobacillus brevis</i> 2 (95,1%)
LA 4	nd
LA 5	nd
LA 6	<i>Lactobacillus brevis</i> 2 (99,9%)
LA 7	<i>Lactobacillus buchneri</i> (94,9%)
LA 8	<i>Lactobacillus brevis</i> 3 (99,7%)
LA 9	<i>Lactobacillus brevis</i> 2 (99,9%)
LA 10	<i>Lactobacillus brevis</i> 3 (99,8%)
LA 11	<i>Lactobacillus brevis</i> 3 (78,9%)
LA 12	<i>Lactobacillus brevis</i> 3 (97,3%)
<i>L. brevis</i> ATCC 14869	<i>Lactobacillus brevis</i> 3 (99,7%)
<i>L. buchneri</i> 4005	<i>Lactobacillus buchneri</i> (95,4%)

**Table 7:** specie of lactobacilli identified with the analysis of metabolic profile and elaborated by APIWEB software.

STRAIN	Identification %
L 1	<i>Candida utilis</i> (99,8%)
L 2	<i>Candida utilis</i> (94,8%)
L 3	<i>Candida utilis</i> (97,3%)
L 4	<i>Candida krusei/inconspicua</i> (84,5%)

**Table 8:** specie of yeasts identified with the metabolic profile elaborated by APIWEB software

Most of the *Lactobacillus* fermented L-arabinose, ribose, D-xylose, galactose, glucose, fructose, mannose, melibiose, gluconate and 5-cheto-gluconate. Six of the isolates (LA1, LA4, LA5, LA13, LA14 and LA20) fermented only galactose, glucose, fructose after five days incubation. Six strains (LA6, LA9, LA15, LA17, LA22, LA25) could also grow on mannitol, maltose, melezitose, raffinose and D-arabitol. In addition LA6 and LA25 ferment melibiose and sucrose. A group of six isolates was positive to arabinose, ribose, D-xylose, galactose, glucose, fructose and gluconate. The remaining seven strains ferment L-arabinose, ribose, galactose, glucose, fructose, gluconate and 5-ketogluconate and hydrolyze esculin; five of them were also positive to  $\alpha$ -methyl-D-glucoside, cellobiose, maltose, melibiose and raffinose, while the other two to sucrose and xylitol.

### PCR – genre identification

The identification of lactobacilli and aerobic bacteria was performed with a method of molecular identification, based on a PCR clustering aggregation. Previously the extraction of genomic DNA was necessary. DNA extracted from isolated strains was quantified and reported in the following table:

**Table 9:** Concentration of Lactobacilli.

Lactobacilli DNA		
Strain	Concentration ng/ $\mu$ l	RATIO
LA 1	41.8	2.15
LA 2	66.8	1.89
LA 3	32.7	2.16
LA 4	414.3	2.11
LA 5	152.4	1.90
LA 6	246.9	2.17
LA 7	-	-
LA 8	97.3	1.97
LA 9	57.8	2.06
LA 10	62.8	2.01
LA 11	49.9	1.89
LA 12	673.7	1.95

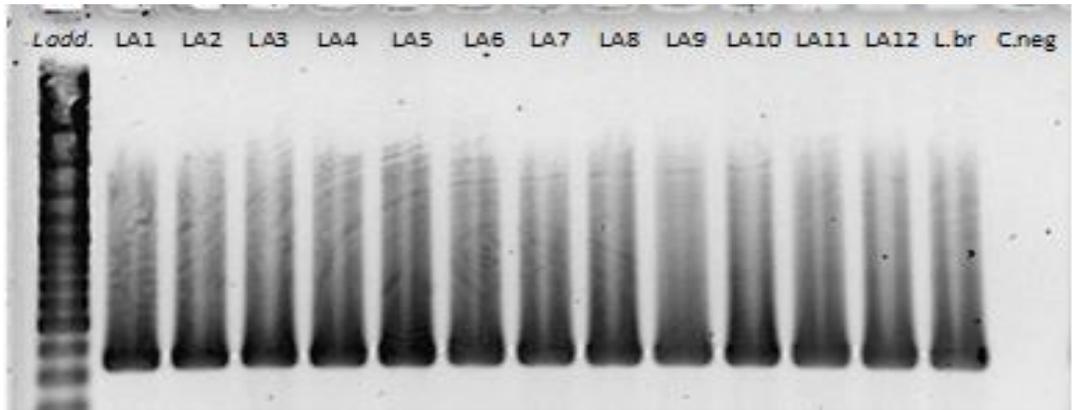
**Table 10:** Concentration of aerobic bacteria.

Aerobic bacteria DNA		
Strain	Concentration ng/ $\mu$ l	RATIO
A 1	243.7	2.06
A 2	92.9	2.01
A 3	666.7	1.48
A 4	374.5	2.06
A 5	116.2	2.06

The DNA extraction conducted allowed obtains a high purity degree of genomic material, with main values between 1.89 and 2.17. Only the strain A3 had a value significantly lower (1.48), which has indicated a contamination by protein and the subsequent exclusion from future tests.

The identification of lactobacilli by API® test has decreed the belonging to the *Lactobacillus* genus from many strains. The confirmation was obtained running a PCR-genre specific (specific for *Lactobacillus*) performed on

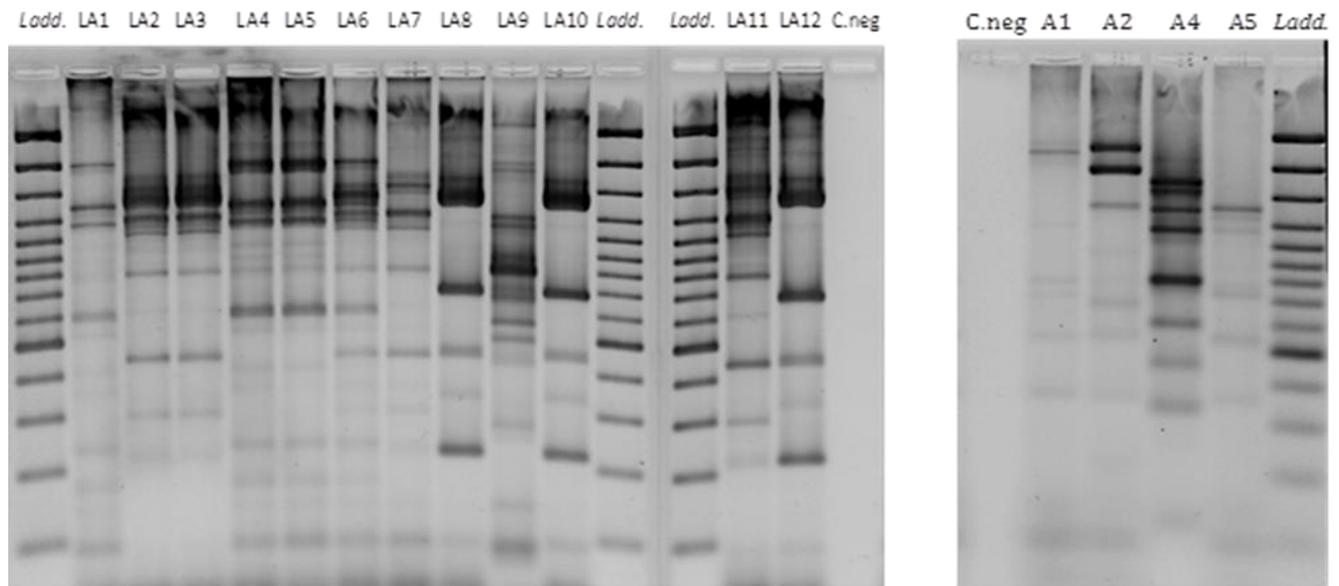
all isolated strains and the positive control *L. brevis* ATCC 14869 (*L. br*). The amplification confirmed the presence of a single band and then the belonging to the genus *Lactobacillus* for all the strains (Figure 33).



**Figure 33:** PCR-genre specific: fingerprints of lactobacilli strains.

The isolated of lactobacilli and aerobic bacteria were genetically grouped using ERIC-I and ERIC-II. The amplification of the DNA extracted from lactobacilli was shown by electrophoresis (Figure 34.a). The analysis of fingerprinting allowed to group 12 strains from the EM-1 solution in 6 groups. On the basis of the fermentation patterns and genotypic results a representative isolate from each group (LA1, LA3, LA6, LA7, LA9 and LA12) was selected for sequencing of the 16S rRNA gene.

The amplification of the DNA extracted from aerobic bacteria was shown by electrophoresis (Figure 34.b). The genotypic grouping and the sequencing results allowed the selection of five groups. As for lactobacilli a representative isolate from each group (A1-A5) was selected for sequencing of the 16S rRNA gene.



**Figure 34:** ERIC-fingerprinting. **34.a** lactobacilli electrophoresis. **34.b** Aerobic bacteria electrophoresis.

Strains belonged to *Stenotrophomonas maltophilia* (ten isolates, three different strains), *Microbacterium trichotecenolyticum* (seven isolates) and *Escherichia coli* (eight isolates) (Table 11). Twenty-five yeasts isolated from SDA were identified by API20C AUX1 as *Candida utilis* (15 isolates) and *Saccharomyces cerevisiae* (ten isolates).

**Table 11:** Strains isolated from EM-1 product.

Isolated strain	bp	Accession	Closest match	Accession	S_ab	DGGE <sup>a</sup>	DGGE profile
<b>La1</b>	1371		Uncultured compost bacterium	FN667288	0.972	8	F-EM, EM
<b>La3</b>	809	JX426086	<i>Lactobacillus buchneri</i>	HM162413	0.995	p7	EM•1 <sup>®</sup>
<b>La6</b>	1439	JX426087	<i>Lactobacillus parafarraginis</i>	AB262734	1.000		
<b>La7</b>	808	JX426088	<i>Lactobacillus buchneri</i>	AB425940	1.000		
<b>La9</b>	1390	JX426089	<i>Lactobacillus parafarraginis</i>	AB262734	1.000	p5, 3	EM, EM•1 <sup>®</sup>
<b>La12</b>	1441	JX426090	<i>Lactobacillus diolivorans</i>	HM218272	0.996		
<b>A1</b>	1382	JX426091	<i>Stenotrophomonas maltophilia</i>	HM355743	0.999		
<b>A2</b>	1386	JX426092	<i>Stenotrophomonas maltophilia</i>	HM355743	1.000		
<b>A3</b>	1395	JX426093	<i>Stenotrophomonas maltophilia</i>	HM355615	0.999		
<b>A4</b>	1385	JX426094	<i>Escherichia coli</i>	CU928161	0.996		
<b>A5</b>	1370	JX426095	<i>Microbacterium trichotecenolyticum</i>	HM032796	0.994		

## Microbial analysis from the samples of roots and soil

Utilizing the Power Soil DNA Isolation Kit (MO – BIO) the microbial DNA was extracted from the roots and the growing medium. With this isolation kit was obtained a good level of purity of genomic DNA as shown in the following table:

**Table 12:** Concentration of the DNA extracted from roots (left) and from soil (right), with purity ratio.

Roots	Concentration (ng/μl)	RATIO	Soil	Concentration (ng/μl)	RATIO
CTR r	54.0	1.86	CTR s	5.20	1.37
NFI r	71.0	1.86	NFI s	3.20	1.68
NFNI r	45.6	1.83	NFNI s	3.00	2.50
FI r	57.9	1.82	FI s	3.50	1.40
FNI r	51.1	1.82	FNI s	2.80	3.50
GG2 r	32.6	1.88	GG s	2.40	2.00

The DNA extracted from the roots shown a good quality level, with a ratio between 1.81 and 1.88. The DNA extracted from the sand had a very low concentration and shown a low quality with ratio values diverging from the optimal range values.

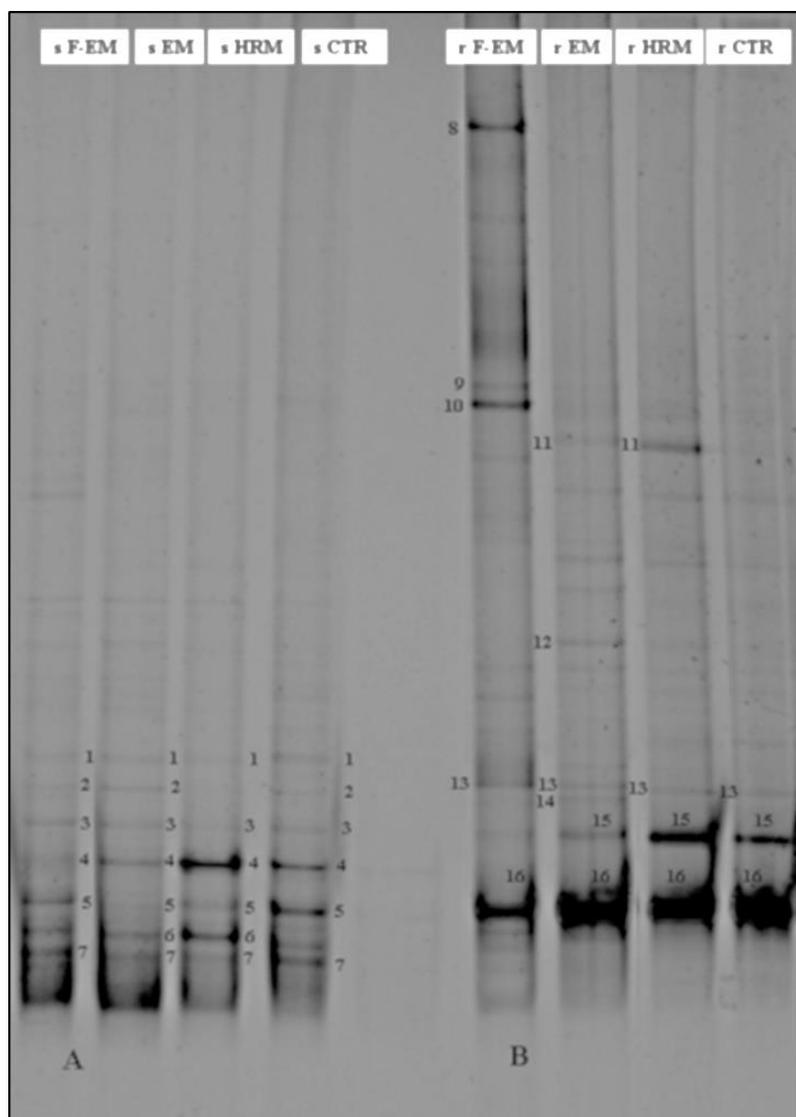
### **Characterization of bacterial communities by PCR-DGGE**

Genomic DNA from sand and surface-sterilized root was used to evaluate the impact of the different thesis on their microbial profile using a PCR-DGE. Sixteen PCR-DGGE bands have been identified by sequencing (Table 13).

The PCR-DGGE analysis revealed the presence of a complex variety of microorganisms in the roots (Fig X.B) and sand (Fig. 35.A). The roots' profiles revealed a consistent bacterial presence with EM-1 and F-EM-1 thesis (Fig 35.B). The band n°16 was the biggest band in roots' profile PCR-DGGE, corresponding to the chloroplast DNA and the preferential amplification of have reduced the yield of amplification of microbial DNA, which clearly has given rise to fainter bands, difficult to be excised. Band n°13 was present in all root profiles, and subsequent sequencing revealed a clos similarity to *Ohtaekwangia kribbensins*.

Sand and root profile showed high similarity of band n° 14 and 15 (root DGGE) and n°2 and 3 (sand DGGE). Exclusively in Filtered-EM-1 were presence bands n° 8, 9 and 10, corresponding to *Flavobacterium* sp. *Leptospira* spp. and *Cytophaga* sp., microorganisms generally present in the water.

EM-1 treatment showed the presence the bands n°12 and n°14, respectively close related to Bacteroidetes bacterium (bacteria of water) and *Hydrogenophaga* spp.(root endophyte).



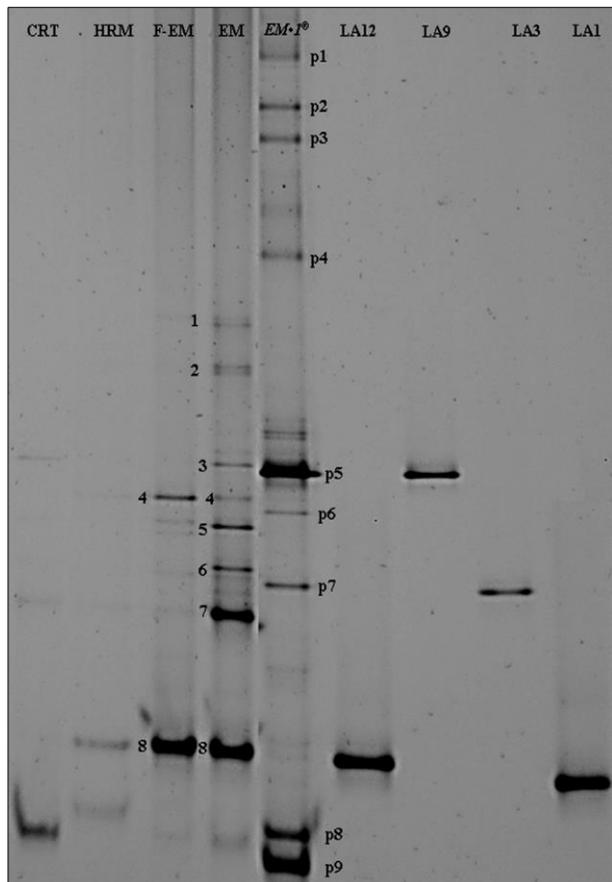
**Table 13:** PCR-DGGE bands from soil treated microorganisms and phytohormons.

DGGE band	Phylogenetic group
1	<i>Lewinella</i> (28%)
2	<i>Hydrogenophaga</i> (100%)
3	Fibrobacter (55%)
4	<i>Vogesella</i> (100%)
5	<i>Polaromonas</i> (97%)
6	<i>Lactobacillus</i> (99%)
7	<i>Acidovorax</i> (50%)
8	<i>Flavobacterium</i> (100%)
9	<i>Leptospira</i> (100%)
10	<i>Cytophaga</i> sp. (55%)
11	Niastella (100%)
12	<i>Ohtaekwangia</i> (90%)
13	<i>Ohtaekwangia</i> (100%)
14	<i>Hydrogenophaga</i> (100%)
15	Fibrobacteres (44%)
16	Chloroplast

**Figure 35:** DGGE bands obtained by amplifying metagenomic DNA with universal primers from sand (A) and roots (B). Treatments was: CTR (control), HRM (hormone), F-EM (Filtered EM-1 solution), EM (EM-1 solution)

A further PCR-DGGE was performed, utilizing specific primers targeting LAB (Lactic Acid Bacteria) to better investigate the presence of lactobacilli within root tissues. The colonization of lactobacilli obtained by CTR, HRM, F-EM and EM-1 treatments and *Lactobacillus* isolates (*Lactobacillus* sp. LA1, *L.buchneri* LA3, *L.parafarraginis* LA9 and *L. diolivorans* LA12) were shown in the PRC-DGGE profiles (Fig X), and in the alongside table are reported the best-match identified bands. *L. parafarraginis* (LA 9) was found in the root tissues of plants treated with EM (bands 3 and 5). *Lactobacillus* spp. (LA 1) was observed in the roots treated with EM and F-EM (bands n°8). Bands 2, 5, 6 and 7, found in roots treated with EM, were closely related to *L. sanfranciscensis*. Band n°1 was found in roots treated with EM and revealed a closely match to *L. pentosus*. Differently by plants treated with EM, the EM-1® activated product did not show the presence of LA1, *L.*

*sanfranciscensis* and *L. pentosus*. Control and HRM treatment did not revealed the presence of microbial profile, due to the absence of lactobacilli in the distilled water and in the phytohormone solution respectively.



**Table 14:** Lactobacilli identified from the root tissues of *Lolium perenne* L.

DGGE band	Phylogenetic group	Closest match
1	Lactobacillus (100%)	Lactobacillus pentosus
2	Lactobacillus (100%)	Lactobacillus sanfranciscensis
3	Lactobacillus (100%)	Lactobacillus parafarraginis
4	Exiguobacterium (100%)	Exiguobacterium sp
5	Lactobacillus (100%)	Lactobacillus sanfranciscensis
6	Lactobacillus (100%)	Lactobacillus sanfranciscensis
7	Lactobacillus (100%)	Lactobacillus sanfranciscensis
8	Lactobacillus (100%)	Uncultured compost bacterium
p1	Lactobacillus (100%)	Lactobacillus diolivorans Lactobacillus farraginis
p2	Lactobacillus (99%)	Lactobacillus parafarraginis
p3	Lactobacillus (100%)	Lactobacillus diolivorans Lactobacillus farraginis
p4	Lactobacillus (100%)	Lactobacillus parafarraginis
p5	Lactobacillus (100%)	Lactobacillus parafarraginis
p6	Lactobacillus (100%)	Lactobacillus parafarraginis
p7	Lactobacillus (100%)	Lactobacillus buchneri
p8	Lactobacillus (100%)	Lactobacillus diolivorans Lactobacillus farraginis
p9	Lactobacillus (98%)	Lactobacillus zeae Lactobacillus casei Lactobacillus paracasei

**Figure 35:** DGGE bands obtained amplifying DNA with Lactobacillus spp. specific primers from root samples, activated EM\_1W and pure cultures (LA1, LA3, LA9, LA12). CTR, control; HRM, treatment with hormone rich solution; F-EM, treatment with filtered/activated product; EM, treatment with activated product; EM\_1W, the activated product.

### 3.4 DISCUSSION

In the first step of the experimental thesis has been studied the effects that different commercial products based on microbial-mixes or bio-stimulants have on the grass essence *Lolium perenne* L. (perennial ryegrass). The plants were sown in a sterilized growing medium and irrigated with a hydroponic system within a climatic chamber in order to simulate the growing condition of a professional sport turf.

The primarily characterization of the EM-1 mix showed the presence of a high number of lactobacilli and yeast in the microbial solution. No significant differences between “activated” and “not activated” microbial solutions were detected. The taxonomic identification of the isolates allow to ascribe the lactobacilli to the species *L. brevis* and *L. buchneri*; while yeasts were principally identified as *C. utilis* and *C. krusei*. These lactobacilli and yeasts species are known for their activity in the bio-control against phytopathogens and pathogens of food (Gaggia et al., 2011). In addition these species were described as bio-degraders of organic substances, and therefore potentially valuable in soils with accumulations of undecomposed organic substances (Lopez et al., 2011; Gao et al., 2008; Valerio et al., 2008).

The morphological and physiological results allowed to determine the main effects of the different microbial treatments on the perennial ryegrass. The commercial microbial mix Embio determined the most vigorous habitus, and shown the highest elongation of roots and leaves if compared with the other microbial treatments. However this pronounced growth determined a substantial lowering of the dry/fresh biomass. On the other hand EM-1 microbial mixes determined a reduction of the growth of roots and leaves, while dry/fresh biomass ratio and DW/L (dry weight biomass/length) were both significantly increased. These results can be related with a protective role against oxidative stresses by effective microorganisms (Higa T. and Parr JF, 1994) and an indirect stimulation of bio-regulators such as the plant hormone's ABA (Arshad and Frankenberger, 1998) that has important role in plant response to abiotic stress (Zhang et al., 2006).

The different growth of the roots treated with the two microbial mixes (EM-1 and Embio) is an interesting result that could be interpreted as a different microbial composition within the two products. Indeed PGPB mainly content in the Embio solution have the attitude to promote the growth of roots with a phytohormone-like activity (Arshad and Frankenberger, 1998), while EM effectiveness would derive by the synthesis of compounds which protect the plants by a stress reduction (Gerhardt *et al.* 2009), such as the degradation of contaminants, protection from pathogens, provision of essential nutrients.

The tear-off test showed the highest values for the Embio-NF and EM-1 treatments. Phytohormone solution determined lower values for tear-off although it showed highest values of elongation and biomass for both roots and leaves. This result can be related with a general influence of rhizosphere microorganisms on the root growth and morphology, because some of them not stimulate root elongation but also lateral root (Schönwitz and Ziegler, 1989) and root hair formation (Martin et al., 1989).

The color analysis of the leaves, showed a significant effect given by microbial treatments if compared with phytohormone and control: Embio-NF, Embio-f and EM-1 NF determined the darker coloration and therefore a better visual performance respect to the control, while the phytohormone and the control were definitely two separated entity. This data is a further confirmation that microorganisms, inoculated in a soil lack of microbial biodiversity and with a simple composition can improve the physiological conditions of the turfgrass.

At the end of the two months experimental period, the clods of perennial ryegrass were collected in order to analyze the bacterial colonization within root tissues and in the rhizosphere. Before to proceed with the identification of the bacteria that colonized the root tissues, has been conducted a microbiological characterization of the products containing effective microorganisms. This solution shoed a high presence of lactobacilli and yeasts. The preliminary taxonomic identification of the isolates allowed to ascribe at the lactobacilli group mainly the species *L. buchneri* and *L. brevis*; while yeasts were principally identified as *C. utilis* and *C. krusei*. All these species are known for their bio-control activity against food pathogens (Gaggia et al, 2011) and in addition to producing useful metabolites and degrading the organic substance (Gao et al., 2008). The genomic DNA extraction performed for the root tissues showed a good bacterial colonization; while in the rhizosphere the genomic DNA concentration resulted significantly lower. This difference may be attributed at the initial sterilized condition of the growing medium that determines a higher difficulty for the bacteria to colonize this kind of substrate (Elliot and Des Jardin, 2001).

The real-time PCR analysis showed significant increasing of the concentration of lactobacilli within the root tissues for all theses if compared to the control, indicating good colonization propriety of this group of bacteria. The high concentration of bacteria founded in the plants treated with the phytohormone solution may be determined by the rhizodeposition that stimulate as carbon source the macrobiotic proliferation (Mueller and Kussow, 2005). In addition the low concentration of bacteria detected in the control thesis is probably determined by the initial condition of the seeds that were not sterilized before the sowing.

Lactobacilli are not widespread as plant growth promoting bacteria in the sport turf market but these positive results could lead to a possible use as beneficial bacteria and recycling of the organic matter that represent a objective problem in many golf courses. In addition, considering some in vitro studies on their strong activity against plant pathogens may have a contrast function on the onset of common turf disease (Berg et al, 2006; Kerry, 2000).

The profile of microbial communities performed with the DGGE showed a higher complexity in terms of number of species present in the root treated with EM-1 respect all other treatments. Species isolated in this study (*L. buchneri*, *L. parafarraginis* and *L. diolivorans*) and those identified with PCR-DGGE (*L. pentosus* and *L. sanfranciscensis*) are commonly utilized to improve the stability of silages, thanks to the production of a wide range of metabolites inhibiting moulds and fungi (Holzer et al, 2003; Zhang et al, 2010). The yeasts identified within root tissues are common residents of soil and rhizosphere, with role of growth promotes and soil-borne fungal antagonists.

Concerning the aerobic bacteria isolated in EM-1 solution, some strain belonging to *Stenotrophomonas* sp., and *Microbacterium* sp., were found in strict association with plant host. *S. maltophilia* found in ryegrass' roots has growth promotion properties previously described for some commercial crop: it promotes the production of antifungal metabolites and phytohormons (IAA, GA and ABA).

## **Effects of original biostimulants containing effective microorganisms and growth promoters on an *Agrostis stolonifera* putting green in a semi-field condition**

### **4. 1 SECTION OBJECTIVES**

In the second step of this thesis thanks to the knowledge acquired during the previous experiments, were developed two different original mixes composed by biostimulants and effective microorganisms. These solutions were tested with a water only control and a commercial product on an *Agrostis stolonifera* putting green in a semi-field condition.

The main experiment was performed on a professional golf course putting green within the Modena Golf & Country Club near Maranello (MO, Italy). Furthermore the experimental trial was repeated within a growth chamber adopting the same protocol utilized in previous tests in order to compare the data of the two original microbial stimulants with the first section's results.

The experimental trials were performed during July and August 2012. This two months period was chosen because temperature and intensity of solar radiation typical of the summer period are limiting factors for the quality of a putting green surface. In addition the top soil temperatures of the summer were supposed to allow a range of optimum development for the inoculated bacteria's.

## 4.2 MATERIALS AND METHODS

### 4.2.1 LOCATION

The second step of this experimental thesis was performed testing different treatments principally based on biostimulants and effective bacteria, both within a growth chamber and on a professional creeping bentgrass (*Agrostis stolonifera*) putting green. The putting green is located inside the Modena Golf & Country Club (Modena, Italy), a sport facility spread across an area of over one hundred hectares, that includes two types of trails, an 18-hole “Beanhard Langer” championship Course, par 72, 6423 meters long, inaugurated in 1990 and a 9-hole Executive Course, par 27, 976 meters long.



**Figure 36:** A section of the Modena Golf & Country Club.

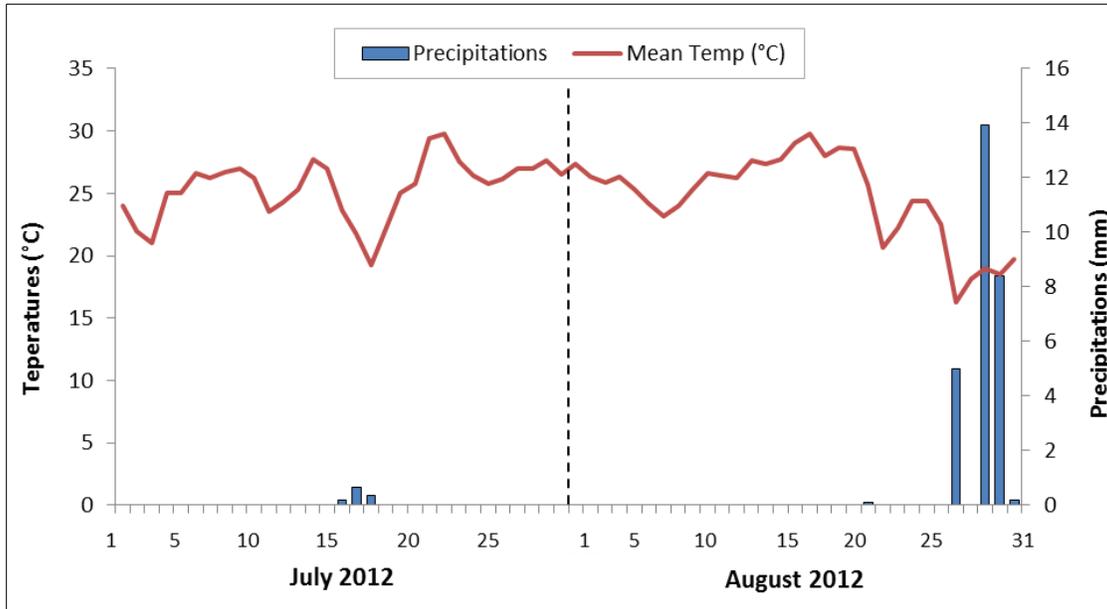
The golf club was designed in the early '80 and the construction began in 1987. It is constituted by wide greens and tees, smoothly winding fairways and numerous bunkers and five large water hazard (Figure 36). Actually the Modena Golf hosts high level competitions such as the International Open, the National Championship and other competitions throughout the sport season.

### Weather conditions

Maranello is located in the North-East of Italy, a temperate sub-continental region, with hot - humid summers and cold - rigid winters. The regime of precipitations is characterized by two peaks, spring and autumn, which do not differ much by them for quantity, while the summer is the dry season.

In the last two decades in Emilia Romagna was measured a wide change of the local climate if compared to the reference period 1961 – 1990, with significant increase of the mean temperatures (+1,1°C) during the whole durate of the year and of the summer peaks (+ 2°C) (Arpa Emilia-Romagna – Weather).

Meteorological data utilized for this thesis were collected with reference to the ARPA weather station of Maranello (MO, Italy), placed 14 Km from the Golf Club of Modena. The temperatures measured during the experimental trial showed values within the means of the period. The precipitations, has been near zero for the whole duration of the test, with a heavy downpour at the end of August as shown by the graph below (Graph 8).



**Graph 8:** Observed temperatures (°C) and precipitations (mm) measured by the meteorological service of the Aeronautica Militare, from the weather station of Modena.

## 4.2.2 TREATMENTS

In this experimental trial were examined two original microbial designed and formulated by the Department of Agricultural Sciences, University of Bologna and identified with the initials DiSTA and DiSTA plus. In order to compare the results a negative and a positive control were tested too (Table 15). The four thesis tested were a negative control (distilled water), a positive control (commercial bacteria solution EM-1, EM-RO<sup>®</sup>, Japan), the DiSTA solution (bacteria only) and DiSTA PLUS solution (bacteria with mycorrhiza and humic acid).

**Table 15:** Treatments examined and dilution applied for the experiment

N° THESIS	TREATMENTS	DILUTION	PRODUCER
1°	Distilled water		-
2°	EM-1 (1:500)	1:500	EM-RO
3°	DiSTA	1:500	DiSTA
4°	D-PLUS	1:500	DiSTA
*No additional fertilizers utilized in the putting trial			
** Nutrient solution (Hoagland) added in the growth chamber trial			

As negative control (Thesis n° 1) was used distilled water with the addition of Hoagland nutrient solution only in the growth chamber trial; indeed no additional nutrients were administered in the field trial in respect of the fertilization schedule of the current season. As positive control (Thesis n° 2) was used the commercial product EM-1 (EM-RO<sup>®</sup>, Japan), containing effective microorganisms lactic acid bacteria, yeasts and photosynthetic bacteria plus Hoagland solution. The "activation" of the product EM-1<sup>®</sup> was performed according to the procedure reported by the manufacturer, which plans to dissolve in distilled water a quantity of product equal to 5% (v / v), together with 5% (v / v) of molasses. The solution thus prepared was left in an incubator at 35 ° for 5 days. Before treatment, the product was further diluted in water to a final dilution of 1:500. The choice of this product has been made on the basis of previous experiments conducted by the research group coordinated by Prof. Dinelli and the Group of Microbiology, coordinated by Dr. Diana Joy (Gaggia et al., 2013).

On the basis of the results obtained in previous growth chamber trials, were formulated two original microbial based mixes, DiSTA (thesis n°3) and D-PLUS (thesis n°4). The mixtures of microorganisms were prepared weekly starting from pure strains reared separately according to their respective needs and growth times and then mixed just before the field/growth chamber inoculation. DiSTA and DiSTA PLUS were composed by the same bacteria matrix but differed by the following addition of furthermore biostimulants. These solutions were prepared weekly, using as the growth medium, respectively, the MRS agar (Merck, Darmstadt, Germany) and the ground Sabouraud Dextrose Agar (Merck, Darmstadt, Germany ). The bacteria were incubated for 4 days at

37 ° under anaerobic conditions, while the yeast for 48 hours at 25 ° aerobically. The different genera of bacteria after the incubation period reached an average concentration of 10<sup>8</sup> ufc. The concentration of bacteria in the microbial mixtures was checked by plate counts.

The colonies were removed and re-suspended in saline (NaCl 0.8%) and then were diluted 1:500 in distilled water. The product DiSTA plus were added 3 g of spores of mycorrhiza and 3 mL of humic acid. Within a few hours after preparation of the products, they were transported to the experimental field for the application.

The bacterial mix of DiSTA and DiSTA PLUS (Table 16) were composed by the following ingredients:

**Table 16:** DiSTA (Blue) and D-PLUS (Red) content.

D i S T A	D - P L U S	Strain's code	Identification	Origin	Probable action	
		<b>Bacteria</b>				
		LB2	<i>L. buchneri</i>	Corn silage	Antagonistic-protective action (competitor against pathogens such as fungal and bacteria)	
		LB6	<i>L. parafarraginis</i>	Corn silage	Antagonistic-protective action (competitor against pathogens such as fungal and bacteria)	
		LB8	<i>L. diolivorans</i>	Corn silage	Antagonistic-protective action (competitor against pathogens such as fungal and bacteria)	
		LB9	<i>L. plantarum</i>	Corn silage	Antagonistic-protective action (competitor against pathogens such as fungal and bacteria)	
		TS1	<i>Stenotrophomona maltophilia</i>	Forest soil	Degrading-destructive action (reduction of the thatch)	
		TS12	<i>Bacillus subtilis</i>	Forest soil	Degrading-destructive action (reduction of the thatch); Anti-bacterial and anti-fungal action against pathogenic species	
		SB1	<i>Candida</i> sp.	Forest soil	Antagonistic-protective action (competitor against pathogens such as fungal and bacteria)	
		<b>Mycorrhizae</b>				
	<i>Glomus intraradices</i>	Forest soil	Protective function, reduction of biotic and abiotic stresses. Increasing of the nutrient up-take and translocation of mineral elements through internal arbuscular structures.			
<b>Humic acid</b>						
	Leonardinte	Biodegradation of lignite	Improving of the electronic cation exchange and physical and chemicals soil's proprieties, promotion of the plant's growth.			

### **Application of the products in growth chamber and on the putting green**

The treatments were applied weekly for a period of two months, between July 10 and September 11 2012. For the growth chamber trial, the different treatments were spilled in relative water tank and brought in circulation from the pumps of the close hydroponic system.

In the golf course's trial the treatments (Figure 36) were applied during the closing day to avoid any leakage or contamination given by the trampling of athletes. Two shoulder-pump were used to spray the different solutions: one pump was first used for spraying of the water only control and then of the EM-1 microbial solution, while the other pump was used for spraying the DiSTA microbial solution and then D-PLUS microbial and biostimulants solution. Within a few minutes from the application of the treatments the irrigation system around the experimental area was turn on for two minutes to promote washing of the products from the leaves and the absorption in deeper layers of the turf.



**Figure 37:** pictures featuring two subsequent steps of the treatment's application on the putting green. Spraying the products on the left and the successive washing by the irrigation system on the right.

### **Growth chamber trial**

For the entire duration of the growth chamber trial (Figure 38) was followed the same protocol utilized in the previous experiment in order to grow up the plants in the same condition and to compare the new data with those collected in the first step. The perennial ryegrass was sown in 12 cm of autoclaved sand growing medium, with 2 cm pumice on the bottom and 0,5 cm of vermiculite as upper layer. Water and nutrient solution (Hoagland diluted 1:1 with distilled water) were supplied with four separated closed hydroponic system, in order to maintain constantly separated the different microbial treatments.

Light was supplemented with an artificial illumination at  $550 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ . Photoperiod was set at 16 hours of light and 8 hour of dark condition. Growth chamber settings were set up  $24^{\circ}\text{C}$  and 70% relative humidity (RH) during the day, and  $20^{\circ}\text{C}$  and 50% RH in night conditions.



**Figure 38:** Perennial ryegrass plants in the early stages of growth inside the growth chamber. In the picture are shown the pots with drip irrigation system and the aluminum which closes the space of light infiltration.

The Hoagland nutrient solution (Table 17) added to each thesis to ensure a balance micro and macro nutrient supply had the following mineral composition:

**Table 17:** Hoagland solution components

Hoagland components	g/L
2M KNO <sub>3</sub>	202g/L
2M Ca(NO <sub>3</sub> ) <sub>2</sub> x 4H <sub>2</sub> O	236g/0.5L
Iron chelate	15g/L
2M MgSO <sub>4</sub> x 7H <sub>2</sub> O	493g/L
1M NH <sub>4</sub> NO <sub>3</sub>	80g/L
H <sub>3</sub> BO <sub>3</sub>	2.86g/L
MnCl <sub>2</sub> x 4H <sub>2</sub> O	1.81g/L
ZnSO <sub>4</sub> x 7H <sub>2</sub> O	0.22g/L
CuSO <sub>4</sub>	0.051g/L
H <sub>3</sub> MoO <sub>4</sub> x H <sub>2</sub> O	0.09g/L
1M KH <sub>2</sub> PO <sub>4</sub>	136g/L

## Putting green trial

The experimental green on which was held the field trial was located in the Modena Golf & Country Club (Modena, Italy). The putting green was built and managed in accordance with the USGA guideline (USGA, 1994). It was placed at the end of the hole n°6 and consisted in a total surface of 250 m<sup>2</sup> (Figure 39).

As well as all other putting green of the Modena Golf club, the only grassy essence contained within was the *Agrostis stolonifera*, variety Penncross, the most common species utilized for this kind of sports turf surface in the northern Italian distribution area. The growing medium of the putting green was composed by an artificially constructed soil. This was textured with a 30 cm layer composed by a mixture of 80% sand and 20% peat, 10 cm of gravel and then the drainage system.

During the two months of the experimental period, the mowing of the grass was reduced from a frequency of five times per week to one time per week. This management changing was decided in order to study the effects of the different thesis on leaves. In addition, even some extraordinary maintenances were interrupted from June until September such as the aeration.

During the test run, all chemical treatments, such fungicides and pesticides were interrupted in order not to interfere with the metabolism of the microbial component present in the applied treatments. Furthermore this interruption has also allowed to verify the effectiveness of the thesis tested against some common diseases of the putting green such as dollar spot (*Sclerotinia homoeocarpa*).



**Figure 39:** The putting green n°6 at the Modena Golf , before the beginning of the treatments (May 2012).

## Fertilization schedule

In agreement with the program scheduled by the green keeper of the Golf Club, the 27<sup>th</sup> March 2012, was made the first treatment of the season using a granular fertilizer starter Lebanon (Herbotech, Italy), based Meth-Ex 40 methylene urea. The nitrogen present in the product was released in times ranging between 8 and 12 weeks after application. Subsequently, a second treatment of fertilizer was applied to the completion of the test, in date 29<sup>th</sup> August 2012. This second treatment was carried out with Nitrophoska® Start 18-24-05 (Compo, Italy) to ensure proper nutrient balance of the turf surface at the end of the summer season. The characteristics of both the fertilizers used are shown in Table 18 below:

**Table 18:** Specs and dosages of treatments.

LEBANON (HERBATECH, ITALY)	NITROPHOSKA® START (COMPO, ITALY)
<b>Title: 16 – 25 – 12</b>	<b>Title: 18 – 24 – 05</b>
Total Nitrogen (N) .....16%	Total Nitrogen (N).....18%
11.8% Ammoniacal Nitrogen	1.6% N Nitric Nitrogen
1.2% Water Insoluble Nitrogen	4.8% N Ammoniacal Nitrogen
0.9% Urea Nitrogen	5.0% Urea Nitrogen
2.1% Other Water Soluble Nitrogen	6.6% N isobutilidendiurea
Available Phosphate (P <sub>2</sub> O <sub>5</sub> )..... 25.0%	Available Phosphate (P <sub>2</sub> O <sub>5</sub> ).....24%
Soluble Potash (K <sub>2</sub> O) .....12.0%	Soluble Potash (K <sub>2</sub> O).....5%
Sulfur (S) .....2.2%	Iron (Fe).....1%
Chlorine (Cl) not more than.....8.0%	Zinc (Zn).....1%
	Manganese (Mn).....0,5%
<b>Dosage: 20 g/m<sup>2</sup></b>	<b>Dosage: 25 g/m<sup>2</sup></b>
Date of application: 27/03/2012	Date of application: 29/08/2012
	

The test was structured according to a randomized block design with three replications. The parcels had dimensions of 1 m wide and 1 m in length, with a total area of one square meter. Each experimental plot resulted spatially isolated from the other, with distances between the various parcels of 2 meters in order to lower the risk of interference between the different microbial treatments (Figure 40).



**Figure 40:** Scheme of the putting green experiment: randomized block design with three replications. Colors white (Control), Blue (EM-1), Yellow (DiSTA) and Red (D-Plus) represent the different treatments.

### 4.2.3 GROWTH CHAMBER ANALYSIS

#### Growth of leaves and biomass production

Every week the growth of leaves and the biomass production were determined. To measure the height of leaves a picture of the plots was taken inside a light-box with cool led lighting and red background to maximize the contrast of the leaves.

A picture for each pot were taken with a Canon Reflex EOS 350D placed on a tripod with an adjustable height in horizontal position relative to the plane of the leaves. With an image analysis process, the heights of the leaves were determined in 10 points transversally along the diameter of the pot. The height of leaves for each pot was measured as average of the 10 transversally points. After the mowing, clipped leaves were collected in paper

bags to be weighed in laboratory. Fresh weight was measured immediately after the mowing, instead dry weight was determined weighting dry biomass after five days at 50°C in a hoven.

### Color of leaves in RGB space

The color of leaves was assessed with a digital analysis process utilizing Assess 2.0 (American Phytopathological Society, APS press), an image analysis software for plant disease quantification. The pictures were taken in a light-box (Figure 41). The background of the light box had the average color values Red (R) =  $195 \pm 9$ , Green (G) =  $72 \pm 7$  and Blue (B) =  $194 \pm 8$ . The shape of the leaves was extrapolated from the background thresholding with red parameters of 105 – 190. The leaves were evaluated over the 4 cm mowing height. The values considered for the image assessment were the RGB (red, green and blue) color space.



**Figure 41:** Acquisition of the RGB values from the leaves of perennial ryegrass. On the left the light-box with red screen on the background and illumination supplied from cool white led. On the right the pot of perennial ryegrass placed within the light-box.

### Chlorophylls and Carotenoids content

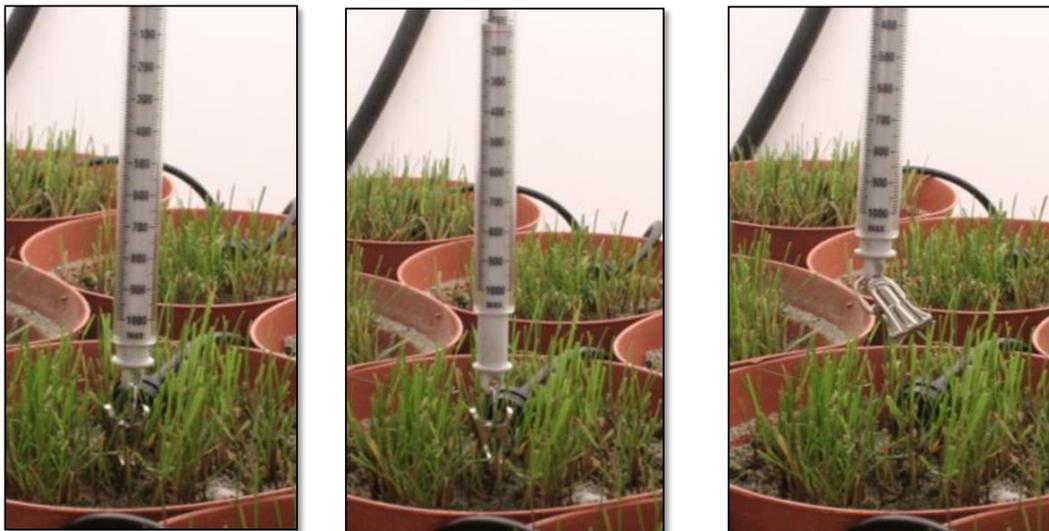
The analysis for the determination of the content in leaf pigments (clofofilla a, chlorophyll b, carotenoids) into the plant tissues, was performed as described by Strckland and Parsons (1972). The leaves were mowed from the pots, weighed (50 mg for sample) and frozen in liquid nitrogen. Afterwards frozen samples were crushed in a

mortar adding 10 mg of magnesium carbonate ( $MgCO_3$ ) to neutralize the acidity of the solutes and prevent the conversion of chlorophyll a in Phaeophytin. At the material crushed were added 10 mL of acetone. The solution thus obtained was placed in dark condition for 12 hours. After the extraction period, the sample were vortex and then centrifuged at  $10^\circ C$  for 10 minutes at 1000 ppm. An aliquot of the supernatant was collected and added to cuvettes with optical path lengths of 1 cm for reading spectrophotometer. The reading was performed at wavelengths of 661.6 nm (which is the maximum absorption peak of chlorophyll a), 644.8 nm (which is the maximum absorption peak of chlorophyll b) and 470 nm (which represents the maximum absorption peak of carotenoids). To calculate the concentration of the leaf pigments have been used the formulas described by Lichtenthaler et al., (2001):

- $c_a$  ( $\mu g/mL$ ) =  $11,24 * A_{661,6} - 2,04 * A_{644,8}$
- $c_b$  ( $\mu g/mL$ ) =  $20,13 * A_{644,8} - 4,19 * A_{661,6}$
- $c_{(c+x)}$  ( $\mu g/mL$ ) =  $(1000 * A_{470} - 1,90 * c_a - 63,14 * c_b) / 214$ .

### Tearing

Tear shoot resistance was tested by dynamometer (PCE Instrument, Lucca, Italy) at the end of the experimental period. The method used consists in measuring the force required to tearing out of the aerial apparatus with the dynamometer (Figure 42). The clamp placed at the base of the dynamometer, composed by small not sharp teeth, was attached to the base of the stem of Lolium. A red marker, placed inside the dynamometer, pointed out the maximum force utilized at the moment of the tear out of the stem from the soil. 4 measures were sampled for each plot.



**Figure 42:** measuring sequence of the needed force to tear out a plant of ryegrass

### **Growth of roots and biomass production**

The determination of the root biomass was performed at the end of the experimental period. The sandy clod was removed from the pot and washed gently dipping the sand and roots within in a tank filled with water. Shaking gently the plant the sand was removed. The residues of sand remained adherent to the roots were removed brushing lightly with hands the surface of the roots. The roots were the dried on an absorbent sheet and then fresh weight was measured with a precision balance. Roots were successively placed in an oven at 50°C for 5 days and therefore the dry biomass was detected.

### **Mycorrhizal analysis**

Mycorrhizal colonization within root tissues was analyzed at the end of the experimental trial on the plant of *Lolium perenne* L. following the same protocol described in the Material and Method, section 1.

## **4.2.4 PUTTING GREEN'S ANALYSIS**

### **EC and pH of soil**

Analysis on the electroconductivity and pH of soil were conducted on sample of putting green topsoil. A portion of soil 5x2,5x2 cm volume was extracted from the putting green to be analyzed in laboratory. The volume of soil were weighted and adjusted at XX grams removing vertical slices.

Sample of soil was placed in a 500 mL beaker and 100 mL of deionized water were added. The sample was mixed for 15 minutes with a magnetic stir bar. Values of EC and pH were analyzed with INSTRUMENT 1 AND 2 respectively.

### **Chemical analysis (C/N) of soil**

Soil portion of 2.5x2.5cm section and 8cm depth was collected from the putting green's topsoil. From all the samples was discarded the first 5 mm layer constituted principally by stolons. The remaining portions of topsoil w utilized for the analysis of the Carbon and Nitrogen content. The samples were completely dried in a oven at 50°C for one week. The samples were finely crushed in a ceramic mortar grinding until obtaining a homogeneous powder. The samples were stored until further analysis, inside a glass bell reduce the moisture content. The elemental analysis of the content of carbon and nitrogen was conducted with a ECS 4010 CHNSO analyzer, according to the method Dumas (1831). The carrier gas (helium) circulates inside the analytical circuit

which consists in a combustion reactor for CHNS. The carrier gas carries the combustion gas to a separation column gas chromatography (GC) and a thermal conductivity detector (TCD) for the CHNS analysis. 35mg (+/- 1mg) of sample were placed in a tin capsule and at intervals fell automatically by gravity in the combustion reactor. The gas flow was 100 ml / min for a time of 50 seconds. The sample and tin capsule reacted with a volume of oxygen and burned at a 1700-1800°C. The gas separated from the GC column is detected sequentially by TCD. The TCD generates a signal proportional to the amount of each element in the sample. The software compares the ECS elemental peak with a peak of a compound standard and generates a report of analysis for each element expressed as a percentage by weight.

### Moisture of soil

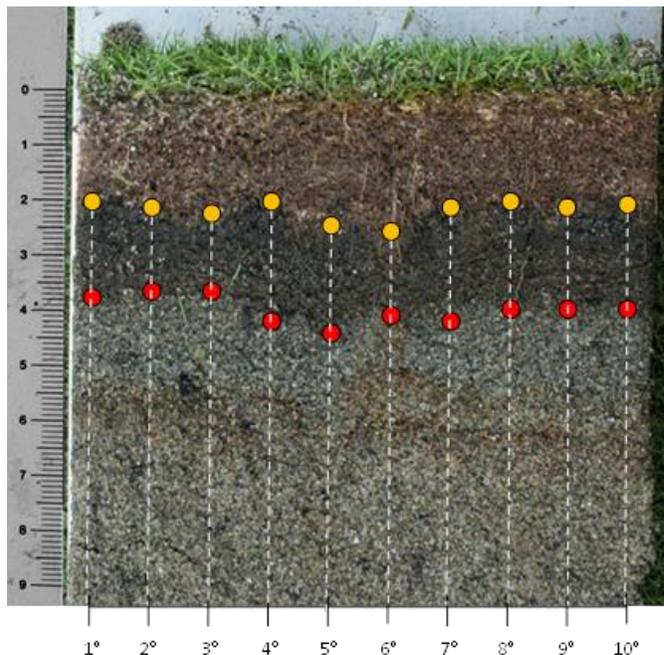
In order to detect the soil moisture determination was a collected a sample of soil of 2.5x2.5 cm section and 8 cm of depth from each plot. Samples were immediately weighted, coarsely fragmented and then placed in semi-closed aluminum containers. Samples were dried in a hoven for approximately one week until the completely desiccation. Finally, the value of soil moisture was calculated as the difference in initial weight – final weight of the soil sample.

### Profile of soil

The thickness of the principal layers of the putting green's soil was measured weekly for the whole durate of the experimental trial. The effect of the different treatments on the thatch and on the humus layers was determined measuring the respective depth from the stolons layer, 5mm below the leaf surface (Figure 43).

From each plot was extracted a vertical section of the soil utilizing an extractor tool with a section of Width 2.5cm x Length 10cm x Depth 10cm.

The thickness of thatch and humus was calculated as mean value of 10 points along the horizontal section of the respective layer.



**Figure 43:** Soil profile analysis. Thatch (yellow) and humus (red) thickness was measured sampling 10 points along the horizontal axis.

### **Biomass of thatch and roots**

The fresh and dry biomass of roots and thatch was measured from sods collected in the experimental putting green. Thatch and roots biomass were measured in a single value. Samples were cut at 0.5 mm from the surface in order to remove leaves and stolons. Samples of sods were washed on a lab sieve (Retsch®, sieve mesh 0.5 mm). Portions of roots and thatch were collected to measure the fresh weight. Samples were dry for 5 days at 50°C to determine the dry biomass.

### **Organic component**

The method applied for the determination of the organic component in the soil was described in Milne et al. 1992, with some adjustment. The soil organic matter was analyzed by portions of soil extracted from a depth ranging between 1 and 2 cm from the grassy surface. From each plot were collected three replicates in random position. In order to remove the humidity contained in the soil, the extracted portions were weighed and then dried at 50°C for 4 days, until complete desiccation. At the end of this phase, the sample was weighed again and then incinerated in a muffle furnace at 550°C for 3 hours. Once removed from the muffle, the samples were cooled in a bell in absence of moisture and then weighed to determine the amount of organic carbon incinerated.

### **Evapotranspiration**

Evapotranspiration was evaluated utilizing subparts of sods removed from the different experimental plots (Figure 44). These portions of soils had a surface area of 2.5 x 2.5 cm and a depth of 5 cm. Each sample was immersed for 15 minutes to a depth of 4.5 cm in water, avoiding to soak the parts constituted by leaves and stolons. Subsequently samples were drain off for a few seconds and then wrapped with parafilm. A further insulating tape was placed on in order to keep the parafilm adherent to the portion of soil. The samples were weighted with a precision scale and placed in a growth chamber artificially lighted with a photoperiod of 16h light and 8 h dark and a temperature of 28 ° C / 20 ° C day/night.

The weight loss of each plate was measured every 24 hours, up to total desiccation of the soil.



**Figure 44:** Clods of soil processed for determination of evapotranspiration

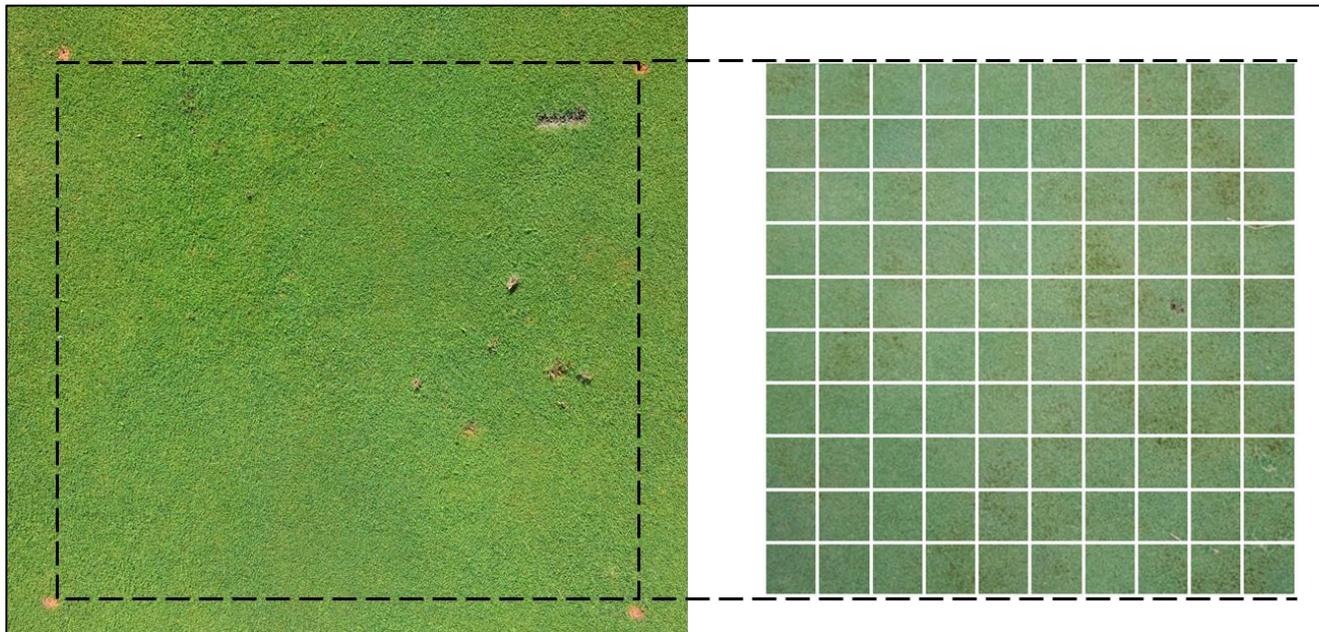
### **Production of leaf biomass**

The analysis of the leaf biomass was conducted taking into consideration both the fresh and the dry biomass of the leaves. Once a week a patch of lawn was sampled and, at the return in the laboratory, leaves were cut and weighed to obtain the weight of fresh biomass. Subsequently the leaves were placed in a oven at 50 °C for 4 days for drying. When all the moisture contained in the leaf tissues was evaporated, the leaves have been re-weighed to determine the dry biomass.

### **Tearing**

Ever on the green, was performed to determine the resistance to tearing of the leaf using a dynamometer for measuring mechanical (PCE Instruments, Lucca, Italy). The end of the dynamometer, equipped with a pair of tweezers, was attached to the base of the stem of a plant of *Agrostis stolonifera* for each plot, 16 by performing sampling and replicating the test in three sessions (beginning, middle and end of trial). The method used consists in measuring the force required to tear seedling from the soil.

## Color analysis



**Figure 45:** Analysis of the putting green's color.

The color of the grass surface was measured taking pictures with a camera Canon EOS 350D in RAW image format. The pictures had a pixel dimension X: 2304 and Y: 3456. The homogeneity of captured picture was obtained by maintaining the same settings of exposure and aperture: 1/800 sec, aperture f/16, focal length 18,0 mm and ISO 1600. With software Adobe Photoshop Lightroom 4, each picture relative at field plot was divided in 10x10 squares (Figure 45). Each square was analyzed singularly for the values of the color in RGB scale and mean for each plot was calculated as mean of 100 sub-squares. To analyze the digital pictures was used the software APS ASSESS 2.0.

## Chlorophylls and Carotenoids content

The clods removed from the field were transported to the laboratory within a frozen container. In laboratory the leaves were mowed and immediately frozen with liquid nitrogen. Thereafter the extraction and the analysis of the photosynthetic pigments were performed following the same protocol utilized for the growth chamber test (as reported in “growth chamber analysis - leaves pigment content”).

### **Inoculation of *Sclerotinia homeocarpa***

At the end of the experimental trial (1<sup>st</sup> September 2012) *Sclerotinia homoeocarpa* fungus (*Sclerotinia homoeocarpa* F.t. Benn., *Annals of Applied Biology*: 24: 236, 1937) was inoculated in each plot of the putting green (Figure 46). Sand carriers infected with *S. homeocarpa* were placed in 2.5 x 2.5 holes in each plot.

The emergence of dollar spot on the green's surface was checked during the months of September and October 2012 and every month from April until October 2013. Each patch of dollar spot was marked with white pickets as shown in Fig 46. The density of dollar spot was calculated as percentage value of n<sup>o</sup> of patches / m<sup>2</sup>.



**Figure 46:** Inoculation of the *Sclerotinia homoeocarpa* within the thatch layer of the experimental putting green.

### **4.2.5 MYCORRHIZAL ANALYSIS**

Mycorrhizal colonization within the root tissues of the Creeping bentgrass was analyzed at the end of the experimental trial. A portion of the putting green's topsoil was extracted from each plot and stored in a portable cooler in order to preserve the samples for the subsequent laboratory analysis. The protocol utilized for the identification and the quantification of the mycorrhiza within root tissues was previously described in the Material and Method, section 1.

## 4.3 RESULTS

### 4.3.1 GROWTH CHAMBER RESULTS

Perennial ryegrass (*Lolium perenne* L.) clods were extracted from pots 60 days after the first treatment. Before to proceed with morphological and physiological analyzes, perennial ryegrass habitus was observed and following reported (Figure 47).



**Figure 47:** *Lolium perenne* L. plants after the 60 days experimental period within growth chamber. Left to right are shown plants treated with Control (distilled water and Hoagland solution), EM-1, DiSTA and D-PLUS.

Preliminary observation of Perennial ryegrass plants showed an effectiveness of microbial treatments in determine observable changes in the habitus of the whole plants. Different biological products tested on plants resulted a lengthening of the roots if compared with the control. Also growth and color changes of leaves demarcate a different effectiveness of microbial treatments. In this particular case EM-1 shown a greenness leaf apparatus with a slight difference in height if compared to the control whereas microbial mixes developed in the Faculty of Agronomy of Bologna, DiSTA and D-PLUS, shown leaves with a brighter color and higher if

compared with Control and EM-1. Observing leaves of plants treated with DiSTA and D-PLUS seems that the only microorganisms product (DiSTA) led to a increased growth if compared to the full treatment (D-PLUS).

### Growth of leaves and biomass production

**Table 19:**

Effects of different treatments (Control = distilled water; EM-1 = activated EM•1®; DiSTA = Department of Science and Agronomical Tech original bacterial mix; D-PLUS = DiSTA + Mycorrhiza + Humic Acid. Leaf analysis on Leaf Length (LL), Leaf Fresh Weight (LFW), Leaf Dry Weight/Leaf Fresh Weight percentage ratio (LDW/LFW), Leaf Dry Weight/Leaf Length (LDW/LL).

LEAF ANALYSIS	Control	EM-1	DiSTA	D-PLUS
LL (cm)	4,82 ± 0,87 (c)	5,43 ± 0,76 (b)	8,42 ± 0,87 (a)	8,05 ± 0,87 (a)
LFW (g)	3,76 ± 0,61 (c)	3,44 ± 0,59 (c)	5,34 ± 1,16 (a)	4,65 ± 1,02 (b)
LDW/LFW (%)	12,95 ± 1,77 (b)	18,53 ± 5,66 (a)	21,46 ± 6,60 (a)	20,28 ± 6,37 (a)
LDW/LL (mg/cm)	2,04 ± 0,63 (b)	2,31 ± 0,61 (a)	2,60 ± 0,49 (a)	2,25 ± 0,50 (a)

The effects of inoculation of the microbial based products in controlled environment showed significant statistically differences within different microbial preparations and compared to the control too. Leaf length was increased with DiSTA and D-PLUS treatments. In agreement with data reported in the first trial, EM-1 led to a growth similar to the control. Leaf weight fresh biomass was greater in microbial only DiSTA, with a weekly production 2 grams higher that control. The microbial treatments EM-1, DiSTA and D-PUS had an homogenous effect on the dry/fresh biomass ratio, increasing the ratio respectively 43%, 65% and 56% compared to the control. Dry biomass produced per length leaf unit (LDW/LFW) was in accord with precedent values showing homogenous values between three microbial treatments.

### Growth of roots and biomass production

**Table 20:**

Root parameters in perennial ryegrass pots treated with different ammendands.

ROOT ANALYSIS	Control	EM-1	DiSTA	D-PLUS
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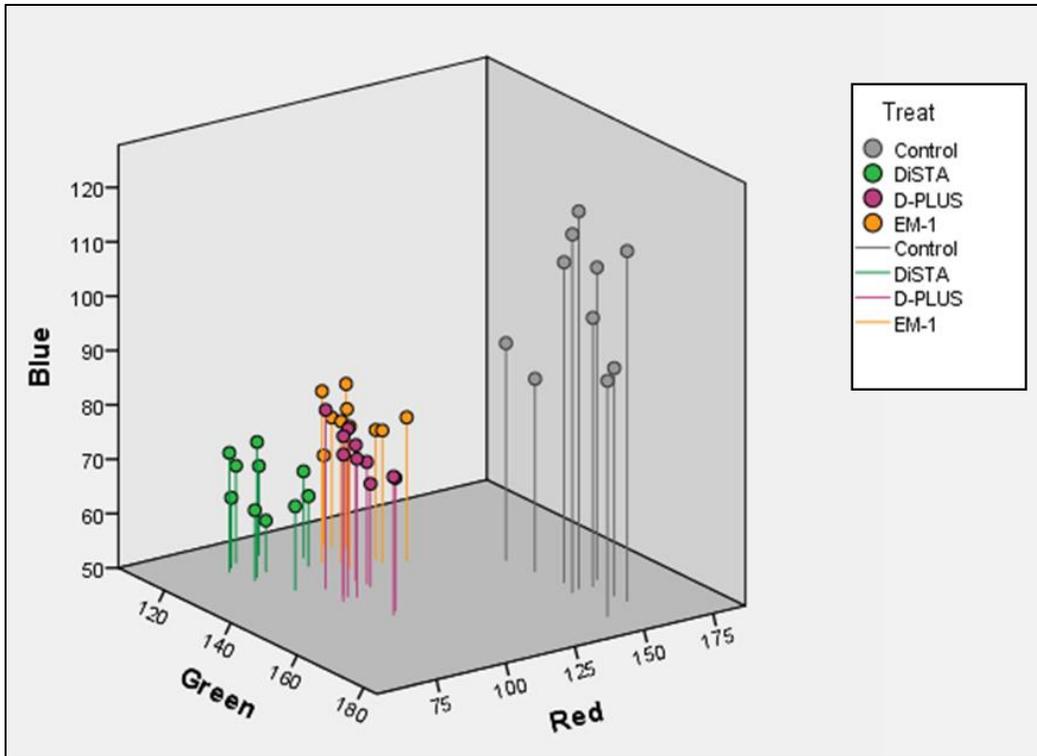
<b>RL (cm)</b>	10,29 ± 2,29 (b)	13,55 ± 2,44 (a)	10,72 ± 1,96 (b)	14,07 ± 1,68 (a)
<b>RFW (g)</b>	5,62 ± 1,35 (c)	8,19 ± 1,52 (a)	7,70 ± 1,55 (b)	7,81 ± 0,99 (ab)
<b>RDW/RFW (%)</b>	17,43 ± 5,43 (a)	19,84 ± 3,91 (a)	16,71 ± 5,34 (a)	18,05 ± 5,11 (a)
<b>RDW/LR (mg/cm)</b>	0,94 ± 0,30 (a)	1,23 ± 0,38 (a)	1,23 ± 0,55 (a)	1,01 ± 0,30 (a)

Morphological and physiological parameters of the roots were acquired at the end of the experimental, after 60 days from the first application. The root apparatus elongation was principally stimulated from D-PLUS and EM-1 that had respectively determined an increasing of 37% (a) and 32% (a) compared to the control. Microbial only DiSTA didn't show a significant elongation respect to the control. Root fresh biomass production were higher in the samples inoculated with EM-1 (+45% to the control), while DiSTA and D-PLUS have increased the fresh weight from 37% (b) to 39% (ab) respect to the control. No statistical difference was detected between four treatments for dry/fresh weight ratio. Dry biomass produced per length unit of leaf (LDW/LFW) didn't result in statistical significant difference between the four treatments tested, although EM-1 and DiSTA had increased the LDW/LFW ratio by 31,5 to 31,1% respectively compared to the control.

### **Color of leaves in RGB space**

The image analysis was conducted measuring individually the values of the three color channels in RGB space. The pictures were captured within a light-box in order to homogenize the lighting conditions of the acquisition environment and were afterward analyzed with Assess software (for a complete description of the picturing process and analysis refer to the relative Materials and Method section 3.2).

The analysis of the color of the leaves (Graph 9) showed an intensification of the color due to the tested product: all three color channels (R,G and B) have indeed shown a decrease of the values respect to the plants treated with Control (water and Hoagland solution) thesis.

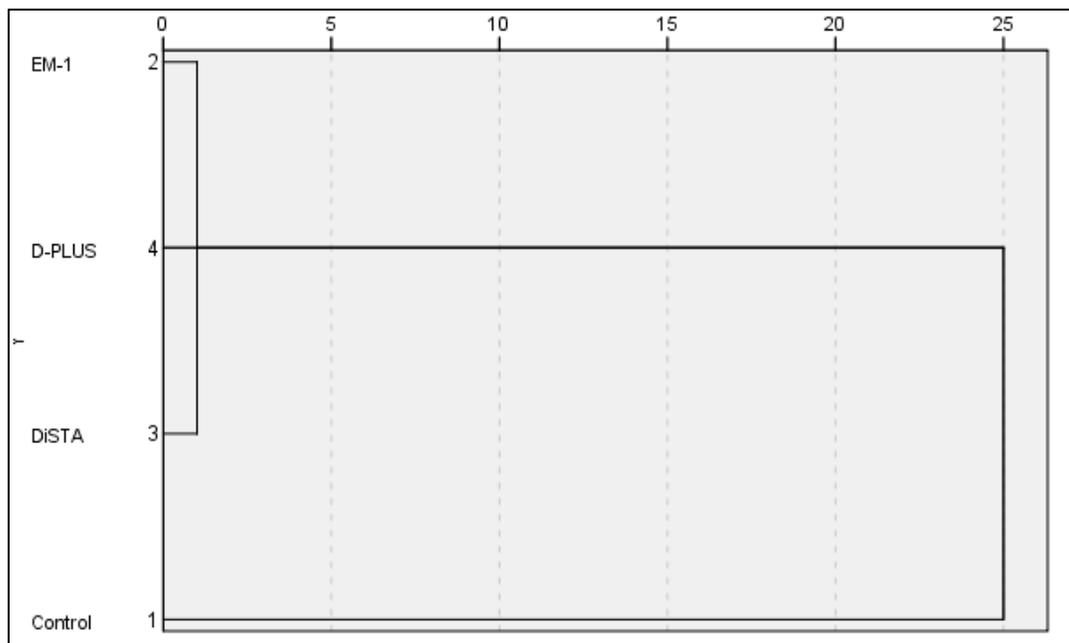


**Graph 9:** 3D scatter plot of RGB color space. The axes correspond to the R, G and B color channel. The points are the average color values for each plot at 60 DAT.

In order to assess the statistical differences between RGB colors showed in Graph 9, a MANOVA test has been conducted:

The red, green and blue color values measured from different pots (130 sampling points for each pot) have been used as dependent variables, while the treatments / control as fixed factors. The test reported a significant difference between the factors ( $F(9, 180) = 54,78, p < 0,0005$ ), and it was therefore followed by a Tukey's post-hoc analysis. The post-hoc test confirmed that the Control was significantly different from all the treatments in the whole color space. D-PLUS resulted not significantly different to EM-1 for all three color channels. DiSTA showed a statistical difference only for Red channel but not for Green and Blue.

A cluster analysis (squared distance, single linkage) has been conducted in order to obtain a graphic representation of the differences suggested by MANOVA test. The following plot (Graph 10) shows a dendrogram of growth chamber trial where the different treatments / control were clustered on the basis of the RGB variables.

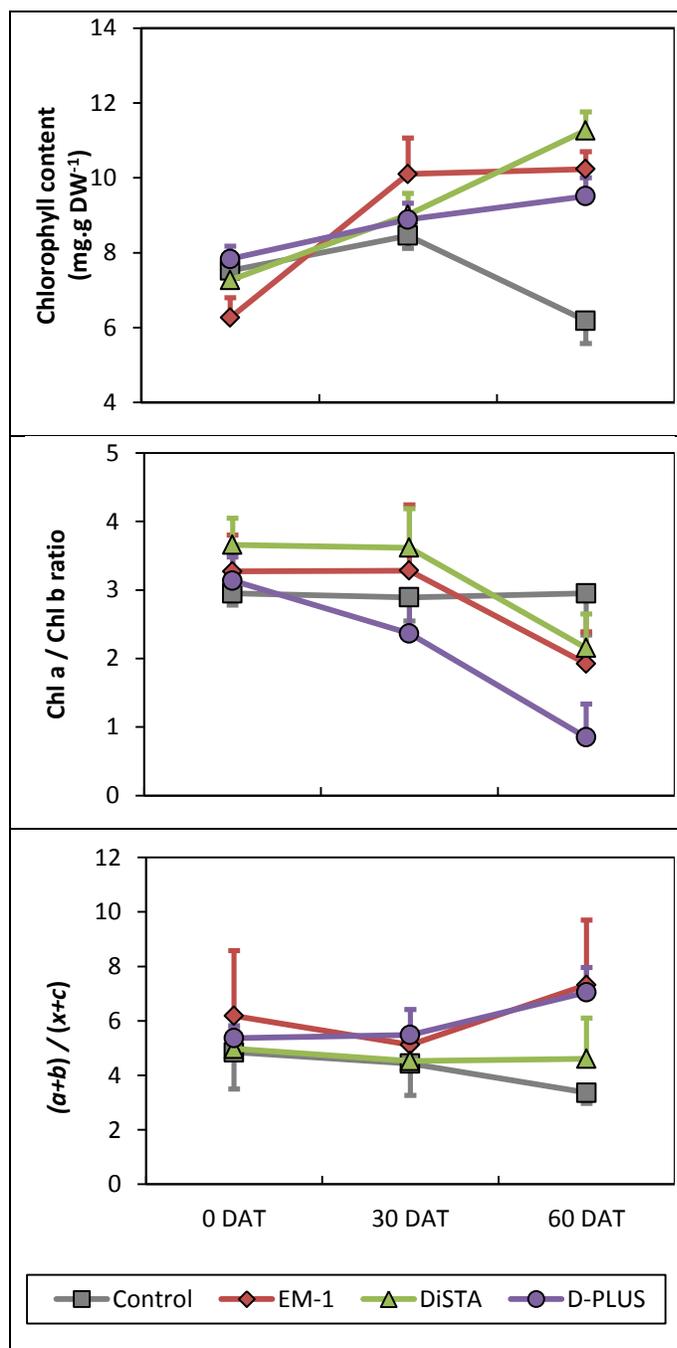


**Graph 10:** Dendrogram representing the differences between mean colors obtained for different treatments within growth chamber.

The treatments EM-1, D-PLUS and DiSTA form a well distinct cluster if compared to the Control, well separate with a high Euclidean distance separating by the treatments' grup.

## Chlorophylls and Carotenoids content

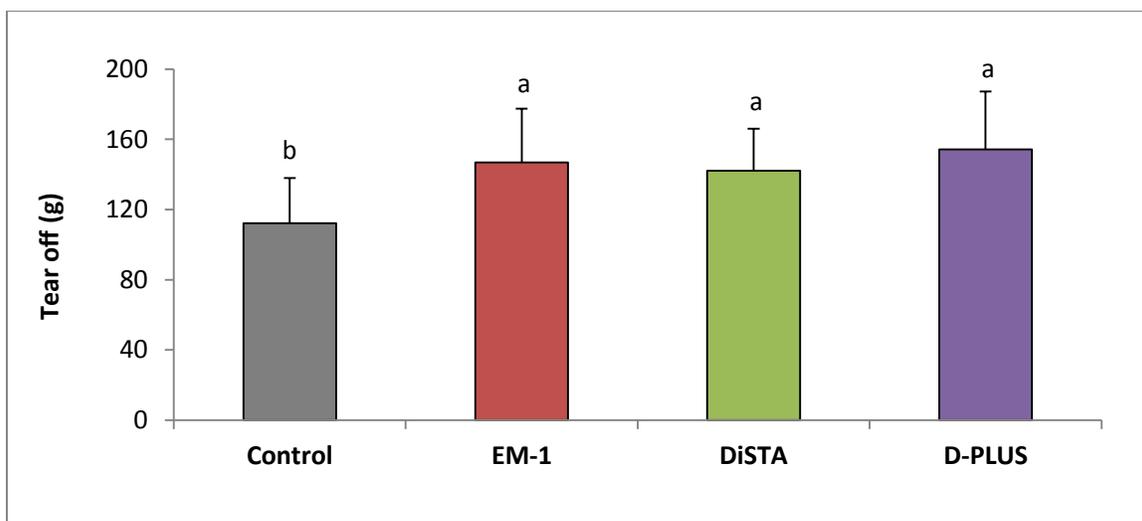
Results of chlorophyll and carotenoids extraction of *L. perenne* leaves are showed in Graph 11. The total chlorophyll content (mg of photopigment *Chl a* and *Chl b* per grams of leaf dry biomass) indicate an increasing with time with all microbial treatments. Instead only Hoagland solution treatment determined an increase of total chlorophyll at 30 DAT and a final diminution of 18% if compared at time 0. Total chlorophyll increase determined by microbial treatments given significant values between EM-1, DiSTA and D-PLUS with difference with the control of +63%, +55%, +21% respectively. The ratio between Chlorophyll a and Chlorophyll b content showed a similar effect of the microbiological treatments comparing with control effect. D-PLUS determined a significant decreasing of *Chl a/b* ration of 75% (0-60 DAT). Only bacteria solution EM-1 and DiSTA determined a similar diminution of *Chl a/b* ration around 38-44% respectively (0-60 DAT). The ratio of *Chls a* and *b* to total carotenoids  $(a+b)/(x+c)$  utilized as indicator of the greenness of the leaf tissues, indicated higher values for D-PLUS and EM-1 treatments with, both statistically different compared to control and DiSTA that determined a ratio reduction of 7 and 31% respectively from time 0.



**Graph 11:** The graphs summarize the parameters related to leaf pigments content in the leaves of *Agrostis stolonifera*. Chlorophyll content is the sum of mg of Chlorophyll a and Chlorophyll b per gram of dry biomass of leaf.  $(a+b)/(x+c)$  represent the ratio between total chlorophyll content and xanthophyll and carotenoids content.

## Tearing

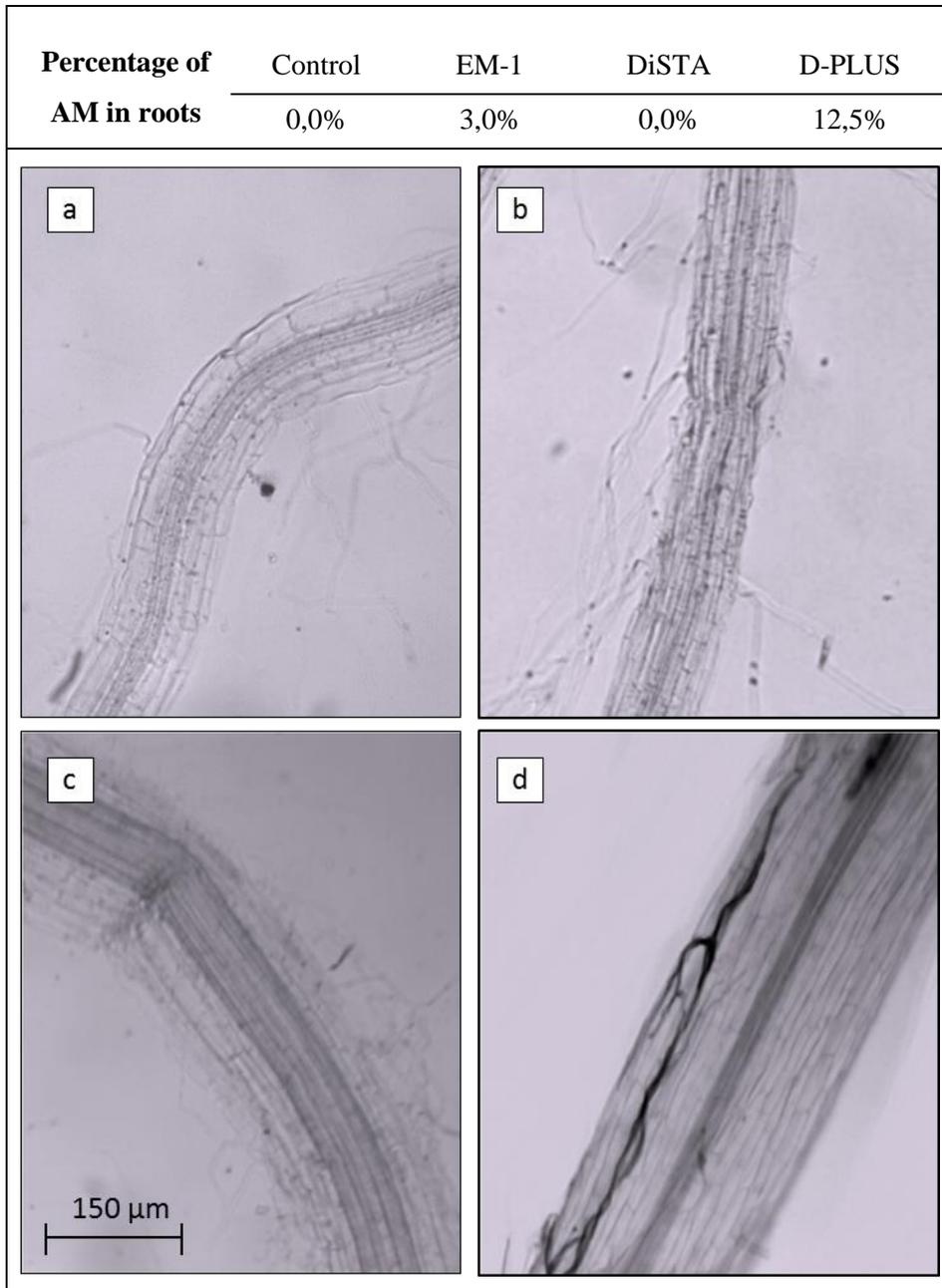
Tear off analysis was conducted with a mechanical dynamometer at the end of the experimental period of 60 days. In the following graph (Graph 11) was reported the values expressed as means of the single treatment. Results between treatments are compared with an analysis of variance (Duncan's means test, Significance Level 0,05). The treatments based on microbiological mixtures determined an increase in tear resistance compared to the control constituted by distilled water. No statistically significant differences were detected between the three different biological treatments applied.



**Graph 12:** Tear out values for *Lolium perenne* L. plants. Tear out was tested with a mechanical dynamometer at 60 DAT. Plants were treated with four different products: Control (Water + Hoagland solution), EM-1 (EM-RO® commercial microorganisms + Hoagland solution), DiSTA (only microorganisms formulate + Hoagland solution) and D-PLUS (microorganisms formulate + *Glomus intraradices* + Hoagland solution).

## Mycorrhizal analysis

Perennial ryegrass roots were analyzed for quantify the inoculum occurred after the different thesis treatments. At the end of the experimental trial, perennial ryegrass roots were washed gently and then treated with CBE, a specific colorant for endomycorrhiza as specified in the concerning paragraph. The presence of fungal hyphae was expressed as percentage of units in which hyphae were present on the total of the squares of the petri dish. As is shown in Figure 48, plants treated with water only (Control), EM-1 and DiSTA did not show mycorrhizal within root tissue. In pots treated with EM-1 were found a very low percentage of mycorrhizal spores but probably as a remnant of the sand, which was sterilized before sowing of perennial ryegrass. D-Plus treatment determined the emergence of fungal hyphae within root tissues, showing a 32,5% presence on the petri dish grid.



**Figure 48:** Portions of *Lolium perenne* roots bleached with KOH and treated with Chlorazol Black E to evidence the AM (Arbusculus Mycorrhizae) within the root tissues. In order are shown roots of Control (a), EM-1 (b), DiSTA (c) and D-PLUS (d).

### 4.3.2 PUTTING GREEN RESULTS

#### EC and pH of soil

**Table 21:**

pH and Electrical Conductivity (EC) values from putting green topsoil. Electrical conductivity was expressed as instrumental scale milliSiemens per cm (mS/cm). Treatments effects were compared with ANOVA, Duncan's test p: 0,05.

Soil Analysis		Control	EM-1	DiSTA	D-PLUS
pH	0 DAT	7,1 ± 0,7 (a)	7,5 ± 0,9 (a)	7,2 ± 0,9 (a)	7,0 ± 0,4 (a)
	30 DAT	7,2 ± 0,9 (a)	7,6 ± 0,9 (a)	7,3 ± 0,8 (a)	7,3 ± 0,5 (a)
	60 DAT	6,9 ± 0,6 (a)	6,8 ± 0,4 (a)	7,1 ± 0,6 (a)	7,1 ± 0,7 (a)
ECe (mS/cm)	0 DAT	1,86 ± 0,44 (a)	1,77 ± 0,24 (a)	1,71 ± 0,16 (a)	1,58 ± 0,21 (a)
	30 DAT	1,71 ± 0,25 (a)	1,69 ± 0,27 (a)	1,73 ± 0,15 (a)	1,89 ± 0,27 (a)
	60 DAT	1,74 ± 0,24 (a)	1,86 ± 0,31 (a)	1,71 ± 0,17 (a)	1,74 ± 0,17 (a)

pH analysis indicate that the putting green top soil had a neutral – slightly alkaline characteristics. Around 30 DAT pH values were slightly higher 0 and 60 DAT in all treatment but no statistical differences were found. Furthermore none of the treatments applied had statistical significant effect on the soil pH during after the 60 days experimental period. Electrical conductivity indicated that the four different thesis didn't result changes in soil salinity. Soil ECe measured showed typical values for putting green soils, comprised between 1,58 and 1,89 mS/cm. ANOVA test didn't found significant difference both between treatments and date.

#### Chemical analysis (C/N) of soil

**Table 22:**

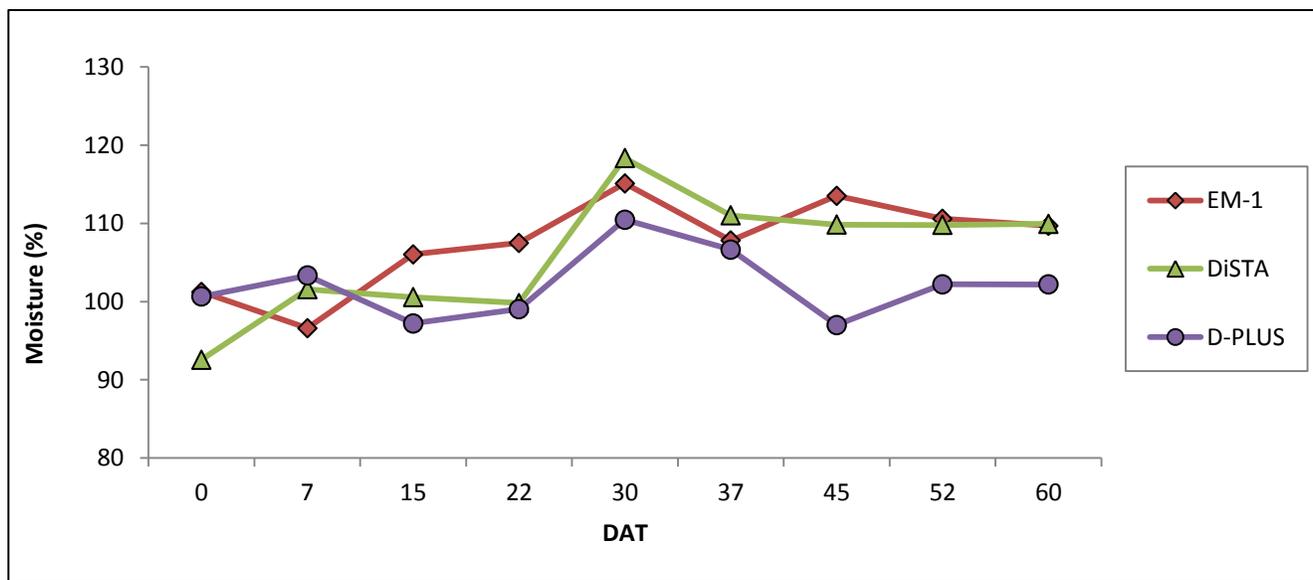
Total Carbon (C) and total Nitrogen (N) content in putting green soil. Thesis tested was Control (distilled water and Hoagland solution), EM-1 = activated EM-1®; DiSTA = Department of Science and Agronomical Tech original bacterial mix; D-PLUS = DiSTA + Mycorrhiza + Humic Acid).

SOIL C/N ANALYSIS		Control	EM-1	DiSTA	D-PLUS
C	0 DAT	5,76 ± 0,56 (a)	4,96 ± 0,91 (a)	5,81 ± 0,47 (a)	4,83 ± 0,51 (a)
	30 DAT	5,81 ± 0,73 (a)	4,89 ± 0,73 (a)	4,43 ± 0,46 (a)	6,01 ± 0,69 (a)
	60 DAT	6,85 ± 0,51 (a)	7,07 ± 0,55 (a)	6,0 ± 0,47 (a)	7,65 ± 0,76 (a)
N	0 DAT	0,45 ± 0,09 (a)	0,38 ± 0,07 (a)	0,44 ± 0,08 (a)	0,40 ± 0,07 (a)
	30 DAT	0,43 ± 0,08 (a)	0,35 ± 0,08 (a)	0,32 ± 0,07 (a)	0,46 ± 0,09 (a)
	60 DAT	0,53 ± 0,07 (a)	0,55 ± 0,07 (a)	0,45 ± 0,08 (a)	0,62 ± 0,08 (a)
C/N (%)	0 DAT	13,1 ± 2,66 (a)	13,30 ± 1,53 (a)	13,53 ± 2,35 (a)	12,40 ± 2,36 (a)
	30 DAT	13,7 ± 1,94 (a)	14,11 ± 2,63(a)	14,24 ± 3,29 (a)	13,24 ± 1,70 (a)
	60 DAT	12,93 ± 1,69 (a)	12,85 ± 1,58 (a)	13,54 ± 2,16 (a)	12,40 ± 1,08 (a)

The total soil nitrogen and carbon of the soil samples were analyzed with ECS 4010 CHNSO system. Total nitrogen and carbon were analyzed at the date 0, 30 and 60 day after treatments (DAT). The values obtained were represented as percentage on the control (Table 22). During the entire duration of the experimental trial, no significant differences were revealed between different treatments and the variation of the values during the two-month period were probably determined by seasonal conditions.

### Moisture of soil

The moisture content of soil was analyzed weekly for the entire period of the experimental trial. The hydric content of each plot was calculated as the percentage difference between the fresh and dry weight of a putting green clod.

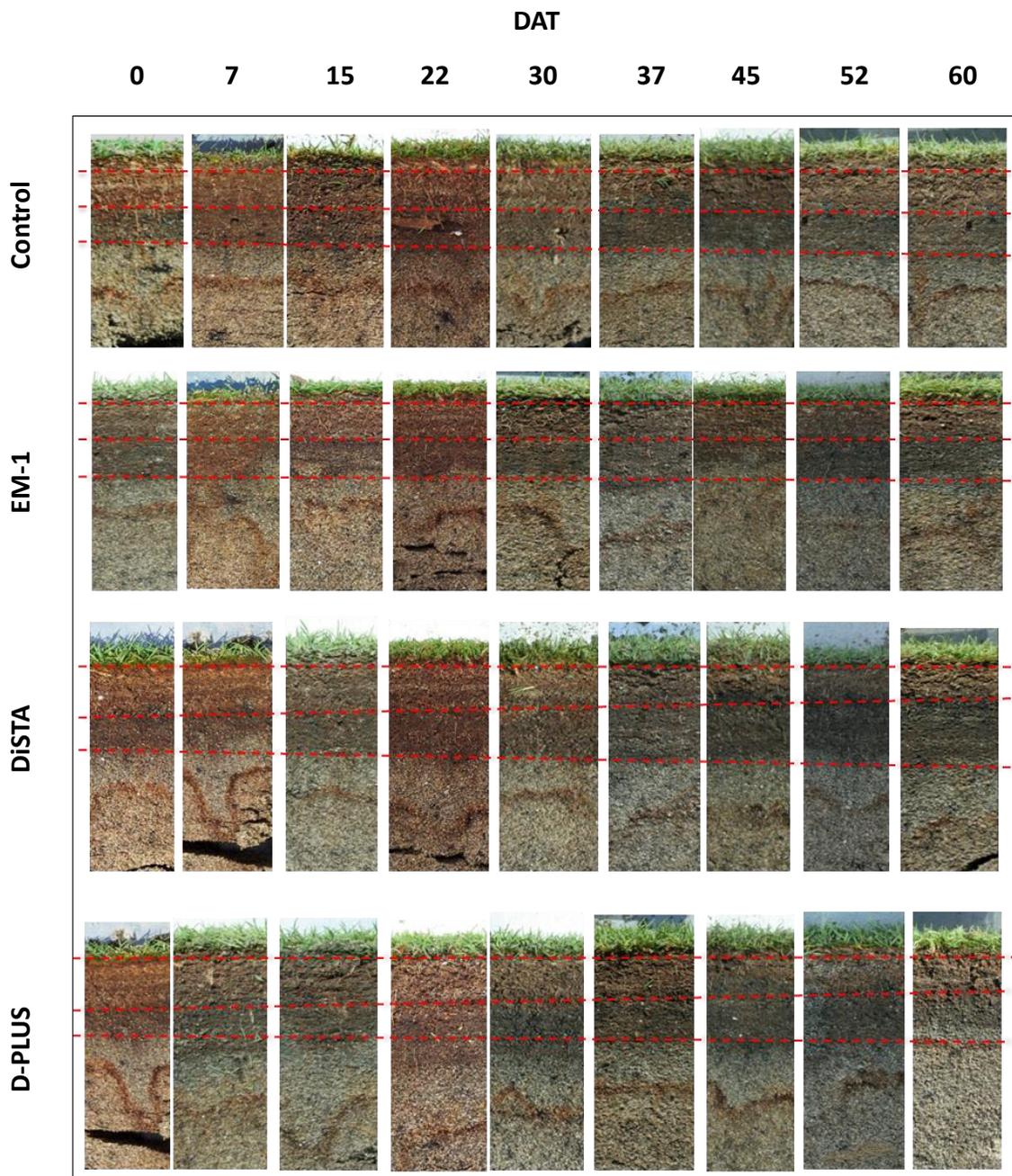


**Graph 12:** Moisture content in a 10 cm section of top soil during the 60 days period of the experimental trial. Values are expressed as percentage of hydric content relative to the control.

Analyzing the data of moisture that were collected from the experimental trial was possible to denote a similar behavior of the plots treated with the EM-1 and DiSTA. The D-PLUS treatment began to determine different moisture content since 45 DAT. The inoculum with DiSTA and EM-1 determined a progressive increasing of the hydric content in the first month, reaching a significant difference compared to control between +18,2 and +15,4% respectively. These values were maintained constant within the following 30 days until the end of the test, when DiSTA and EM-1 reached values 10,4 and 9,9% respectively. Although three microbial treatments showing a different trend respect to the control, were not detected significant differences between the four treatments, for the entire duration of the test.

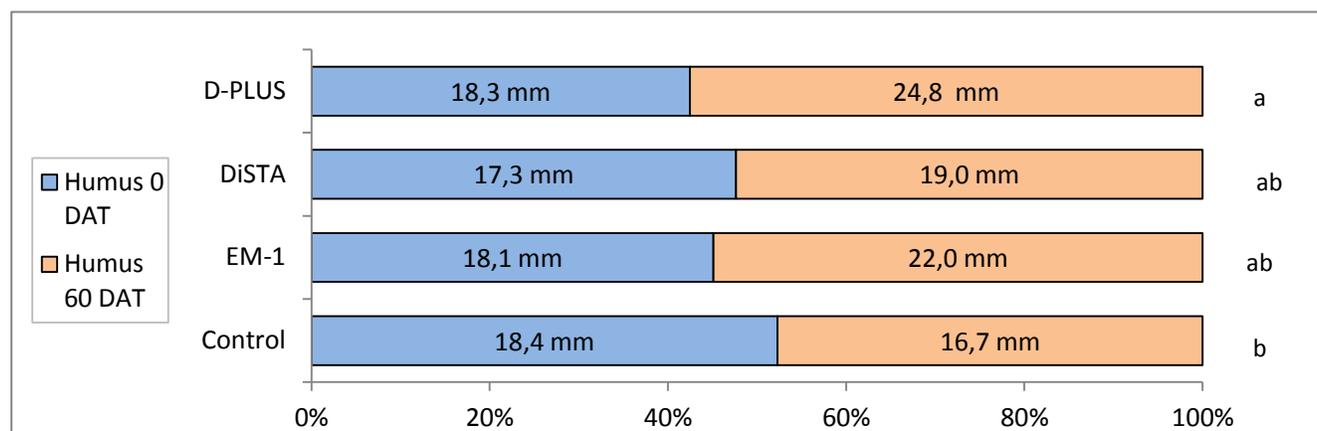
## Profile of soil

The stratigraphy of the soil was detected weekly by the extraction of a section of soil and taking pictures with a metric reference system. Thicknesses of the layers that compose soil profile were measured with a digital image analysis (Figure 49). These values have allowed to study changes in soil profile caused by inoculation of microorganisms and biological substances in a soil that is generally deficient in microbial flora.



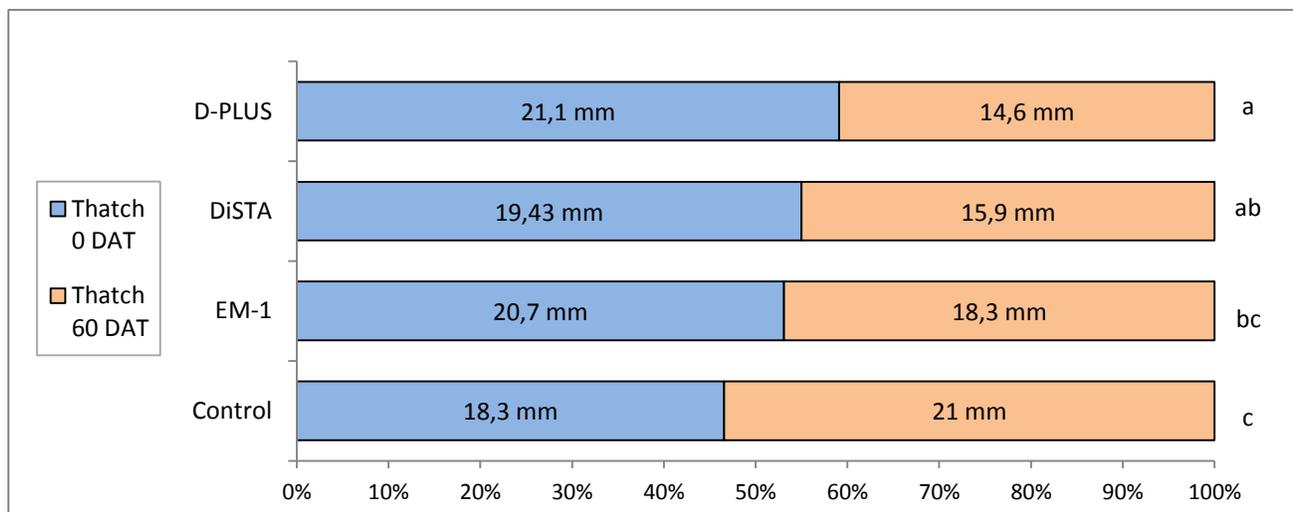
**Figure 49:** Effects of different treatments on the soil layers. Thatch and the underlying decomposing layers were evidenced with two red lines.

The soil profile showed a significant trend in soils where inoculum occurred. As is shown in Graph 13 the inoculum of D-PLUS determined a thickening of the humic layer by 41,4% at 60 DAT if compared with related 0 DAT value. DiSTA and EM-1 applications determined an increasing of the humic layer by 20,4% and 23,5% compared to the respective 0 DAT values. In control plots the humic layer thickness remained essentially unchanged (-1,24 to 0 DAT). The effects offered by the microbial inoculants on the thickness of humic layer was found statistically significant only for D-PLUS treatment by an ANOVA test (Duncan's means test, significant level: 0,05).



**Graph 13:** Effects of the four thesis inoculum on the thickness of the “humus layer.

Regarding the changing of the thickness of the thatch layer, the inoculation of microbial product showed statistical significant differences for all treatments if compared with the control. In the plots treated with water only (Control), thatch layer had an increasing of 18,4 % during the two month experimental period. Only bacteria solution EM-1 determined a reduction of 6,1 %, while DiSTA had an effect statistically different compared to control bringing the thinning of the thatch at 15,9 %. The greater result was obtained from the application of D-PLUS that determine a thatch reduction of 26,74 % in two month of weekly treatments.



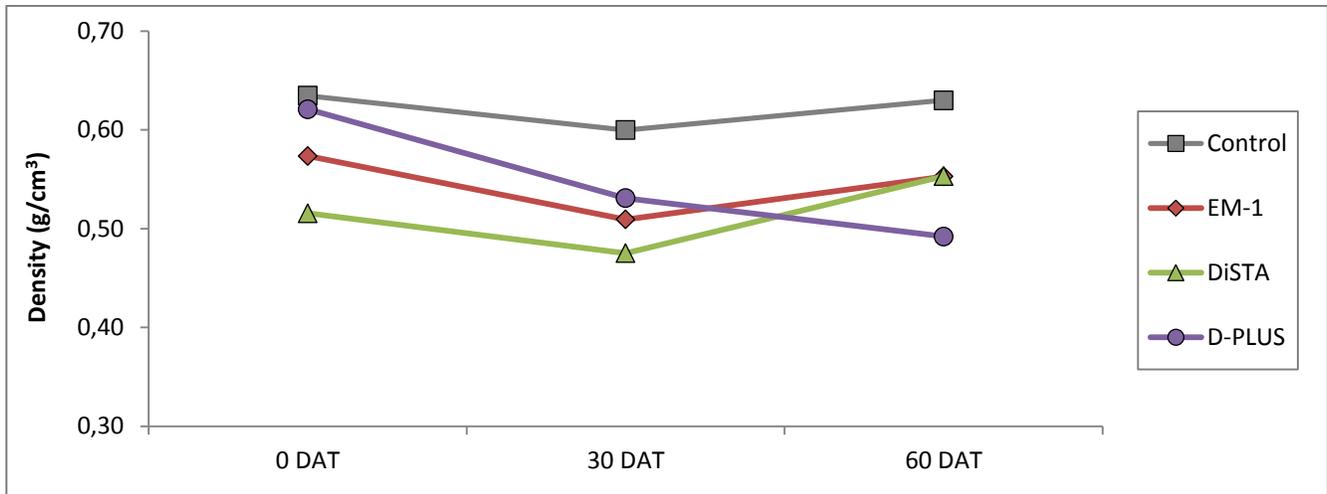
**Graph 14:** Effects of different treatments on the reduction of the thickness of the thatch.

### Biomass of thatch and roots

**Table 23:** Thatch values of Fresh Weigh (FW), Dry Weight/Fresh Weight-ratio (DW/FW) and Density.

Thatch results		Control	EM-1	DiSTA	D-PLUS
FW (g/cm <sup>2</sup> )	0 DAT	0,59 ± 0,05	0,58 ± 0,07	0,53 ± 0,12	0,61 ± 0,02
	30 DAT	0,64 ± 0,12	0,60 ± 0,12	0,54 ± 0,11	0,55 ± 0,08
	60 DAT	0,68 ± 0,14	0,55 ± 0,14	0,52 ± 0,08	0,49 ± 0,12
DW/FW (%)	0 DAT	20,3 ± 1,0	20,7 ± 3,6	19,4 ± 8,1	21,3 ± 3,6
	30 DAT	19,9 ± 4,8	18,9 ± 4,7	18,6 ± 4,6	19,0 ± 3,3
	60 DAT	19,2 ± 1,9	20,3 ± 3,2	16,9 ± 3,5	15,8 ± 2,3
Density (mg/cm <sup>3</sup> )	0 DAT	0,63 ± 0,15	0,57 ± 0,08	0,52 ± 0,20	0,62 ± 0,12
	30 DAT	0,60 ± 0,10	0,51 ± 0,07	0,48 ± 0,10	0,53 ± 0,14
	60 DAT	0,63 ± 0,14	0,63 ± 0,25	0,55 ± 0,12	0,49 ± 0,11

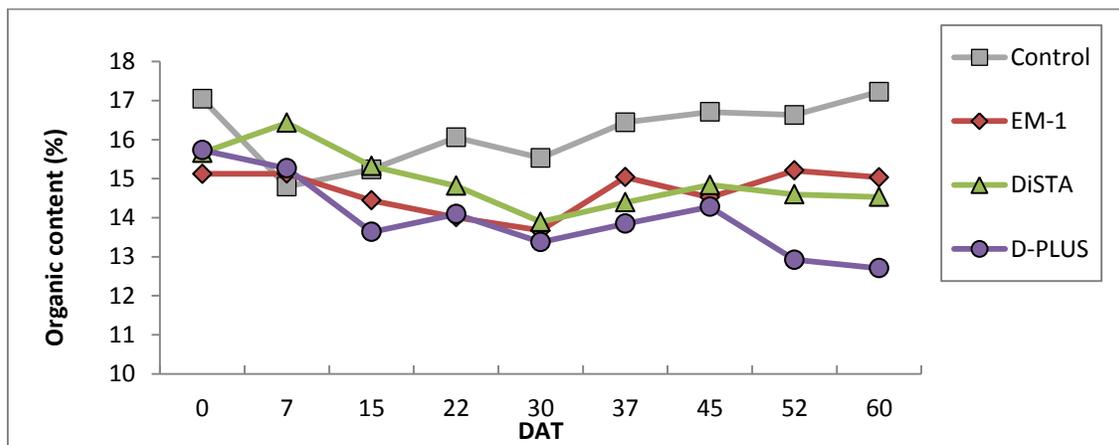
Microbial treatments determined different responses on thatch during the experimental trial. EM-1 and DiSTA decreased the fresh biomass of 3,9% and 1,2% respectively comparing at the control but significant differences were not detected. D-PLUS treatments determined a significant thatch fresh biomass diminution of 19,2% compared to control. Dry/Fresh-Ratio was not modified by only water treatments (Control) and EM-1 but was gradually decreased by DiSTA and D-PLUS with percentage values of 12,9% and 25,9% respectively. The density of thatch layer, expressed as dry biomass (mg) per unit of volume (cm<sup>3</sup>) didn't show significant differences from 0 to 60 DAT intra-treatments. Anyway, although a significant difference was not verified, D-PLUS determined a trend diminution of the density of 15,1% if compared to the control (Graph 15).



**Graph 15:** Effects of different treatments on thatch density ( $\text{g}/\text{cm}^3$ ) parameter during the 60 days trial period.

### Organic content

Organic content in the top soil of the experimental putting green was evaluated weekly. A  $12,5 \text{ cm}^3$  portion of soil from 0,5mm of the surface was extracted for quantification of the organic component. Graph 16 shows the trend of top soil organic component during the 60 days of experimental period, expressed as percentage on the biomass of soil.



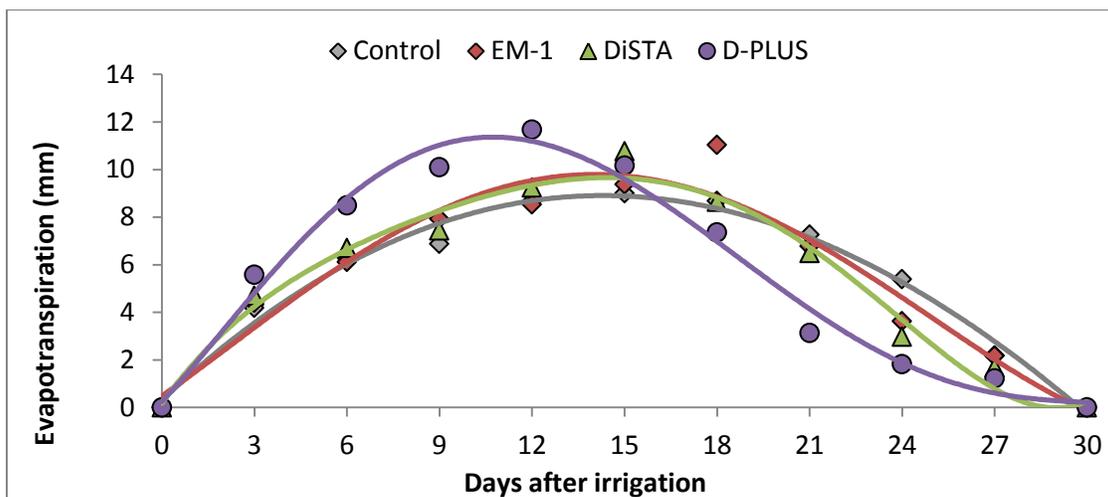
**Graph 16:** Trend of organic matter content in the top soil of the experimental putting green during the 60 days experimental period.

In the plots treated with water only (Control) the soil organic matter did not significantly changed during the test. Bacteria solutions EM-1 and DiSTA determined reduction on the control of 12,7% and 15,6% respectively. D-

PLUS determined the highest reduction of organic content in the soil of putting green with a statistically significant reduction of 26,2% comparing at the control and reduction from 0 DAT of 19,21%.

### Evapotranspiration

Initially all clods were brought at field capacity and then sealed with parafilm on the bottom and on the side faces. Real Evapotranspiration was determined from each sample measuring the daily loss of water from the initial time.



**Graph 17:** Graph of evapotranspiration expressed as mm of water lost daily from time 0.

Plots treated with D-PLUS showed an evapotranspiration capacity greater than control and other microbial treatments. With D-PLUS plots loss half of the water content in 10 days whereas in control plots needed 15 days. Even microbial only product brought a slight increased evapotranspiration relatively to the control but significant differences were not determined.

### Production of leaf biomass

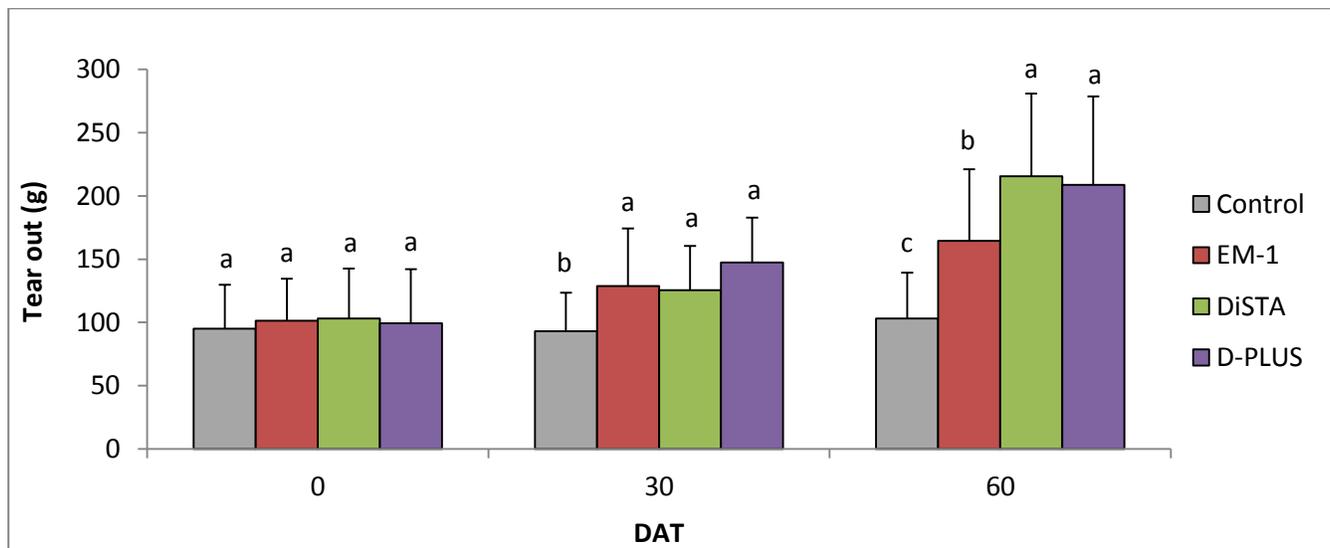
**Table 24:** Effects of different treatments (Control, EM-1, DiSTA and D-PLUS) on leaf biomass parameters. ANOVA test was conducted comparing the four thesis at the same date.

LEAF ANALYSIS		Control	EM-1	DiSTA	D-PLUS
Fresh Weight (Kg)	0 DAT	813 ± 153 (a)	768 ± 170 (a)	810 ± 101 (a)	752 ± 141 (a)
	30 DAT	850 ± 120 (ab)	815 ± 101 (c)	836 ± 191 (b)	867 ± 118 (a)
	60 DAT	822 ± 153 (a)	630 ± 113 (b)	833 ± 99 (a)	948 ± 99 (a)
Dry Weight (Kg)	0 DAT	207 ± 70 (a)	219 ± 46 (a)	127 ± 48 (a)	227 ± 69 (a)

	30 DAT	236 ± 35 (b)	260 ± 72 (b)	288 ± 155 (b)	257 ± 104 (a)
	60 DAT	223 ± 66 (b)	210 ± 45 (b)	255 ± 29 (ab)	305 ± 33 (a)
	0 DAT	27,0 ± 12,5 (a)	29,0 ± 5,8 (a)	27,1 ± 6,6 (a)	30,7 ± 9,2 (a)
<b>Dry/Fresh Weight (%)</b>	30 DAT	28,4 ± 6,0 (a)	31,5 ± 5,5 (a)	32,9 ± 11,4 (a)	31,0 ± 15,3 (a)
	60 DAT	27,3 ± 6,9 (b)	32,2 ± 2,8 (a)	30,1 ± 1,7 (ab)	32,4 ± 4,0 (ab)

At the end of 60 days experimental period, EM-1 application determined a decreasing of leaf fresh biomass production of 18% from date zero and a significant diminution of 23,3% comparing at the control. DiSTA treatments did detect any changing on fresh biomass from 0-60 DAT and comparing with control. D-PLUS increased the leaf fresh biomass production but no significant differences were detected. Dry biomass content was not significantly influenced by EM-1 and DiSTA treatments but D-PLUS determined a significant increasing of 36,9% comparing to the control. EM-1 determined a significant increasing of Dry/Fresh Weight-ratio of 21,5% at 60 DAT in respect to the control while DiSTA and D-PLUS didn't had significant effects.

## Tearing



**Graph 18:** Tearing out values at 60 DAT.

Tearing analysis showed an increasing effect of microbial inoculum on improving a mechanical resistance at tear out parameter. At 30 DAT all microbial treatments determined an increasing between 38% and 58% in respect to the control. At 60 DAT DiSTA and D-PLUS redoubled tear values comparing to the control with a significant increasing of 109% and 102% respectively. At 60 DAT, EM-1 measured significant differences between both DiSTA and D-PLUS and to the control.

## Color analysis

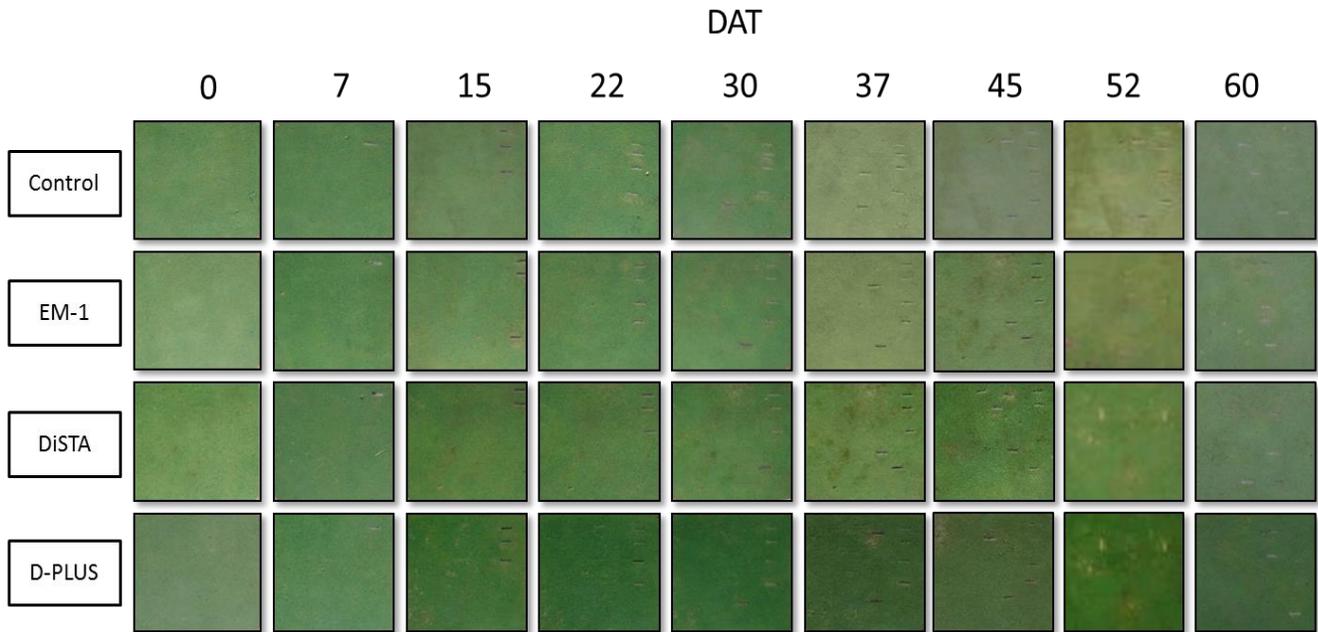
Aesthetic values are the first parameters taken in consideration to evaluate the quality of putting green. Aesthetic assessment was conducted measuring the color changing of the grassy surface during the 60 days experimental trial. During this period color parameters were measured in RGB color space acquiring pictures with a digital camera EOS 350D. Observing the Figure 51, the surface of the putting green has visibly changed color in D-PLUS treated plots. EM-1 and DiSTA did not induce appreciable aesthetic changes but with software measure of RGB data were conducted more accurate determinations.



**Figure 51:** Picture of the putting green at 60 DAT. Foreground is shows a plot treated with D-PLUS.

D-PLUS microbial product determined significant changes of the surface color during the experimental period. Other microbial treatments EM-1 and DiSTA determined less pronounced responses. A visual assessment of EM-1 effect did not determine aesthetic variation compared to the control. DiSTA determined a partial change in the color that has veered towards darker shades, an important aspect in the sport field area. First color differences compared to the control were determined by D-PLUS from 14-21 days after treatments. A visual difference between D-PLUS and Control was maintained until the end of the test (Figure 52).

Pictures taken from each plot were digitally analyzed with the software APS ASSESS 2.0. Colors parameters in RGB color space were evaluated between different treatments within the same date in order to minimize the lighting differences given by different weather conditions across the two months experimental period (for a complete description of the picturing process and analysis refer to the relative Materials and Method section).



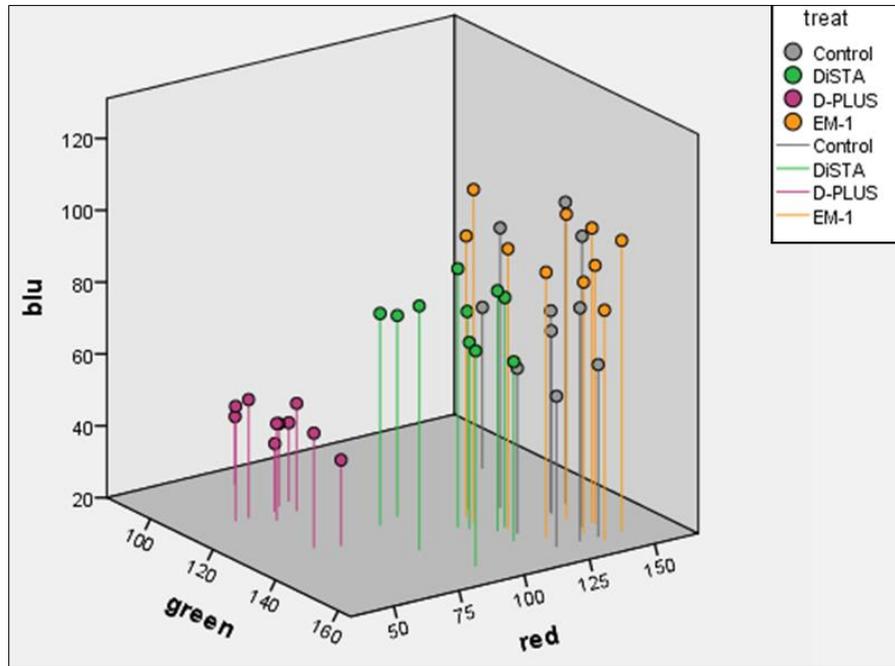
**Figure 52:** Plots color changing during the 60 days experimental test.

The digital color analysis confirmed the visual assessment conducted on the field: D-PLUS determined a substantial separation of the mean points in the scatter plot graph, while EM-1 and DiSTA appear more aggregate position relatively to the control (Graph 18). D.PLUS treatment intensification of the color at 60 DAT was 50%, 21% and 42% for R,G and B respectively if compared to the control.

In order to assess the statistical differences between RGB colors showed in Fig X, a MANOVA test has been conducted.

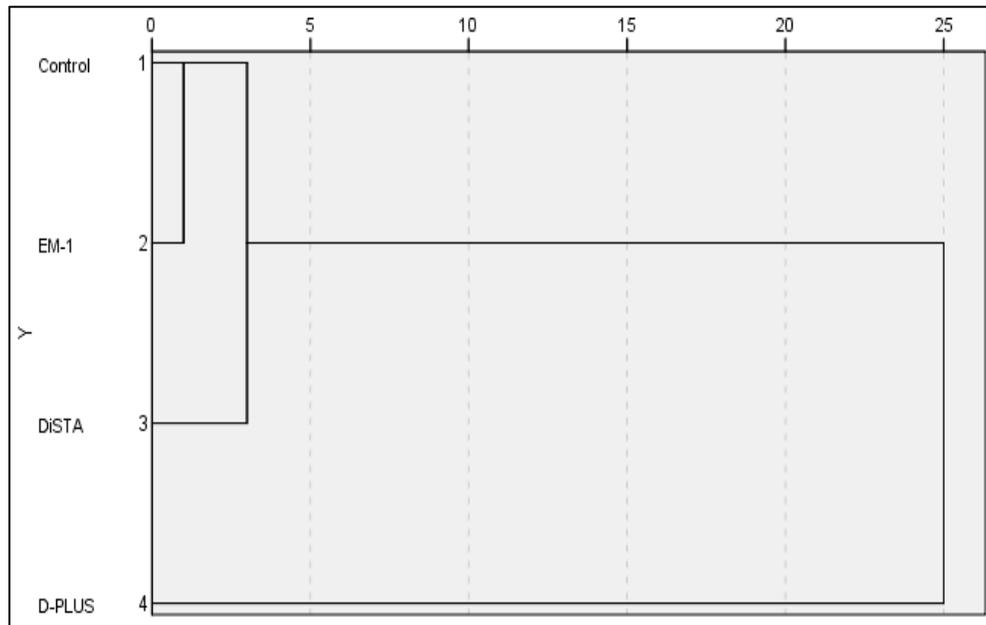
The red, green and blue color values measured from different pots (130 sampling points for each pot) have been used as dependent variables, while the treatments / control as fixed factors. The test reported a significant difference between the factors ( $F(9, 180) = 49,83, p < 0,0005$ ), and it was therefore followed by a Tukey's post-hoc analysis.

The post-hoc test confirmed that the D-PLUS was significantly different from all the treatments in the whole color space. DiSTA resulted not statistically different for Green channel to Control and EM-1 and for Blue Channel to the Control. EM-1 showed a significant difference from other treatments in the Blue channel, but resulted not statistical different for Red and Green respect the other treatments. For a detailed report of the test results refer to Table X in the Appendix.



**Graph 18:** 3d scatterplot where the axes correspond to the R, G and B colour channel and the points are the averaged color values for each parcel at the end of the treatment

A cluster analysis (squared distance, single linkage) has been conducted in order to obtain a graphic representation of the differences suggested by MANOVA test. The following plot (Graph 19) shows a dendrogram of growth chamber trial where the different treatments / control were clustered on the basis of the RGB variables.



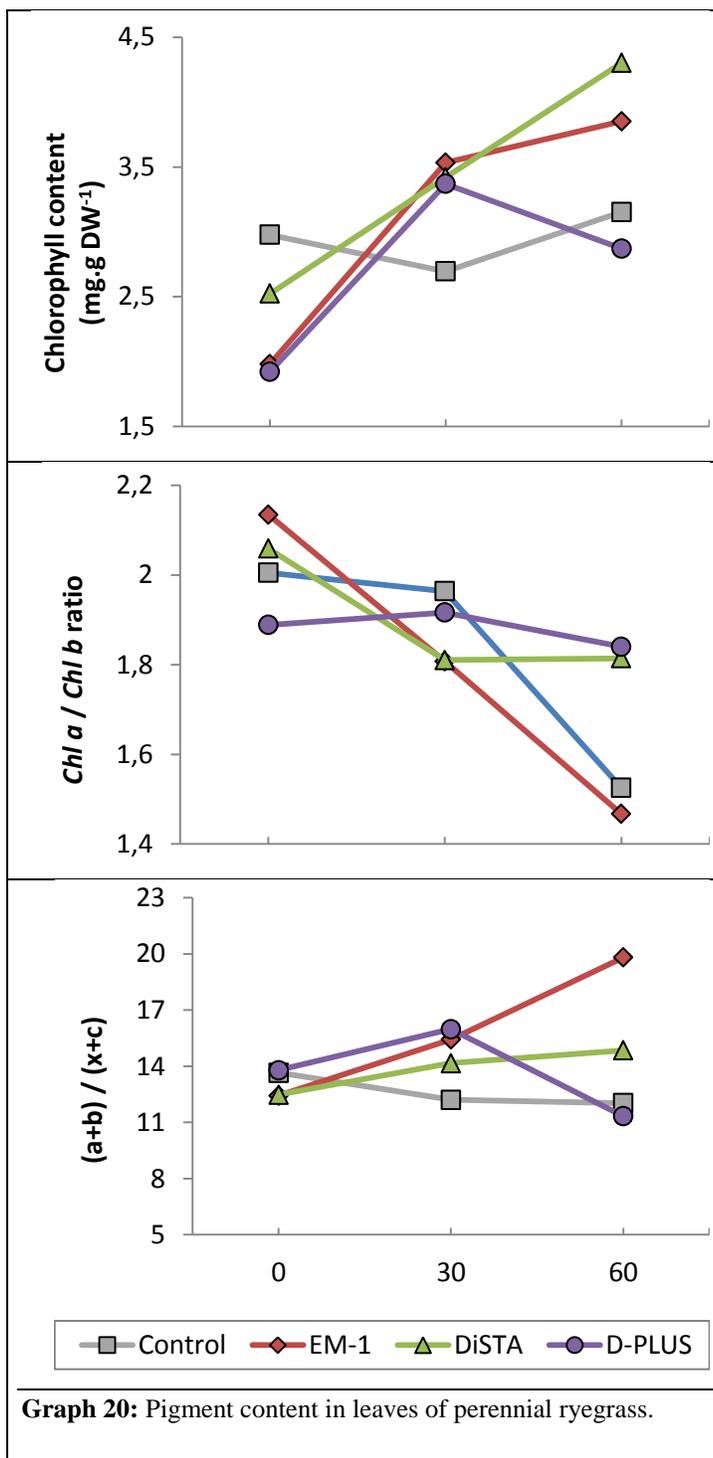
**Graph 19:** Dendrogram representing the differences between mean color obtained for different treatments within growth chamber.

The treatment EM-1 and Control form a well close cluster with a secondary proximity also with DiSTA. Control is a clearly separate entity with a much longer clustering distance if compared with the treatments applied.

### Chlorophylls and carotenoids content

Total chlorophyll content, calculated as sum of Chl *a* and Chl *b*, did not reported statistical differences between different treatments and control. Anyway, all microbial treatments showed an increasing of the total chlorophyll content from 0 DAT to 60 DAT if compared to respectively time 0. Indeed EM-1, DiSTA and D-PLUS increased the Chl content in foliar tissues of 95%, 71% and 50% respect to 0 DAT, while control thesis showed an increase of 6% compared at time 0. Differences of chlorophyll content determined by EM-1, DiSTA and D-PLUS from 0DAT to 60 DAT resulted statistically significant both at 30 and 60 days after treatment, while control did not determined differences in different sampling dates.

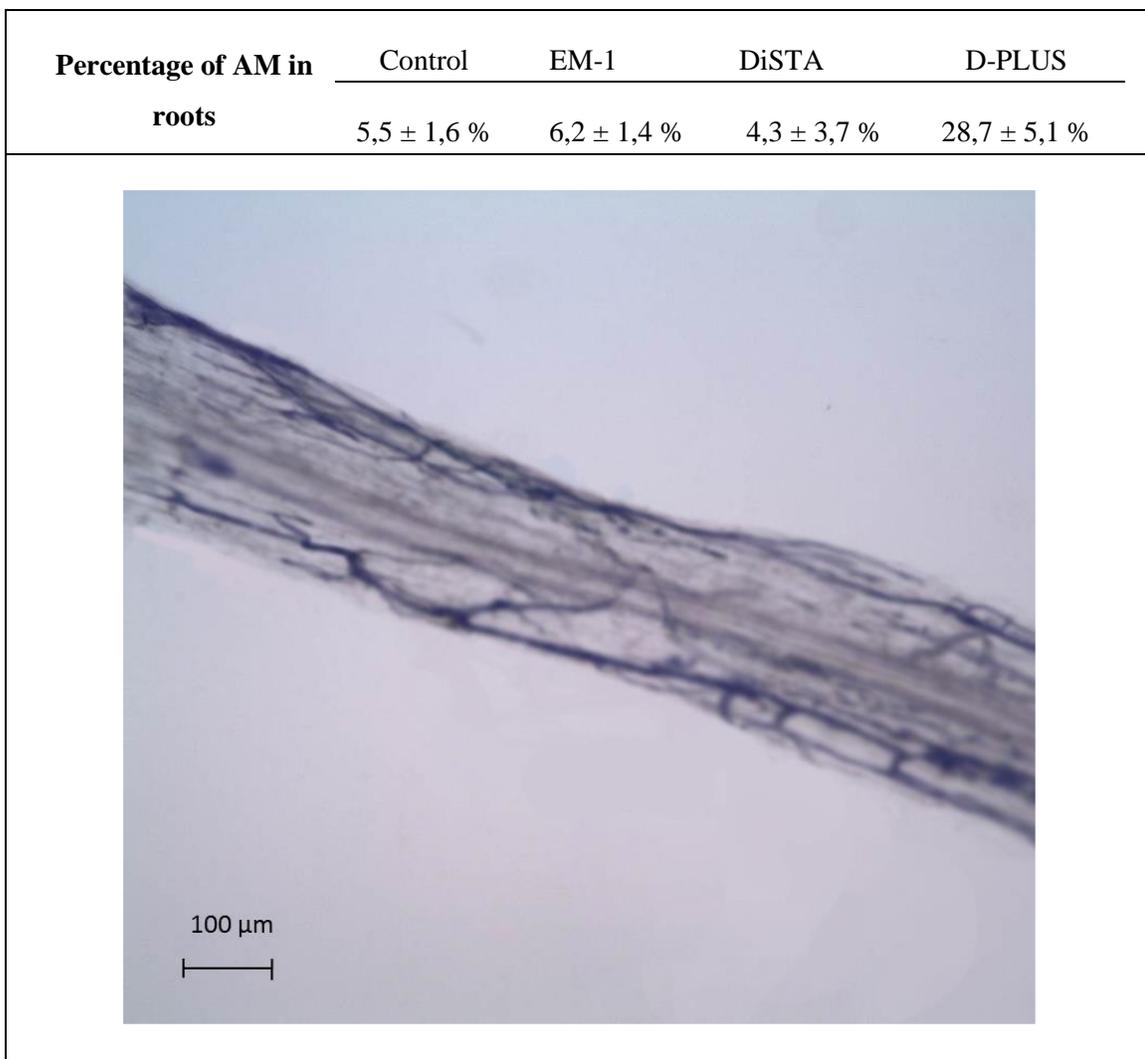
Chl *a*/Chl *b* ratio showed a decreasing effect determined by EM-1 microbial mix of 31% respect to time 0, similarly to only water treatment that showed a reduction of 24% during the experimental period. Plots treated with DiSTA and D-PLUS the Chl *a*/Chl *b* ratio was maintained constant. The weight ratio of Chls *a* and *b* to total carotenoids  $(a+b)/(x+c)$  indicated a large base ratio on this experimental field. Chls/carotenoids showed no significant differences in any sampling date.



## Mycorrhizal inoculation

The analysis of the mycorrhizal presence within the root tissues were conducted at the end of the experimental trial. The portion of thatch was removed because was impossible to separate death material (roots and leaves) from alive roots. The portion of root tissues analyzed was below the thatch layer. The density of fungal hyphae evidenced in roots was expressed as percentage of units in which hyphae were present on the total of the squares of the petri dish.

Plants treated with water only (Control) shown the lower presence of hyphae, while EM-1 and DiSTA treatments determined a significant increasing if compared to the control. D-PLUS (Figure 53), shown an high percentage of hyphae within the root tissues, statistically higher if compared with the other treatments.



**Figure 53:** 1mm<sup>2</sup> pictures of a portion of *Agrostis stolonifera* root. The mycorrhizae within root tissues was evidenced by the dark-blue coloration

## 4.4 DISCUSSION

On the basis of the previously results, in the second step of this thesis two original microbial mixes (DiSTA and D-PLUS) were formulated and then tested in a golf course putting green. A further trial was conducted within a growth chamber in order to compare these data with the result obtained in the first step's experiment. The application in a semi-field condition aimed to verify the effectiveness of the two different microbial solutions. DiSTA and D-PLUS were formulated specifically to be inoculated in a putting green that represents the highest technical level in the world of sport turf. The EM-1 commercial biostimulant and water only were respectively used as positive and negative controls. The complex microbial content of DiSTA and D-PLUS composed by lactobacilli, yeasts, aerobic bacteria, humic acids and mycorrhizae aimed to promote the growing of root tissues, to prevent the occurrence of diseases and pathologies, and to enhance the healthiness of the putting green turfgrass. In order to assess the effectively action of the inoculations, all output such as chemicals and fertilizers were suspended since two months previously the experimental trial (July and August).

The growth chamber trial has shown a set of values comparable to the first trial: Control and EM-1 determined similar effects on the *Lolium perenne* L. essence without showing significant differences between the two experiments. DiSTA and D-PLUS determined an increasing of the ryegrass leaves tissues for both dry and fresh biomass, while the ratio between dry/fresh biomass was similar to EM-1 treatment. Root growth shown similar values between EM-1 and D-PLUS while DiSTA did not determined positive results determining effects of root length and biomass production comparable to the control. Remaining in the growth chamber's results, the analysis of the color of the leaves showed intensification by three microbial treatments, whereas the only control treatment determined a brighter coloration and as consequence a lower quality leaves.

The distinct group given by the darker coloration in microbial theses seems to be correlated at the chlorophyll content in perennial ryegrass leaves, confirming the trend shown in previous researches where brighter color was due to an increasing of total chlorophyll.

The field trial was conducted on an *Agrostis stolonifera* putting green during July and August 2012, two month that generally determine stress condition for cool-season grass (Beard, 1973). The soil parameters of pH, electro-conductivity, and total carbon-nitrogen content were bi-weekly monitored in order to verify eventually changing in the soil given by applied treatments, but no significant effects were detected.

The analysis of the layer of the putting green substrate shown important modification of the profiles between different theses, especially for the thickness of the thatch. Indeed, due to the inoculum of the different microbial mixes based on beneficial bacterial, the thatch layer has been progressively degraded. The EM-1 and DiSTA (both based on only bacterial components) determined a reduction of the thatch by 6,1% and 15,9% comparing to the control, while D-PLUS given a significative reduction of 26,7%. The underlying humus layer showed a thickening trend, opposite at the thatch reduction probably determined by the microbial degradation of thatch

and the subsequent downward leaching. The growth of the humus layer for each treatment was effectively inversely proportional at the relative thatch degradation: plots treated with EM-1 and DiSTA solutions shown an increasing of the humus layer of 23,5% and 20,4% respectively, while plots treated with D-PLUS had a humus increasing of 41,4% compared at 0 DAT value.

These results confirmed positive effects of the inoculation microorganisms in the soil, that had increased the decomposition of the soil organic matter making nutrients more available for the mineral assimilation by plants (Muller and Kussow, 2005).

A further confirm of the positive effect due to the application of the full treatment D-PLUS on the thatch layer was the reduction of the fresh biomass and the dry/fresh biomass ratio.

In order to assess if the diminution of the thickness of thatch and of the biomass was given by a metabolic degradation of effective microorganisms, the test of the organic content in the putting green top soil has shown interesting results. Indeed, plots treated with D-PLUS shown a progressively diminution of the of the percentage organic content while the total carbon in the soil was maintained constant. This was a positive proof that microbial degradation led to a partial mineralization of the organic carbon content in lignin or cellulose of leaves and roots.

The effective microorganisms applied on the creeping bentgrass surface have determined positive effects also on the physiological wellness of the turfgrass. The dry/fresh ratio of leaves biomass increased similarly in all the treatments and this data, as was seen in previously finding in growth chamber trial, corresponded in an increased tear off resistance.

The quality of the putting green surface was evaluated with the color analysis in RGB mode. The data shown a pronounced effect of D-PLUS to determine a darker color comparing with other treatments, while EM-1 and DiSTA shown only a partial significant difference comparing to the control.

## **Effect of commercial biostimulants containing *Bacillus spp.* plus growth promoters on an *Agrostis stolonifera* putting green in a field condition**

### **5.1 SECTION OBJECTIVES**

The third part of the study was carried out at the Turfgrass Research Center (TRC) of Virginia Polytechnic Institute and State University (Virginia Tech, United States). Different microbial treatments and biostimulants were tested on a creeping bentgrass putting green in a real field condition. Indeed, in this last trial the managing schedule such as fertilization and chemicals applications was maintained for the entire durate of the trial.

The effects of the different treatments were evaluated analyzing physiological, agronomical and morphological parameters. In addition was studied the microbial flora colonization in the roots and rhizosphere throughout molecular techniques.

## 5.2 MATERIALS AND METHODS

### 5.2.1 LOCATION

The last experimental trial conducted in the TRC (Virginia Tech) had a total duration of four months. Previously reliefs of chemicals and physical composition of the soil were conducted between March and April, two months before the starting of the trial. In addition during the period that proceeded the treatments applications were monitored the presence of weeds such as Kentucky bluegrass (*Poa pratensis* L.) and turf's pathologies insurgences such as dollar spot (*S. homoeocarpa*).



**Figure 54:** Experimental putting green in Turf Research Center of Virginia Tech.

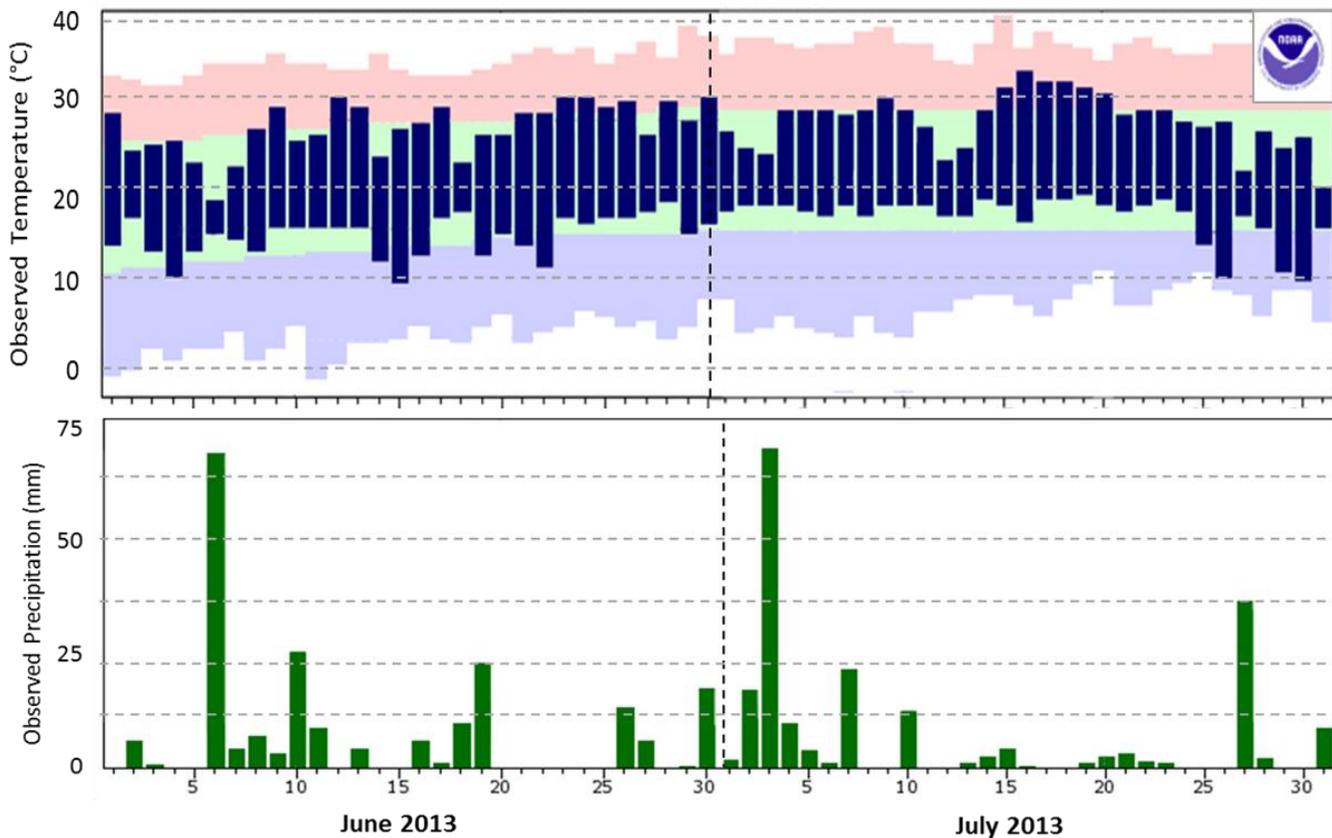
### Weather conditions

Virginia Tech is located in the South West Virginia in the upper transition zone, also termed as “transitional” because placed between the warm climates of the South and the cooler climates of the North. The climate is classified as mild mid-latitude in the subcategory of humid subtropical. Principal characteristic of this area are no dry seasons and hot summers. This is a climate very demanding for growing and quality of plants, influencing turfgrass species selection, culture and pest management.

Weather data were collected with reference to the National Weather Service Climate Forecast Office, Blacksburg, Virginia (Graph 21). Between 1<sup>st</sup> March and 31 May the total rainfall was 324 mm, a value that was 11% above the period mean. In the same period the temperature showed an average value of 9°C in correspondence with season's values, with minimum and maximum respectively 3°C and 15°C.

During the experimental period (1<sup>st</sup> of June until the end of July) the precipitations showed values close to the highest values recorder, with a total precipitation of 449 mm, 49% higher than the season's values.

For the whole experimental period the temperature remained within the season's values, showing an average temperature of 21°C, with a minimum and a maximum that were respectively 15,72°C and 32,17°C.



**Graph 21:** Observed temperatures (°C) and observed precipitations (mm) measured by the National Oceanic and Atmospheric Administration (NOAA), (Blacksburg, Virginia, United States). Mean temperatures for the summer season are included within the green band; highest and lowest temperatures are included in the red and blue band respectively.

### Experimental putting green

The trial was conducted in an experimental putting green inside the TRC, built following the USGA recommendations (2004): the growing medium had a topsoil layer of 450 mm composed by 95% sand, 3% silt and 2% clay. The portion of sand was represented by 55% of medium coarse sand (0,5-0,25 mm diameter), 20% coarse sand (1-0,5 mm diameter) and 25% very coarse sand (2-1 mm diameter). As most of Virginia's Golf Courses, at TRC the irrigation during the summer was settable in function of the daily precipitations. Generally, Virginia's golf courses need 25 – 40 mm of water from rainfall or artificial irrigation per week during the summer's period.

## 5.2.2 AGRONOMICAL MAINTENANCE

This final experiment was carried out following all the managements such as mechanical operations and chemicals applications scheduled for a creeping bentgrass' putting green, in order to operate in a real sport turf situation. The first mechanical management carry out was an aerification, that was performed with a ProCore® TORO, on April 15<sup>th</sup>. The core drilling of the putting green was adjusted to 5 cm deeper, with a density of 400 holes for square meter. A subsequent top dressing closed holes resulting from the mechanical removal of thatch.



**Figure 55:** The experimental putting green during the preparation stages in May.

The mowing of the creeping bentgrass' leaves was set at a 3,2 mm height with a frequency of 5 days per week. Mowing was processed with a Greenmaster® ride-on putting green mower (Figure 56).



**Figure 56:** mowing *Agrostis stolonifera* in Turf Research Center with the Greenmaster® mower for putting green

In order to collect leaves' samples for successive analysis of laboratory, was utilized a Jacobsen pedestrian mower (Figure 57). The mower had to be manually accompanied on the putting green's surface and every plot was mowed singularly. Leaves were collected in the plastic box ahead the mower and were immediately frozen with liquid nitrogen.



**Figure 57:** Jacobsen 500a pedestrian green mower for putting green.

### Fertilizer and chemicals

During the summer 2013, fertilizers and fungicides applications have followed a pre-established schedule but some changes were subsequently brought on the basis of weekly needs of the turf. Fertilizers were applied on a bicycle sprayer that was utilized manually (Figure 58.2) whereas chemicals were applied with a Toro multi-pro sprayer and fungicides were applied with a CHEM PRO prayer (Figure 58.1).



**Figure 58:** On the left the CHEM PRO sprayer. On the right the bicycle sprayer utilized for the applications of fertilizers and microbial solutions tested in the experimental trial.

### **Fertilizer applications**

Fertilizer was applied with foliar spraying throughout the summer on an N rates of 0,024 g per m<sup>2</sup>. They consisted of a Bent Special (28-8-18) applied via foliar spraying the 20<sup>th</sup> of June, a Green Grade granular (16-4-8) applied the 5<sup>th</sup> of June and finally an urea treatment (46-0-0) the 26<sup>th</sup> of July.

### **Fungicide applications**

To suppress the dollar spot presence was utilized Daconil ULTREX® (Chlorothalonil 0,2ml/m<sup>2</sup>), a broad spectrum non-system fungicide. Daconil ULTREX® was applied in date 10 June and 26 July. In date 20 June and 21 August Daconil ULTREX® was administered with the addition of Compass™ (Trifloxystrobin, 0,02ml/m<sup>2</sup>) mixed with Emerald® fungicide (principal active ingredient Boscalid 70%, 0,015 mL/m<sup>2</sup>).

Due to very wet condition of the summer 2013, an application of Banner MAXX® (principal active ingredient Propiconazole 14,3%, 0,05 ml/m<sup>2</sup>) on 15 June and 28 July was done. Branner MAXX® is a broad spectrum ad system disease control specific for turf.

### **Insecticide applications**

In order to control worm proliferation on the creeping bentgrass surface, in date 4 June was applied the synthetic pyrethroid Allectus®. This granular insecticide combine two active ingredients Imidacloprid (Merit®) and Bifenthrin. Allectus® was applied with a concentration of 100 kilograms/hectare. In according with the optimal efficiency was applied before egg hatch of the insects, followed by sufficient irrigation or rainfall to move the ingredient through the thatch.

## 5.2.3 TREATMENTS

The experimental trial was conducted applying different microbial mixtures and growth promoters on a creeping bentgrass putting green. Fertilizers used were chosen on the basis of increasing complexity and composition of growth promoters and soil bacteria. The applying concentration of different treatments was set in order to balance the nitrogen supply at 0,024 g per m<sup>2</sup>. Nitrogen is the main element influencing the quality, color, leaves growth and several other physiological parameters in sport turf. The five treatments tested in this study were applied bi-weekly from 27 May until 15 July 2013 for a total of 4 applying dates.

As control fertilizer was chosen the Nutriculture hi-K (13-0-44) a common fertilizer for a basic maintenance of the high quality sport turf during the summer season. The second thesis was composed by the Nutriculture hi-K with the addition of maltodextrin, a water-soluble complex carbohydrate. A treatment composed by sea weed extracts (SWE) and fulvic acid was the Emerald Isle TF K (2-0-16). A treatment composed only of bacteria was Roots 1-2-3 Premix. A complete treatment constituted by microorganisms and biostimulants was Roots Flex 3-0-20.

**Table 25:** List of the treatments and concentration utilized in the field trial.

TREATMENTS	SUBSTANCE	RATE per plot (30 sq ft) / 14 d	RATE per 1m <sup>2</sup> / 14 d
<b>a</b>	Nutriculture hi-K (13-0-44)	0,51 g	0,183 g (0,024 g N)
<b>b</b>	Nutriculture hi-K (13-0-44) + Maltodextrin	0,51 g + 17 g (=0,068 lb N)	0,183 g (0,024 g N)
<b>c</b>	Emerald Isle K (2-0-16)	3,39 g (=0,068 lb N)	1,190 g (0,024 g N)
<b>d</b>	Roots (1-2-3) Premix	6,78 g (=0,068 lb N)	0,793 g (0,024 g N)
<b>e</b>	Roots Flex® (3-0-20)	2,25 g (= 0,068 lb N)	0,807 g (0,024 g N)

### a) Nutriculture hi-K

The thesis chosen as control was the Nutriculture hi-K (13-0-44). This is a common fertilizer utilized in high quality turfgrass and its application was necessary in order to balance the minerals supplied in other thesis.

### b) Nutriculture hi-K + Maltodextrin

Maltodextrin is a complex hydro-soluble carbohydrate commonly used to provide energy to the microorganisms in the soil inoculant products.

**c) TrueFoliar-K® Nutri-Rational**

TrueFoliar-K® is an Emerald Isle Solution™ mix of mineral and growth promoter. This product in this experimental trial represents a thesis with mineral nutrient, seaweed extracts and fulvic acid.

<b>Nutri-Rational® TrueFoliar-K®</b>	
Total Nitrogen (N)	2%
1.15% Ammoniacal Nitrogen	
0.85% Nitrate Nitrogen	
Soluble Potash (K <sub>2</sub> O)	16%
Magnesium (Mg)	1.0%
1.0% Water Soluble Magnesium (Mg)	
Iron (Fe)	0.6%
0.6% Water Soluble Iron (Fe)	
Manganese (Mn)	0.45%
0.45% Water Soluble Manganese (Mn)	
Micronutrients	
Seaweed extract	

**Table 26:** Label of Nutri-Rational® TrueFoliar-K®

**d) Roots® 1-2-3 PreMix Plus®**

Roots 1-2-3 Premix Plus represents the bacteria only thesis tested in this experimental trial. It provides chelated micronutrients in addition with a blend of microbial cultures. The producer declares a deeper roots mass, green color without rapid growth, improved turf health and greener color. This product is particularly indicated for putting greens, tees and other high quality sport turfs. The period of applications can occur between spring, summer and pre-autumn, during installing sod, turf renovation, during fertigation and for injection. Roots 1-2-3 Premix contains 1,0% urea nitrogen, 2,0% phosphate and 3,0% potash. Other minor elements are represent by 2,7% chelated iron and 0,3 manganese. Microbial blend of Roots 1-2-3 is represented by *B. licheniformis* and *B. subtilis* both of the genera Baciullus. Microbial nutrients are supplied by the presence of 9,2% of yucca extracts.

<b>Roots® 1-2-3 PreMix Plus®</b>	
<b>Total Nitrogen (N)</b>	1.0%
1.0% Urea Nitrogen	
Available Phosphate (P <sub>2</sub> O <sub>5</sub> )	2.0%
Soluble Potash (K <sub>2</sub> O)	3.0%
Iron (Fe)	2.7%
2.7% Chelated Iron (Fe)	
Manganese (Mn)	0.3%
0.3% Chelated Manganese (Mn)	
Derived From: Dipotassium Phosphate, Iron Citrate, Manganese Sulfate, Urea	
<b>MICROBIAL CONTENT</b>	0,012%
Also contains non plant food ingredients	
0.012% Bacterial Cultures:	
Bacillus licheniformis	7 x 10 <sup>8</sup> cfu/mL
Bacillus subtilis	7 x 10 <sup>8</sup> cfu/mL
<b>MICROBIAL NUTRIENTS</b>	
Yucca Extract Surfactant	9,200%

**Table 27:** Label of Roots® 1-2-3 PreMix Plus®

e) **Roots Flexx® LebanonTurf®**

Roots Flexx® was the water –soluble full commercial product containing a blend of beneficial rhizosphere bacteria with organic amendments, yucca plant extracts and chelated micronutrients. Lebanon Turf’s specifics indicate a use for grow-in’s as well as during renovation and maintenance with all types of warm and cool season grasses.

Mineral content is composed by 3% content of total nitrogen (N) a 20% content of potash (K<sub>2</sub>O), 4% of sulfur (S) and other minor nutrients as manganese and magnesium.

In addition at the mineral content, Roots Flexx® contains a microbial population of 22 million colony forming unit for gram (cfu/g) composed almost entirely by Bacillus genera. Paenibacillus was classified in genus Bacillus since 1993 and now is reclassified as a separate genus (Paenibacillus genus).

Root Flexx contains also a 3% humic acid derived from Leonardite, the most commonly applied organic amendment in sport turf management. HA shown several positive actions toward sport turfs such as simile-phytohormone activity for promotion of root growth and increase of the cation exchange capacity.

Other bio-stimulants content in Root Flexx are 7,2 %

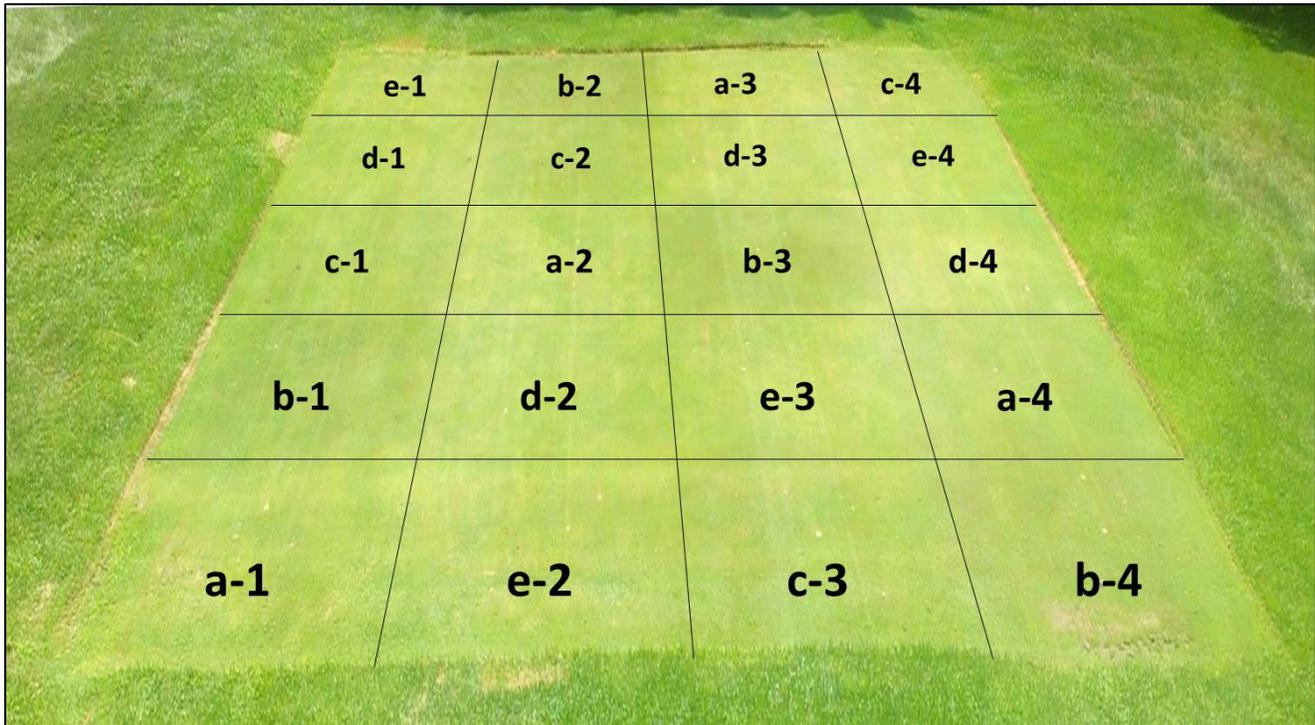
of Maltodextrin that is a hydrosoluble carbohydrate for promoting of bacteria metabolism, 1,8% of Yucca plant extract and seaweed extract that are plant growth promoter. Cytokine-containing seaweed and humic acid extracts are tested on creeping bentgrass (*Agrostis palustris* Huds. A.) in previous works by Zhang and Ervin (2003), where shown an enhanced root mass (21-68%) and foliar α-tocopherol (110%) and zeatin ribose (ZR) contents (38%). Other works demonstrated the capacity of SW extracts and HA to improve cool-season grass drought resistance possibility by hormonal up-regulation of plant defense system against oxidative stress.

<b>ROOTS® FLEXX® - LEBANONTURF</b>	
<b>Total Nitrogen (N)</b>	3%
3% Urea Nitrogen	
Soluble Potash (K <sub>2</sub> O)	20%
Water Soluble Magnesium (Mg)	1.5%
Sulfur (S), combined	4%
Iron (Fe), complexed	7%
Manganese (Mn), chelated	0.2%
Derived from: Urea, Ferrous Sulfate, Potassium Citrate, Manganese EDTA and Magnesium Hydroxide.	
<b>MICROBIAL CONTENT</b>	11 Billion cfu/Lb
1.83 Million cfu/Lb <i>Bacillus licheniformis</i>	
1.83 Million cfu/Lb <i>Bacillus megaterium</i>	
1.83 Million cfu/Lb <i>Bacillus polymyxa</i>	
1.83 Million cfu/Lb <i>Bacillus subtilis</i>	
1.83 Million cfu/Lb <i>Bacillus pumilus</i>	
1.83 Million cfu/Lb <i>Paenibacillus azotofixans</i>	
<b>Humic Acids</b> (derived from Leonardite)	3%
Formononetin	0.15%
<b>MICROBIAL NUTRIENTS</b>	12%
7.2% Maltodextrin	
1.8% Yucca Plant Extract (derived from <i>Yucca schidigera</i> )	
3% Seaweed Extract (derived from <i>Ascophyllum nodosum</i> )	
Fertilizer Ingredients	79.65%
Inert Ingredients	5.2%

**Table 28:** Root® FLEXX® label.

## 5.2.4 SCHEME DESIGN AND STATISTICAL ANALYSIS

A randomized blocks design with four replication's blocks was used.



**Figure 59:** Scheme design of the experimental putting green at TRC.

The statistical analyses were conducted with the software IBM® SPSS® Statistic 20 (IBM, United States). Comparisons between the means were conducted using the Student-Newman-Keuls test. Significant differences ( $p < 0,05$ ) among treatments were marked with different letters.

## 5.2.5 AGRONOMICAL AND MORPHOLOGICAL ANALYSIS

### Leaf production

Every 15 days leaf samples were collected from experimental plots in order to determine the production of dry biomass led by the different treatments. The leaves were mowed with the Jacobsen pedestrian mower with mowing eight set at 3.2 mm from the ground. Each plot was mowed singularly and leaves were caught stopping the mower on the edge of the plot. Leaves were gathered within the box of the mower and then closed in a paper bag 15 cm x 10 cm. Sample were dried in a hoven at 50°C for one week and then dry biomass of leaves were weighed. The value was expressed as grams production of dry leaf biomass per square meter in a day.

### Root biomass and length

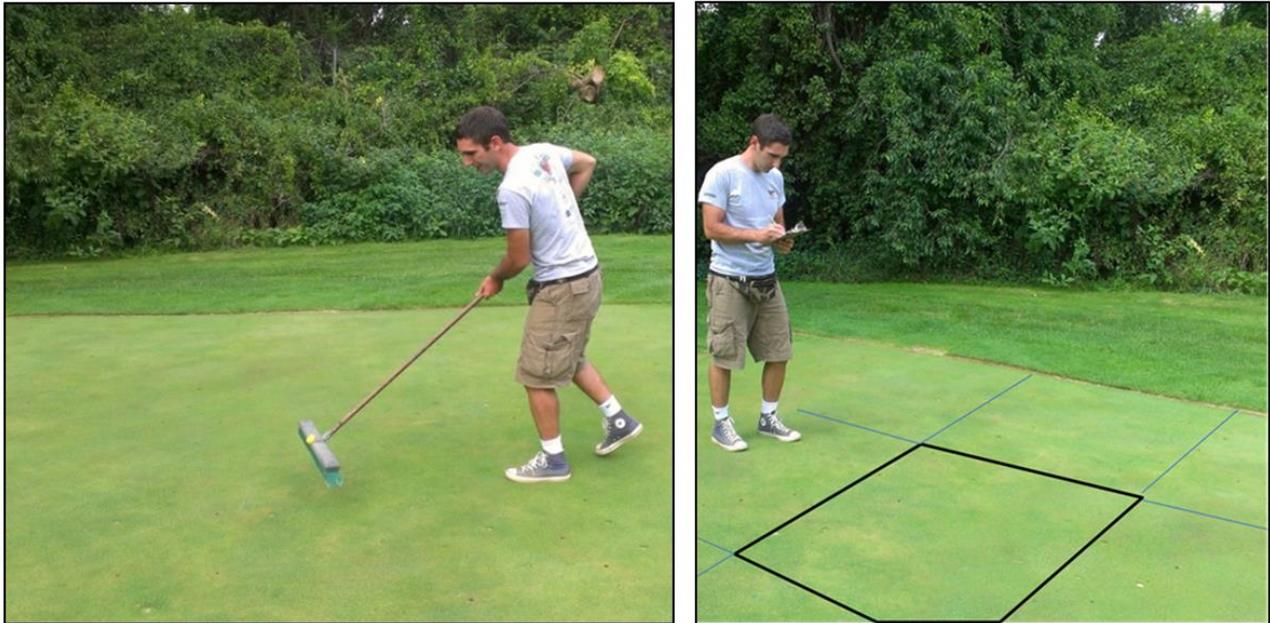
The analyses on the root apparatus were evaluated every two weeks, previously the leaf treatment day. Utilizing a 2cm section coring was extracted one sample from each plot. The samples of soil were collected to a 15 cm depth. Soil was carefully removed with a spatula beginning from the bottom of the sod. Roots were completely uncovered and cleaned from sand and plant residues. Roots apparatus was stretched out on a surface and the length was measured from the base of the leaf collar. In this same point plants were clipped to separate the roots. Roots were weighed and placed in a hoven to determine the dry biomass.



**Figure 60:** Clod extraction and roots after the cleaning from the growing medium

### **Turf quality and color (1-9) evaluation**

The visual assessment of the turf surface, was based on the 1-9 rating scale as reported in the guide lines of NTEP (National Turfgrass Evaluation Program). This kind of evaluation has the peculiarity to be a subjective assessment based on the relative comparison between the different plots that constitute the putting green. This parameter has been successively compared to an optical sensing reading (NDVI) for an objective turf quality and color evaluation.



**Figure 61:** Two steps of the color and quality assessment. On the left the preparation of the turfgrass surface. On the right the relative comparison between plots for the visual assessment.

Visual evaluations were conducted only during cloud-covered days, when shadows and reflection due to sun exposition were minimal. Furthermore, before starting the assessment was necessary to clean the green surface with a brush, always along the same direction, in order to eliminate dew to the leaves and homogenize the surface.

Visual assessment of the color and quality were evaluated as two different parameters. Color rating was measured overall plot color with 1 as worst value being straw brown and 9 as highest being dark green. Quality rating also based on 1-9 scale, considered all aesthetic and functional aspects of the turf such as color in combination with density, uniformity, texture and presence of diseases.

## NDVI color evaluation

Every two weeks was measured NDVI value (Normalized Difference Vegetative Index) that provides an indication of spectral vegetation of the putting green surface. NDVI data was obtained with a specific instrument for the green surface reading: Holland Scientific Crop Circle™, Model ACS-210. The Crop-Circle™ is a commercially available sensor-based system being used for site-specific analysis of plant covered surfaces and indirectly of the chlorophyll content by calculation the NDVI from the Visible Red (VIR) and Near Infrared (NIR) crop reflectance. Using reflectance sensor that was vehicle mounted, a reading was taken every given interval time and depending on vehicle speed (0,15 m/s) at least 90 readings was sampled. Normally NDVI values range from -1 to +1 with negative values for bare soil.



**Figure 62:** Crop Circle, reading the NDVI on the field

## 5.2.6 PHYSIOLOGICAL ANALYSIS

### Chlorophylls and Carotenoids

The determination of the leaf pigments content (Chlorophyll *a*, chlorophyll *b*, carotenoids and xanthophylls) into the plant tissues was performed as described by Strickland and Parsons (1972). After the grass mowing with 500a mower, around 50 grams of fresh tissues were collected in a paper bag and immediately frozen with a little amount of liquid nitrogen.

Before proceeding with the chlorophyll extraction, the leaves were crushed in a ceramic mortar adding 10 mg of magnesium carbonate ( $MgCO_3$ ) to neutralize the acidity of the solutes and prevent the conversion of chlorophyll *a* in Pheophytin. The material crushed in a mortar, were added 10 mL of acetone. The solution thus obtained was placed in the dark for 24 hours. Subsequently, the samples were shaken for 30 seconds with a vortex and then centrifuged at 10 ° C for 10 minutes at 1000 ppm. An aliquot of the supernatant was collected and added to cuvettes with optical path lengths of 1 cm for reading spectrophotometer. The reading was performed at wavelengths of 661.6 nm (which is the maximum absorption peak of chlorophyll *a*), 644.8 nm (which is the

maximum absorption peak of chlorophyll b) and 470 nm (which represents the maximum absorption peak of carotenoids). To calculate the concentration of the leaf pigments have been used the formulas described by Lichtenthaler et al., (2001):

- $c_a$  ( $\mu\text{g/mL}$ ) =  $11,24 * A_{661,6} - 2,04 * A_{644,8}$
- $c_b$  ( $\mu\text{g/mL}$ ) =  $20,13 * A_{644,8} - 4,19 * A_{661,6}$
- $c_{(c+x)}$  ( $\mu\text{g/mL}$ ) =  $(1000 * A_{470} - 1,90 * c_a - 63,14 * c_b) / 214$ .

### **Proline content**

The proline content of the foliar tissues was analyzed every two weeks. The protocol utilized was based on the work of Claussen (2005) and Bates (1973) with some adjustments. For the quantification of the antioxidant were collected about 10 grams of leaf tissues from each plot. The different samples were immediately frozen with liquid nitrogen and then stored at  $-80^\circ\text{C}$ . 50 mg samples were successively pulverized with liquid nitrogen within a mortar. 1.2 mL of 3% solution of 5-sulfosalicylic acid – hydrate. The samples were well vortexed for 30 seconds and then centrifuged at 1400 g for 15 minutes at  $22^\circ\text{C}$ . After the centrifugation 1 mL of supernatant was collected and to this was added 1 mL of reagent with ninhydrin. In parallel were prepared the standard of proline at different concentration in order to construct the relative calibration curve: 1, 5, 10, 20 and 30  $\mu\text{L}$  of 10 mM proline. These standards of proline were brought to 1 mL volume with 3 % of 5-sulfosalicylic acid – hydrate, and then were added 1 mL glacial acetic acid and 1 mL of reagent of ninhydrin. Being the ninhydrin light sensitive to prolonged exposure to light, from this step all the procedure were conducted in semi-dark conditions. All the samples, including the standards, were incubated for 1 hour at  $90^\circ\text{C}$  and subsequently the samples were cooled in ice for 5 minutes. 2 mL of toluene were added to all samples in order to separate the chromosphere. The samples were then vortexed for 10 seconds. In 30 second the upper phase was stabilized. The upper phase that contained the chromosphere was utilized for the reading in a spectrophotometer at a wavelength of 525 nm.

### **Superoxide dismutase (SOD)**

Superoxide dismutase (SOD) activity was measured from samples of leaves collected bi-weekly by the creeping bentgrass putting green. Leaves were mowed with the pedestrian mower Jacobsen 500A, immediately frozen with liquid nitrogen and stored at  $-20^\circ\text{C}$  for the successive analysis. 0.2 g of frozen were weight and then grinded in a mortar. Sample was homogenized in 10 mL of 0.05 M  $\text{Na}_2\text{PO}_4 / \text{NaH}_2\text{PO}_4$  pH 7 buffer and grinded in the mortar accurately. The resulting homogenate was filtered through four layers of cheesecloth and then centrifuged

at 4°C for 20 min at 15,000 g. The supernatant was collected to determine SOD activity photochemically using the assay system described by Giannopolitis and Ries (1977) with some adjustments.

Working in a semi-dark condition, in a glass tube was added the supernatant and a reaction mixture composed by 10 mM EDTA (Sigma-Aldrich), 130 mM L-Methionine (Sigma-Aldrich), 6.3 mM nitroblue tetrazolium (NBT – Sigma-Aldrich), 130 uM Riboflavin. Two more tubes blank composed by reaction mixture and extraction buffer without leaf homogenate were prepared too.

The samples were poured in a glass test tube 150mm x 18mm. Tubes were closed in a lighting apparatus composed by a rotating surface on the bottom where the glass tubes were placed on, and closed with a lid equipped with a circular fluorescent lamp (Sylvania Circular Fluorescent Lamp). The lighting apparatus was lined with an aluminum foil. Samples were subjected to lighting time of 30 min, with the rotating bottom activated. One blank was conserved in a dark condition meanwhile the other one was subjected at the illuminating treatment. After the 30 min samples and two blanks were read in a spectrophotometer at 560 nm wavelength. SOD activity was defined as the amount of enzyme required to cause 50% inhibition of NTB.

## **IAA and ABA**

Phytormones IAA and IBA were extracted from the leaves tissues using the procedure described by Zhang et al. (2009) with some adjustments and quantified using a LC-MS/MS linear ion trap quadrupole liquid chromatography-tandem mass spectroscopy (2300 Q Trap model; Applied Biosystem, Foster City, CA). 50 mg of fresh tissues of leaves were grinded in a ceramic mortar with liquid nitrogen. The frozen and grinded tissue was transferred in a 2 mL eppendorf and then added 1.6 mL Na-Phosphate buffer (50 mM pH 7.0) containing 0.02% sodium diethyldithiocarbamate as an antioxidant. Eppendorf were closed and enveloped with aluminum to be maintained in dark condition and slurried for 1h at 4°C. Within eppendorf was added 5 µL of an internal standard (<sup>13</sup>C<sub>6</sub>-IAA, 50 ng), 96 µL of HCl 1M and 150 mg of Amberlite XAD-7HP (Sigma, St. Louis, MO). Eppendorf was closed again, enveloped with aluminum and slurried 30 minutes at 4°C. The solution was completely collected with a pipette, paying attention to not pick up beads of amberlite. Amberlite on the bottom of the eppendorf was washed twice with 1% acetic acid, shaking gently with hands and washing water was removed paying attention to not pick up amberlite that had to remain on the bottom of eppendorf. After washing, in the eppendorf was added 1 mL of Methylene Chloride and amberlite was shaken in this reagent for 30 min at 4°C in dark condition. The solution was retained in a glass vial, without picking up amberlite from the falcon. This last step was replicate twice in order to be sure to extract with Methyl Chloride all the Phytormones. The methylene chloride was evaporated with nitrogen (Fig X) to dryness. The samples were dissolved in 150 µL methanol with 0.1% formic acid and diluted to 1 mL with 300 µL d.i. water with 0.1% formic acid. The samples were filtered using 17 MM Teflon syringe filters (0.2 µm) prior the reading in LC-MS/MS.

The analysis in LC-MS/MS was performed with a Prodigy 5  $\mu\text{m}$  OD83 100 to 150 mm by 1 mm (Phenomenex, Torrance, California) column. For each sample was injected 25  $\mu\text{L}$  of solution. The mobile phase consisted by 1 min of sample loading at 10/90 (vol/vol) methanol-ammonium oxaloacetate (MeOH-NH<sub>4</sub>OAc; 0,01 M; pH7), from 1 to 5 minutes a linear gradient from 10/90 to 95/5 (vol/vol) MeOH-NH<sub>4</sub>OAc (0,01 M; pH 7); from 5 to 7,5 min isocratic at 95/5 MeOH-NH<sub>4</sub>OAc (0,01 M) and from 7,5 to 11 min isocratic equilibration at start conditions at 10/90 (vol/vol) MeOH-NH<sub>4</sub>OAc (0,01 M; pH 7). A Kontron BIO-TEK P522 (Kontron Instrument, Milan, Italy) was used to obtain a constant flow rate of 60  $\mu\text{L}/\text{min}$  for gradient pump. The effluent was introduced at a rate of 60  $\mu\text{L}/\text{min}$  inside the MS source.

The LC system was linked to a Quattro II mass spectrometer (Micromass, Ltd., Manchester, United Kingdom) equipped with an electrospray (ES) interface and Z-spray (Micromass). The source temperature was 80°C, the nebulizing gas flow was 20 liters/h, the drying gas flow was 400 liters/h, and the capillary voltage was 3.5 kV. The cone voltage depended on the appropriate compound (45, 46). Collision activated dissociation of the protonated molecular ion ([MH<sup>+</sup>]) was obtained by using argon as a collision gas at the appropriate compound-specific collision energy, which ranged between 10 and 20 eV (46), and a PAR of 4.10 to 3 mbar. Quantification was done by multiple reactant monitoring of the [MH<sup>+</sup>] ion (dwell time, 0.05 s; interchannel delay, 0.01 s; span, 0 atomic mass unit) and the appropriate product ion. All indole compounds present in one sample were analyzed simultaneously during a single LC-MS/MS run. All mass spectra were background subtracted and smoothed once. All data were processed by using Masslynx 3.5 software.

### Gas exchange analysis – Licor 6400 XT

Photosynthetic carbon assimilation (Photo), stomatal conductance (Cond), leaf transpiration (Trmmol) and intracellular CO<sub>2</sub> concentration (Ci) were measured simultaneously utilizing the portable photosynthesis system Licor-6400XT (LiCor Inc. Lincoln, NE, USA) equipped with a leaf chamber fluorometer 6400-40. Within the leaf chamber photosynthetically active radiation (PARi) allows to compare the gas exchange in leaves under the effect of different treatments applied.

In order to obtain long enough leaves to be read within the foliar chamber, putting green sods were extracted from the experimental field with a metallic cylinder at 0 day after



**Figure 62:** Sods of creeping bentgrass during the growing within the hydroponic system

treatments (0 DAT), 30 DAT and 60 DAT, and grown for two weeks within a growth chamber. Sods had a diameter of 4 cm and a depth of 4 cm. Sods were placed inside plastic cones and then vertically tiled in a suitable support inside the hydroponic bath. From each plot were extracted three sods. 12 sods of the each treatment were placed in separated tanks in order to keep separate the hydroponic solutions. The different groups of sods were kept separately within the tank by insulating sidewalls. Growth chamber were set at 30°C with 16 hours of light and 20°C with 8 hours of dark as described by Xu and air humidity was set at 75% as described by Xu (2000).

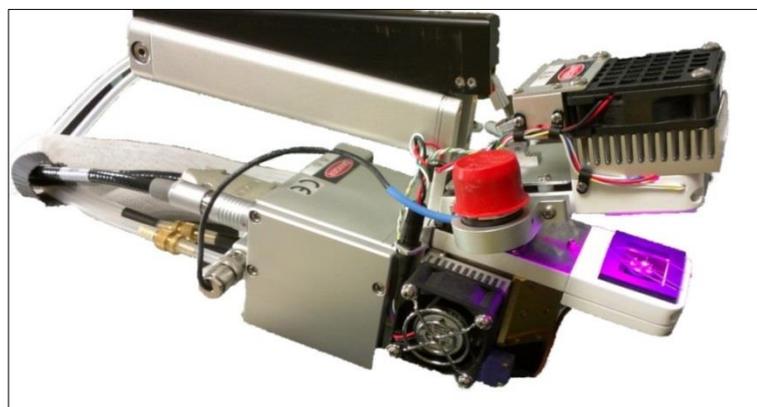
LI-6400 was initially connected to the IRGA (Infra Red Gas Analyzer), taking care to uncoiling the cable to prevent cable wing. Batteries were put within the Licor console. CO<sub>2</sub> cartridge was attached in the specific position and a new check-O-ring was positioned to ensure the connection. Desiccant and CO<sub>2</sub> external source was positioned in bypass position. The console was turned on with on/off switch. In main menu, parameters of leaf chamber was set at initial calibration: CO<sub>2</sub> flow inside the leaf chamber was set up at 400  $\mu\text{mol mol}^{-1}$ , leaf temperature was controlled by the integrated Peltier coolers and was set at 23°C, lighting intensity of the Red/Ble LED light source was set at 800  $\mu\text{mol m}^{-2}\text{s}^{-1}$  PAR.

Leaf area subjected to analysis was set at 10 mm (4 leaves x 2,5 mm width). In this way there was not necessary to fill the entire area of the leaf chamber and the software calculated values adjusted accordingly. Utilizing four leaves instead one, the accuracy of the detection increased because detection precision it is directly proportional to the leaf area. Humidity value within the leaf chamber was maintained constant with through the use of specific desiccant flow input.

Gas exchange analysis were analyzed at 12:00 – 13:00 at 0, 30 and 60 DAT, in order to respect the diurnal plant response changing in function of temporal and environmental conditions (Bernacchi et al., 2006).

From each sample were cut four leaves to be placed within the Li-6400XT chamber system. Leaves were placed with the superior page facing upward, parallel with each other and non-overlapping. The leaves used for the gas exchange reading should have similar shape and length, in order to cover a similar section within and across completely the leaf chamber (3 cm length).

The exactly leaf surface analyzed in the chamber was successively detected scanning leaves on a graph paper and measure, obtained with the software image J. The total foliar surface analyzed for each sample was utilized to adjust relative data. Measurements for each plot were taken in three replicates. Each measurement was taken with four leaves placed simultaneously within the leaf chamber.



**Figure 63:** Leaf in the Licor's chamber during gas change's analysis

## 5.2.6 MICROBIAL ANALYSIS

Samples of the putting green top soil were extracted in three dates (0 DAT, 30 DAT, 60 DAT) and shipped at the Department of Agricultural Science of Bologna (Italy) in order to analyze the microbial composition. The clods of the experimental green were extracted with a clod-sampler, sealed with parafilm in a plastic container and then placed in a polystyrene container, submerged in dry-ice and lastly shipped after a couple of hours from the extraction. In Italy all sample were frozen at -80°C for the successive analysis.

### DNA extraction from soil

Metagenomic DNA from approximately 250 mg soil was extracted using the PowerSoil DNA kit (Mo Bio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions with some adjustments. In particular, 5 µl of mutanolysin (100 U/ml, Sigma-Aldrich) and 195 µl of lysozyme (50 mg/ml, Sigma-Aldrich) were added to the soil in the bead solution supplied with the kit. The suspension was then incubated at 37 °C on a rotary shaker for two hours, prior to chemical (with SDS-containing solution supplied with the kit) and mechanical (bead beating on vortex at maximum speed for 10 min) cell lysis. DNA was eluted in 100 µl of TE buffer pH 8.0. DNA extraction was performed in duplicate. The purity and quantification were determined by measuring the ratio of the absorbance at 260 and 280 nm (Infinite® 200 PRONanoQuant, Tecan Switzerland). Extracted DNA was stored at -20°.

### 16S rRNA gene amplification

For Bacterial DGGE analysis performed on soil samples, PCR amplification of 16S rDNA was performed with universal primers HDA1 with GC clamp (5'-CGCCCGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCAGCAGT-3') and HDA2 (5'-GTATTACCGCGGCTGCTGGCAC-3') (Walter et al., 2000). The use of these primers generates a PCR fragment of about 200 bp and suitable for a subsequent DGGE analysis. All reactions for PCR-DGGE were carried out in a 50 µl volume containing 1.5U AmpliTaq Gold DNA polymerase (Applied Biosystem), 5 µl of 10X PCR Gold Buffer (Applied Biosystem), 200 µM of each deoxynucleotide triphosphate (Fermentas GmbH, Germany), 1.50 mM MgCl<sub>2</sub> (Amersham Biosciences), 0.45 µM of each primer (MWG Technologies, Germany), 2.5% (w/v) bovine serum albumin (BSA; Fermentas), 4 µl DNA template, and sterile MilliQ water for adjustment of the volume to 50 µl.

The PCR reaction was performed on a Biometra Trio-Thermoblock (Biotron, Gottingen, Germany) under the following thermocycling program: 5 min initial denaturation at 95 °C; 35 cycles of 95 °C for 30 s, 54 °C for 60 s,

72 °C for 40 s; followed by a final elongation step of 72 °C for 7 min. The expected size (~200pb) and amount of the PCR products were estimated by analyzing 2 µl samples by agarose gel (1.5% w/v) electrophoresis and ethidium bromide staining.

### **PCR-DGGE analysis**

The DGGE analysis was basically performed as first described by Muyzer et al. (1993), using a DCode System apparatus (Bio-Rad). Polyacrylamide gels [7% (w/v) acrylamide:bisacrylamide (37.5 : 1) (Bio-Rad)] in 1X Tris-Acetate-Edta (TAE) buffer were prepared using a Bio-Rad Gradient Delivery System (Model 475, Bio-Rad), using solutions containing 35–60% denaturant [100% denaturant corresponds to 7 M urea (Sigma-Aldrich) and 40% (v/v) formamide (Sigma-Aldrich)]. The electrophoresis was run at 55 V for 16 h at 60 °C. The gels were stained in a solution of 1× SYBR-Green (Sigma–Aldrich, Milwaukee, WI) in 1× TAE for 20 min and its image captured in UV transillumination with a digital camera supported by a Gel Doc™ XR apparatus (Bio-Rad).

By inclusion of the same reference pattern, previously described, twice on each DGGE gel, resulting band profiles could be digitally normalized by comparison with a standard reference, using the Gel Compare, version 6.1 software package (Applied Maths, Kortrijk, Belgium). Similarity matrix and dendrogram of the DGGE profiles were generated on the base of Pearson correlation coefficient and unweighted pair-group method average (UPGMA), respectively.

Selected dominant bands were cut from the gel with a sterile scalpel and DNA was eluted by incubating the gel fragments for 16 h in 50 µL of sterile deionized water at 4 °C. 2 µL of the solution were then used as template to re-amplify the band fragment using the same primers without the GC-clamp and the same PCR conditions described above except for primer concentration of 200 nM and PCR cycle annealing at 55 °C for 30 sec. The obtained bacterial amplicons were then sequenced with primer HDA2 as described below.

Sequencing was performed after amplicon purification with PCR clean-up (Macherey-Nagel GmbH & Co. KG, Germany) according to the manufacturer's instructions. Sequencing reactions and runs were performed by Eurofins MWG Operon (Ebersberg, Germany). The sequences obtained were subjected to taxon classification using RDP classifier, an available tool at the RDP-II website (<http://rdp.cme.msu.edu/classifier/classifier.jsp>). Moreover, seqmatch search was used to find the closet match for each 16S rRNA fragment ([http://rdp.cme.msu.edu/seqmatch/seqmatch\\_intro.jsp](http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp)).

## 5.3 RESULTS

### 5.3.1 AGRONOMICAL AND MORPHOLOGICAL RESULTS

#### Leaf production

**Table 29:** Leaves biomass production. Fresh weight, dry weight and dry /fresh weight ratio (DW/FW %) at 0, 30 and 60 days after treatments (DAT).

Leaf production	DAT	Control	Control + MD	SWE	Bacteria only	Full
Fresh Weight	0	15,21 ± 2,38 a	14,20 ± 3,02 a	13,31 ± 4,29 a	15,26 ± 1,01 a	14,86 ± 1,96 a
	30	11,90 ± 5,37 a	13,41 ± 1,00 a	12,19 ± 3,36 a	11,81 ± 1,34 a	12,14 ± 1,76 a
	60	13,85 ± 3,04 a	13,00 ± 4,63 a	11,79 ± 2,04 a	11,50 ± 2,45 a	12,67 ± 2,60 a
Dry Weight	0	1,99 ± 0,25 a	1,81 ± 0,28 a	1,81 ± 0,73 a	1,89 ± 0,29 a	1,95 ± 0,30 a
	30	1,46 ± 0,66 a	1,70 ± 0,15 a	1,47 ± 0,49 a	1,37 ± 0,25 a	1,47 ± 0,31 a
	60	1,70 ± 0,47 a	1,55 ± 0,55 a	1,39 ± 0,48 a	1,23 ± 0,46 a	1,69 ± 0,62 a
DW/FW (%)	0	13, 12 ± 0,66 a	12,90 ± 1,12 a	13,34 ± 1,26 a	12,31 ± 1,25 a	13,13 ± 0,95 a
	30	12,25 ± 0,77 a	12,65 ± 0,15 a	11,95 ± 0,72 a	11,56 ± 0,89 a	12,05 ± 1,08 a
	60	12,23 ± 0,90 a	12,20 ± 2,98 a	11,52 ± 2,78 a	10,93 ± 4,18 a	13,26 ± 3,98 a

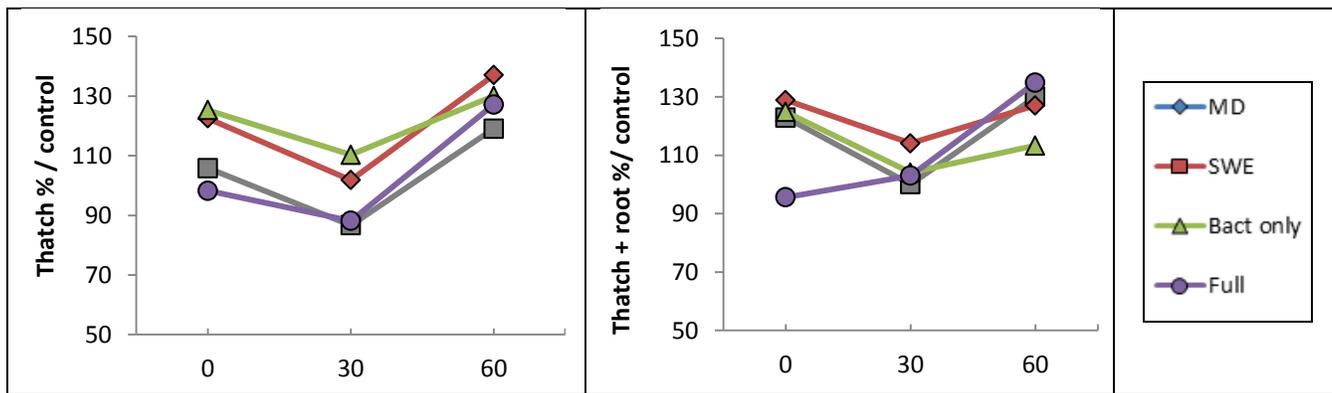
The leaf biomass was not significantly affected by the different product tested on the experimental putting green during the two month period. Fresh weight and dry weight leaves production diminished homogeneously during the experimental period, in line with the seasonal trend due of the solar radiation and summer temperatures. Any tested thesis, compared with the control within the same DAT, achieved a statistical difference for both dry and fresh biomass production. Furthermore, DW/FW ratios did not showed changings due different treatments but it maintained constant values without showing statistical differences.

#### Root biomass production

**Table 30:** Thatch and total biomass (thatch + roots) at 0,30 and 60 DAT.

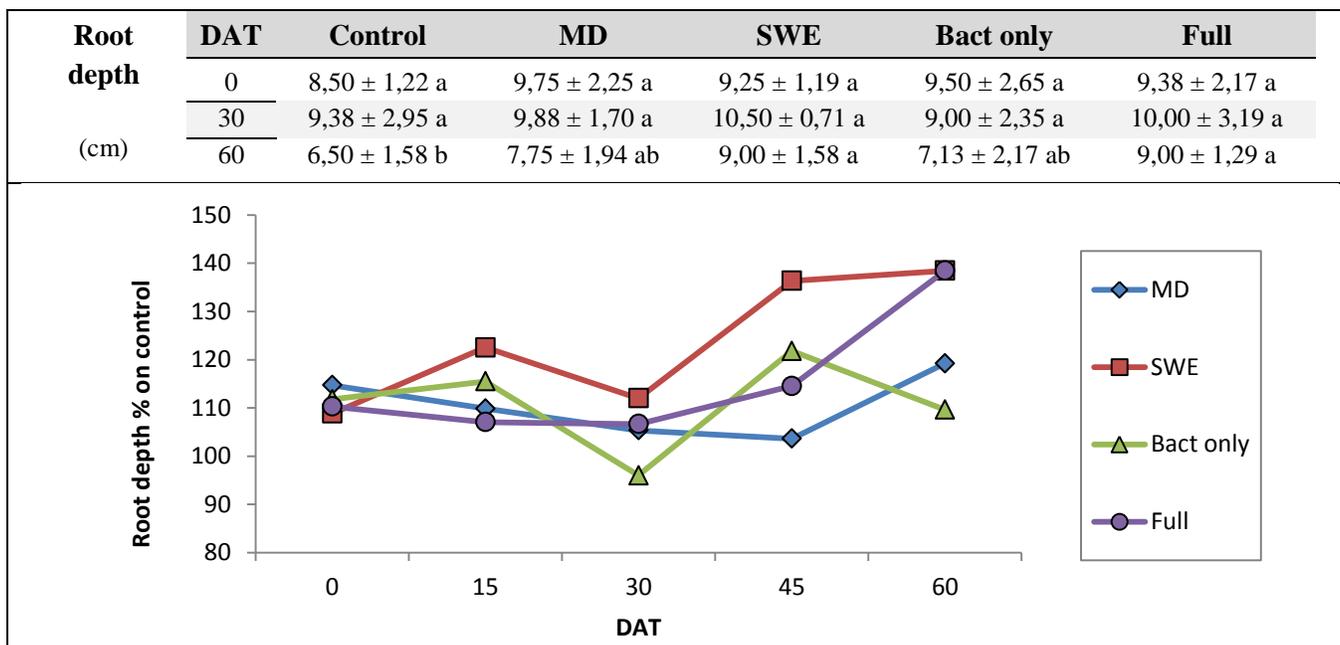
Dry Weight		Control	Control + MD	SWE	Bacteria only	Full
Thatch	0 DAT	0,10 ± 0,03 a	0,11 ± 0,03 a	0,12 ± 0,02 a	0,13 ± 0,02 a	0,10 ± 0,01 a
	30 DAT	0,11 ± 0,01 a	0,10 ± 0,01 a	0,12 ± 0,02 a	0,12 ± 0,04 a	0,10 ± 0,02 a
	60 DAT	0,08 ± 0,02 a	0,10 ± 0,02 a	0,11 ± 0,01 a	0,11 ± 0,02 a	0,11 ± 0,01 a
Thatch + Roots	0 DAT	0,24 ± 0,05 ab	0,29 ± 0,04 ab	0,31 ± 0,06 a	0,30 ± 0,05 ab	0,23 ± 0,04 b
	30 DAT	0,29 ± 0,05 a	0,29 ± 0,05 a	0,33 ± 0,07 a	0,30 ± 0,09 a	0,30 ± 0,04 a
	60 DAT	0,28 ± 0,02 b	0,36 ± 0,05 a	0,36 ± 0,04 a	0,32 ± 0,06 ab	0,38 ± 0,06 a

As summarized in the related table, the different treatments did not determined significant effects on the dry biomass of thatch if during the two month experimental period. However considering also the roots biomass under the thatch's layer is possible to appreciate a significant growth given by MD, SWE and Full treatments. The graphs below represent the percentages variations of dry biomass of thatch and total weight comparing to the control.



**Graph 22:** Thatch and thatch + roots dry biomass percentage compared to the control during at 0, 30 and 60 DAT.

### Root length



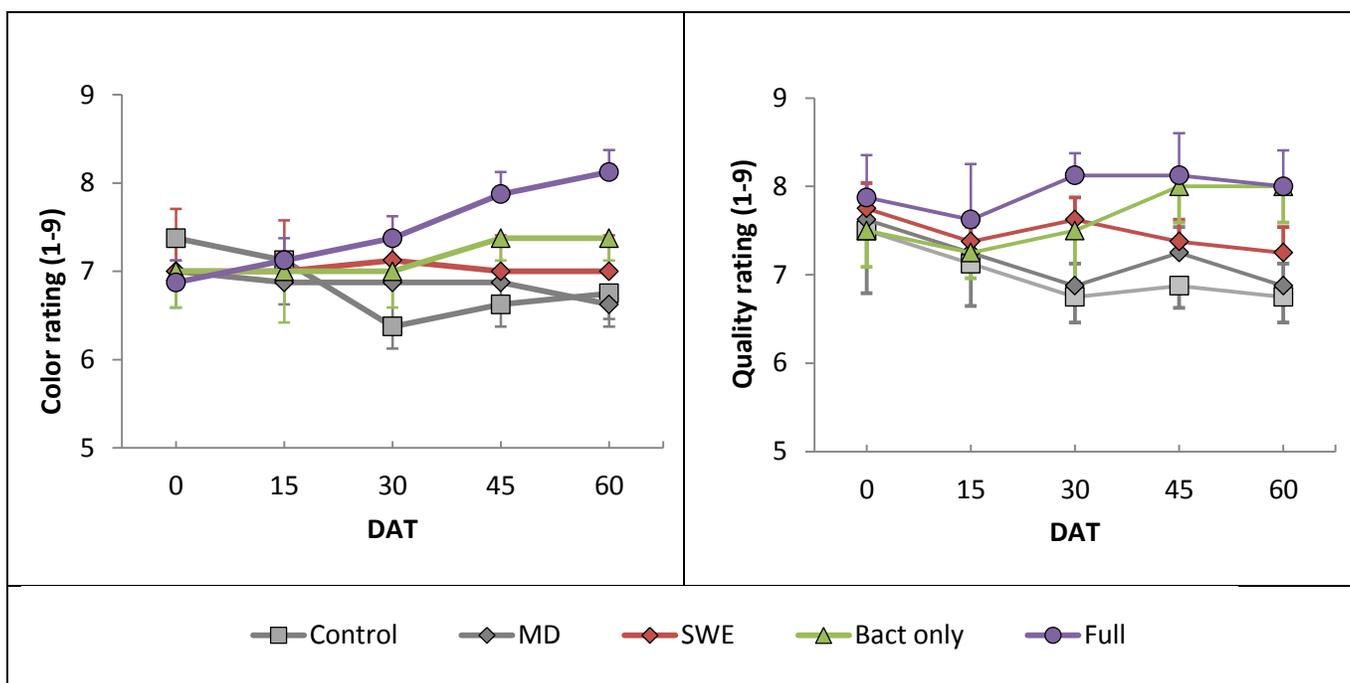
**Graph 23:** Root length (% respect to the control).

Sea weed extract and the Full products determined significant differences respect to the control at the end of the experimental trial. The first valuable effects were visible after 45 DAT, although these did not show significant

differences yet. At 60 DAT SWE and Full treatments determined a significant increasing of the root elongation if compared to control and to the Bact only applications.

### Quality and color (1-9) assessment

The quality and color ratings were conducted bi-weekly utilizing the 1-9 evaluation scale as reported in the relative material and method section. Color and quality ratings were performed measuring the overall plot values as relative comparison between parcels, as explained in NTEP (National Turfgrass Evaluation Program) guideline.



**Graph 24:** Color and Quality results. Data were collected bi-weekly for a period of 60 days. Treatments applied was Control (fertilizer), MD (Fertilizer and Maltodextrine), SWE (Nutriculture hi-K), Bacteria only (Roots 1-2-3 Premix) and Full application (Root®Flexx®).

Full product determined a gradual increase of the color score during the two month experimental period, and showed statistical significant effect on the influencing the color rating from 45 DAT. At 60 DAT Full product determined the highest value of color rating with a mean value of 8,13. Bacteria only solution and SWE determined a color ratings of 7,37 and 7,00 respectively at 60 DAT. Maltodextrin based on treatment did not showed significant differences from the control during all the experimental period.

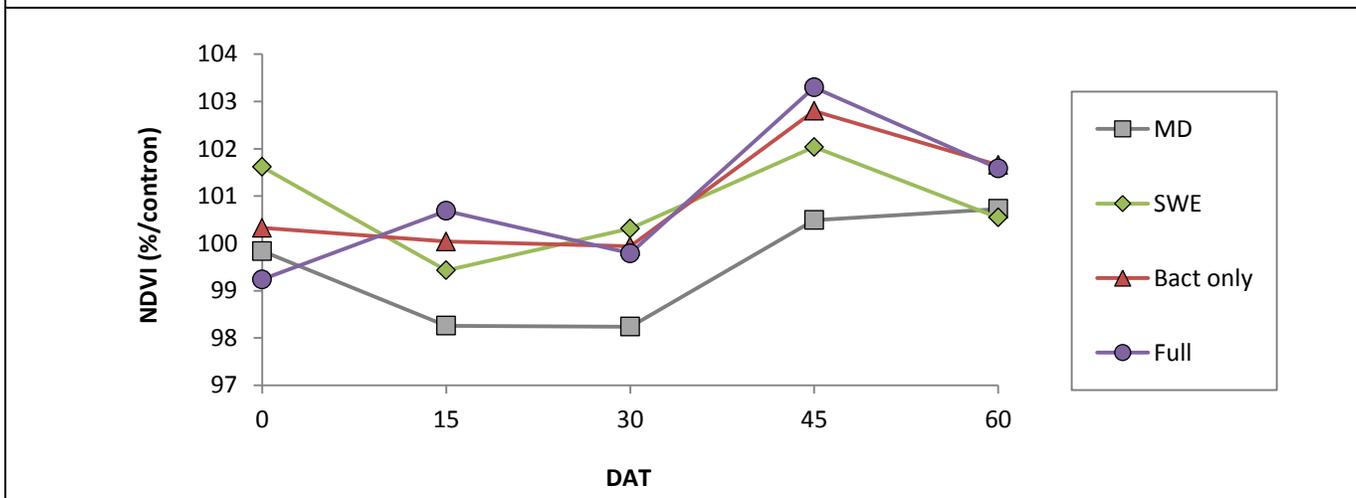
Quality ratings that is not based on a color evaluation alone, but on a combination of color, density, uniformity and texture, has partially met the color values. Also for the quality rating, the Full product determined the highest values with a score of 8,00 at 60 DAT. The same value was obtained by Bact only product, indeed between two microbial based solutions there was not statistical difference at 60 DAT. SWE showed significant

differences comparing to the control and MD thesis at 60 DAT, achieving an evaluation of 7,25 at 60 DAT. Also in this case, there was not statistical difference between MD and fertilizer only theses.

### NDVI color evaluation

Normalized difference vegetation index (NDVI) was evaluated bi-weekly on the experimental putting green with a Crop Circle to evaluate the aesthetic variations of the putting green surface with an objective assessment. In the table below are shown the values obtained from each treatment during the two month experimental period.

DAT	Control	MD	SWE	Bact Only	Full
0	0,660 ± 0,027 b	0,659 ± 0,024 b	0,670 ± 0,024 a	0,662 ± 0,027 b	0,655 ± 0,019 c
15	0,676 ± 0,012 b	0,664 ± 0,016 d	0,672 ± 0,021 c	0,676 ± 0,006 b	0,680 ± 0,008 a
30	0,681 ± 0,012 ab	0,669 ± 0,016 c	0,683 ± 0,021 a	0,681 ± 0,012 ab	0,680 ± 0,014 b
45	0,624 ± 0,025 c	0,627 ± 0,018 c	0,636 ± 0,029 b	0,641 ± 0,028 a	0,644 ± 0,012 a
60	0,635 ± 0,030 c	0,640 ± 0,017 b	0,639 ± 0,043 bc	0,646 ± 0,034 a	0,646 ± 0,026 a



**Graph 25:** Normalized difference vegetation index (NDVI) in an *Agrostis stolonifera* putting green. In the graph are compared percentage values obtained for different thesis respect to the control.

Due to the high instrumental sensitivity, the values were founded to be statistically different early at the beginning of the experimental evidence. At the end of the experimental period Full treatment and Bacterial only solution determined a significant increasing of the NDVI parameter, with a percentage difference to the control of 1,6% and 1,7% respectively.

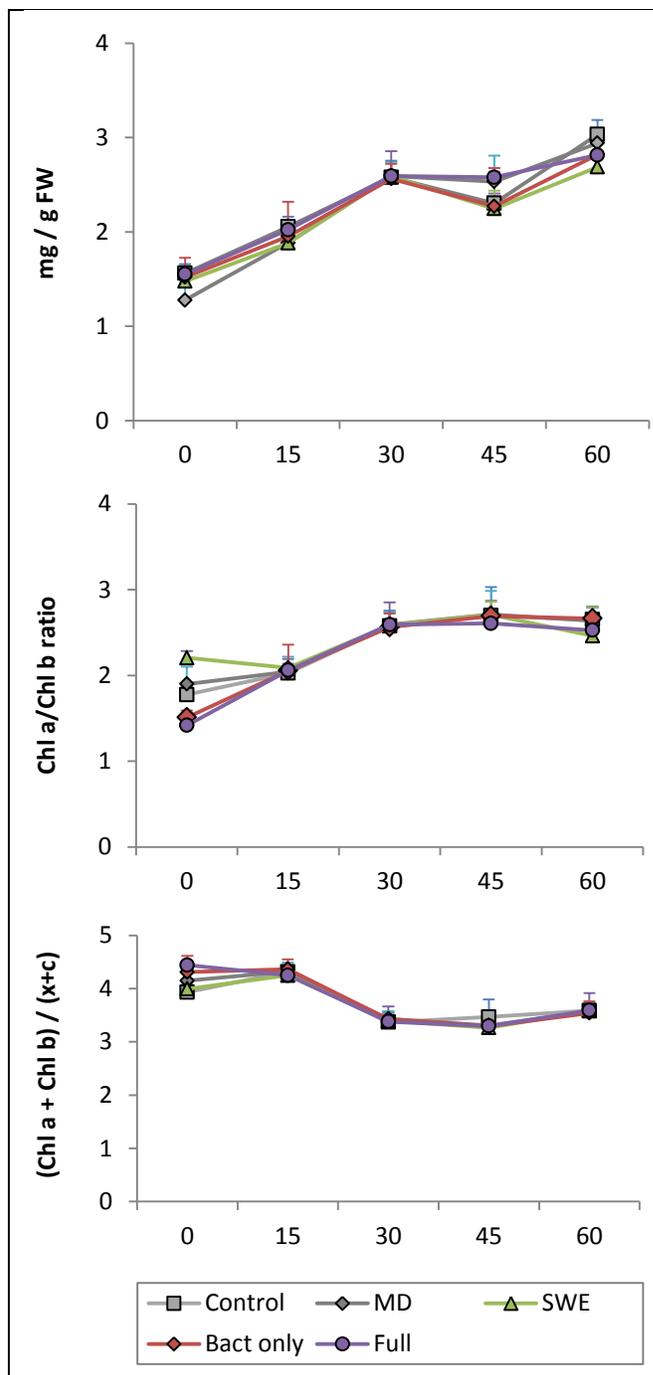
## 5.3.2 PHYSIOLOGICAL RESULTS

### Chlorophylls and Carotenoids content

Foliar pigment content showed that all the treatments applied on the *Agrostis stolonifera* determined similar effects without changing statistical differences during the two month experimental period. The total chlorophylls content in foliar tissues (mg of Chlorophyll a + Chlorophyll b on grams foliar fresh biomass) grown in a range between 81% and 130% from 0 until 60 DAT without showing statistical difference for different treatments. The decline in the growth of the photosynthetic pigments content occurred between 30 and 45 DAT, correspond to a sharp rise of temperatures that reached 35°C at 38 DAT.

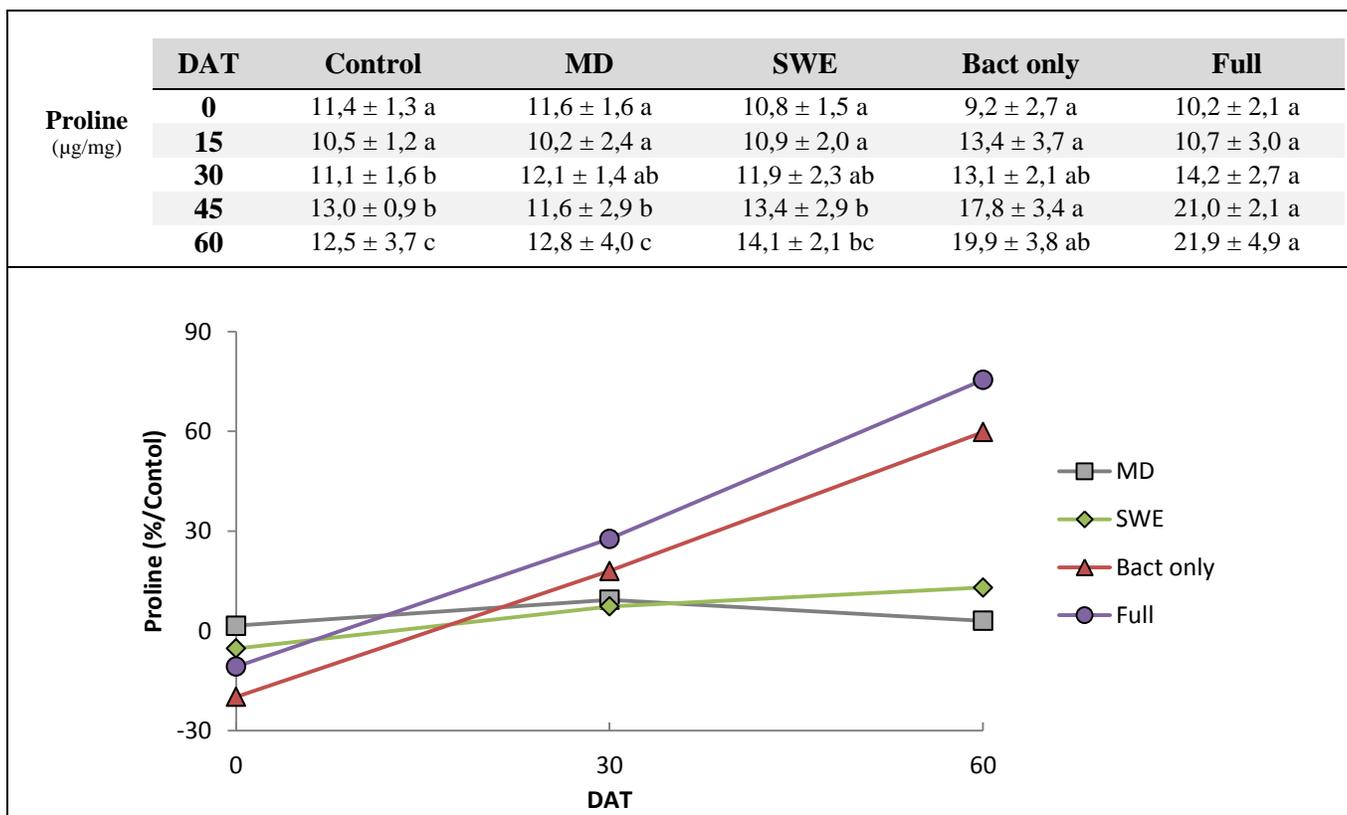
Chlorophyll a/b ratio was utilized as indicator of stress by sun exposing condition. a/b ratio was stabilized at an average of 2,59 after one month of treatments and remained constant until the end of the experimental trial, without showing statistical differences between different treatments.

The weight ratio of Chls a and Chls b to total carotenoids  $(a+b)/(x+c)$  was utilized as indicator of the greenness of the grassy surface. The Chls/carotenoids ratio decreased from 15 and 30 DAT by 16% - 31%, and successively maintained the same values from 30 and 60 DAT, without showing statistical differences between different treatments. From 45 DAT to 60 DAT Chls/carotenoids ratio increase lightly by 3,5% to 6,5%.



**Graph 26:** Leaves pigment content in the leaf tissues of creeping bentgrass.

## Proline

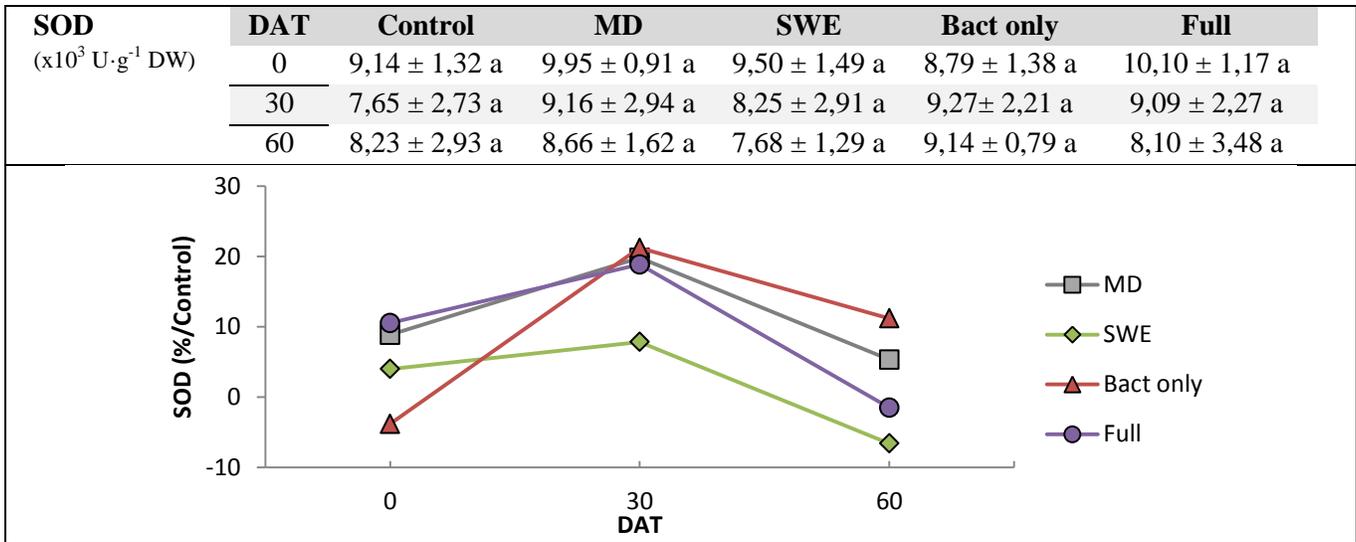


**Graph 27:** Proline contents in leaf tissues of creeping bentgrass.

Full treatment and Bacterial only solution determined a gradual increasing of the proline content in the putting green creeping bentgrass (Graph 27). Full treatment showed a statistical difference comparing to the control from 30 DAT. Bacteria only applications brought increased the content of proline similarly to Full treatment, leading to a percentage increase by 60% comparing to the control. Sea weed extract treatment show a partial statistical comparing to the control improving the proline content by 13% at 60 DAT.

## SOD activity

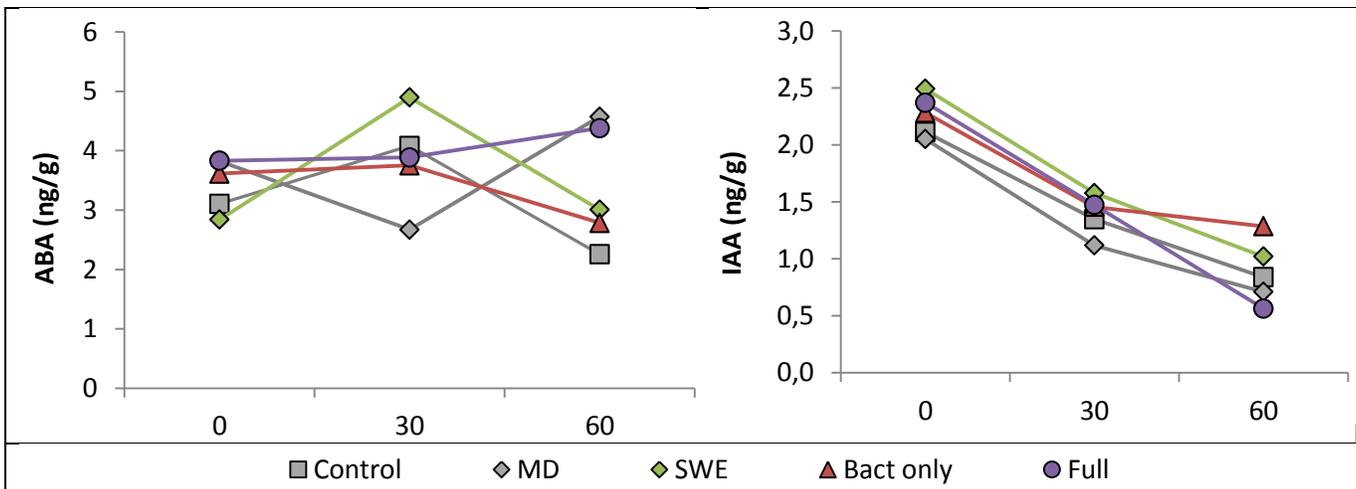
The study of the superoxide dismutase activity was performed analyzing the leaves of *Agrostis stolonifera* putting green. The activity on the SOD activity was quantified as the amount of enzyme required to inhibit 50% nitroblue tetrazolium (NTB), as explained in relative material and method section.



**Graph 28:** Content of superoxide dismutase in the foliar tissues of the creeping bentgrass.

Determination of the SOD activity in leaf tissues did not show significant statistical differences between applied treatments. At 30 days after treatments bacterial based treatments and maltodextrin determined an increased SOD activity by 19%, 21% and 20% respectively if compared to the control (Graph 28). At 30 DAT, bacteria only treatment determined the highest increasing of the SOD, with an increment of 25% if compared with respective 0 DAT values. At the end of the experimental period, Bacteria only thesis shown an SOD activity 11% higher comparing to the control, with a percentage diminution from 30DAT to 60DAT by 10%. SWE, Full treatment and MD shown a decreasing of the SOD activity by 14%, 20% and 14% respectively from 30 to 60 DAT.

### Indole-3-acetic acid and abscisic acid content



**Graph 29:** ABA and IAA content per grams of leaves tissues in *Agrostis stolonifera*.

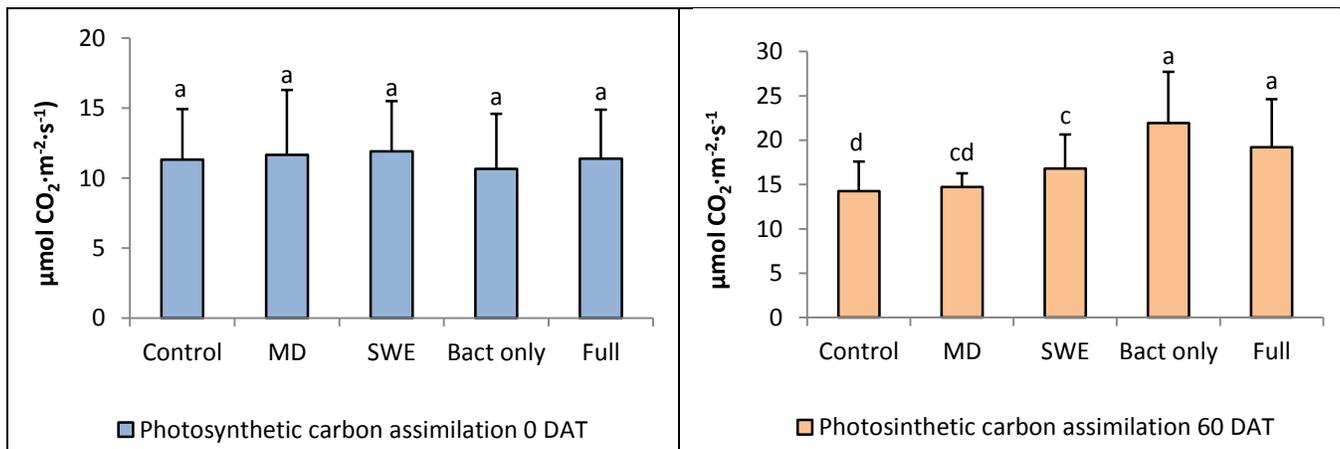
The analysis of the abscisic acid (ABA) content in the leaves of *Agrostis stolonifera* did not show significant effects between the different treatments. The ABA content was maintained in a range of  $3,5 \pm 0,5$  ng of phytohormone per gram of leaf tissues during the two month experimental period, without showing predominant effects of one or more treatments at any sampling date.

Indole-3-acetic acid (IAA) did not show significant differences between the different treatments applied and control. Unlike the contents of ABA, the levels of IAA decreased steadily over the two month experimental period; at 0 DAT IAA contents in leaves tissues were between 2,1 - 2,5 ng per gram of leaf tissue and decreased until a range by 0,8 - 1,3 ng/g.

### Gas exchange analysis - LiCor 6400 XT

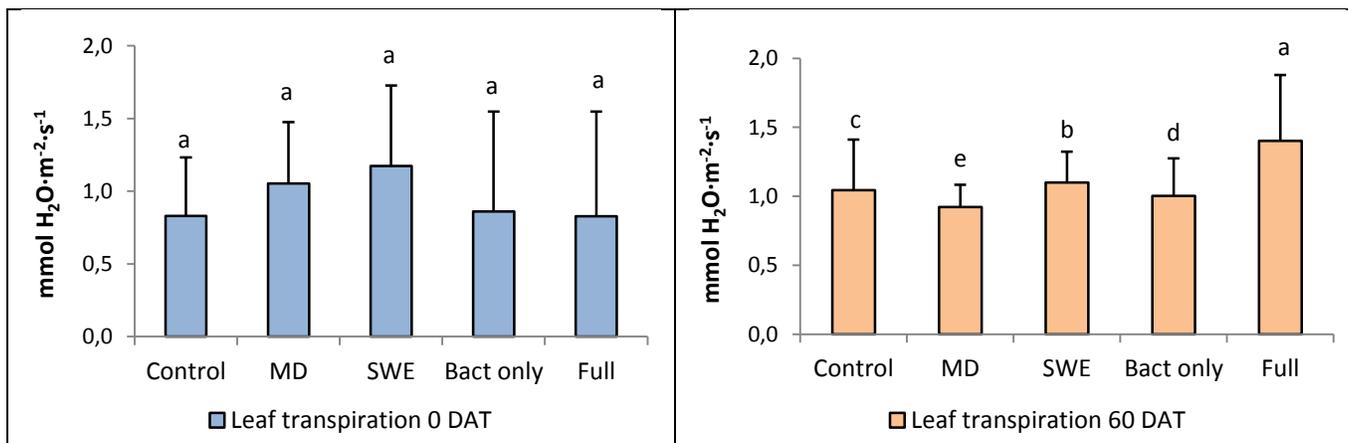
The CO<sub>2</sub> and water flux were measured with the Li-Cor 6400 XT. Determinations were conducted every two weeks on Creeping bentgrass plants, transplanted from the field after each treatment and grown within a grow chamber until the leaf reached 4 cm length. Gases exchanges were measured simultaneously, over the same leaf area. Parameters included: photosynthetic carbon assimilation (A), stomatal conductance (g<sub>s</sub>), intracellular CO<sub>2</sub> (C<sub>i</sub>), leaf transpiration (E).

Photosynthetic carbon assimilation (Graph 30) appeared uniform across different plots before to start the applications at 0 DAT. After two months experimental period the Bacterial only and the Full treatments determined the highest and significant increasing by 54% and 35% comparing to the control respectively. Also SWE induced significant difference comparing to the control increasing the photosynthetic carbon assimilation by 18%, but still lower than Bact only and Full treatments.



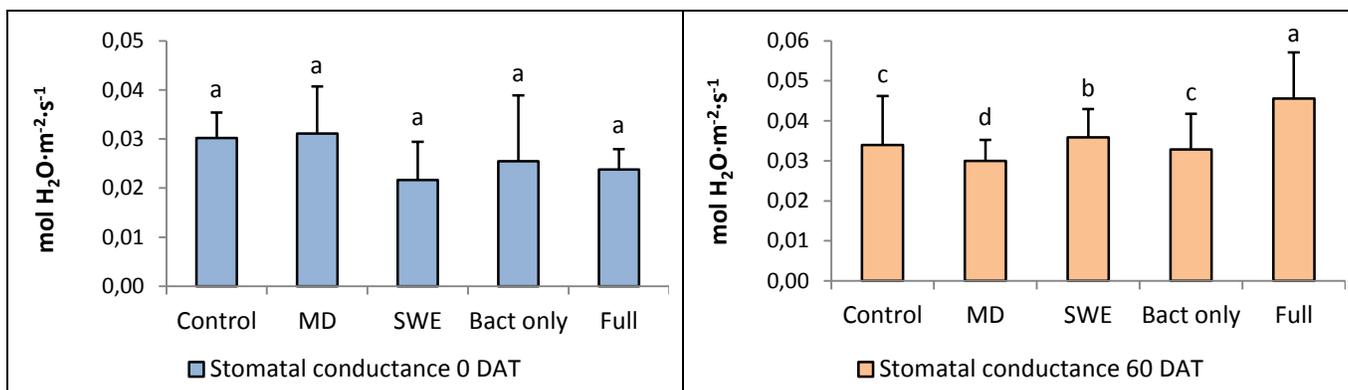
**Graph 30:** Photosynthetic carbon assimilation (A) results at 0 DAT and 60 DAT for applied treatments.

The transpiration of the leaves tissues (Graph 31) shown similar and not statistically different values at 0 DAT. After the two month experimental applications the values of leaf transpiration appeared intensified in Control, bacterial only and Full treatments. Full treatment showed the highest and significant value of leaf transpiration with a difference of 34% if compared to the control. At 60 DAT maltodextrin based treatment shown a decreasing by 12% of the foliar transpiration if compared at the corresponding 0 DAT value.



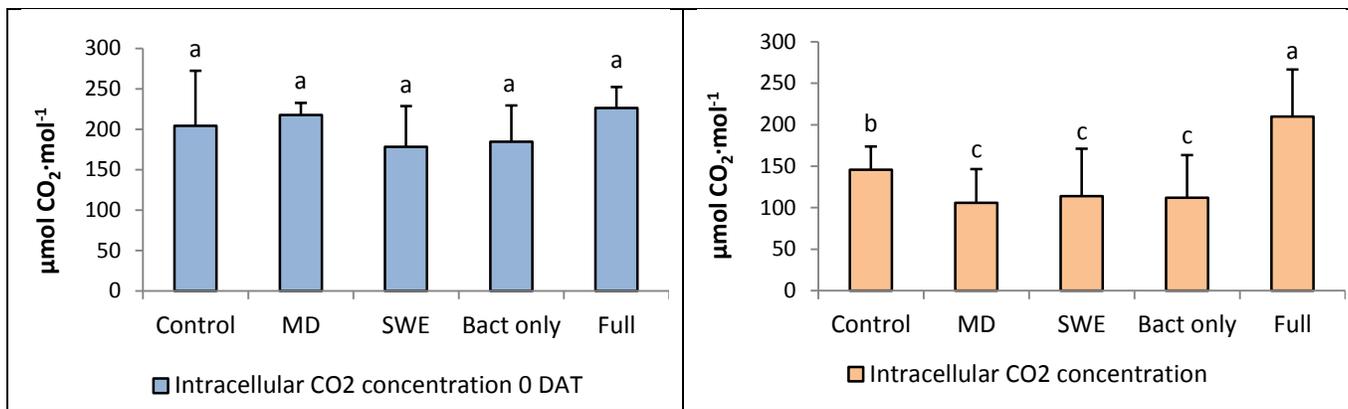
**Graph 31:** Leaf transpiration rate (E) results at 0 DAT and 60 DAT for applied treatments.

The stomatal conductance of the *Agrostis stolonifera* leaves shown similar values at 60 DAT, and no significant differences were found between the different plots before to start the applications. After the two months applications period the Full treatment determined the highest value for the stomatal conductance, with an increasing by 44% respect to the control and by 92% comparing at the relative 0 DAT value. SWE showed an increasing by 66% if compared at the corresponding 0 DAT value a significant difference respect to the control at 60 DAT. Bacterial only based solution showed no significant difference comparing to the control. Maltodextrin determined a decrease of the stomatal conductance by 12% comparing to the control.



**Graph 32:** stomatal conductance (g<sub>s</sub>) results at 0 DAT and 60 DAT for applied treatments.

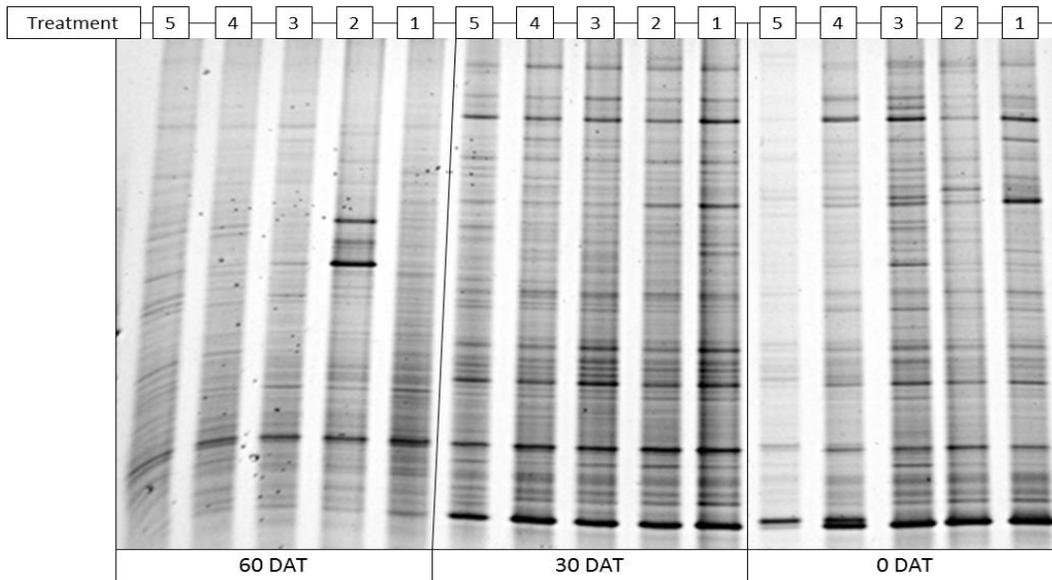
The intracellular CO<sub>2</sub> concentration (C<sub>i</sub>) content in the leaves tissues measured before the first treatments resulted not statistically different. At the end of the experimental trial the mean values of CO<sub>2</sub> concentration within leaf tissues decreased for all these except for the Full treatment. The full treatment maintained the CO<sub>2</sub> content around 200 μmol CO<sub>2</sub>/mol showing a significant difference by 44% comparing to the control. The other treatments brought the CO<sub>2</sub> concentration under the control values showing all a significant difference if compared with the control thesis.



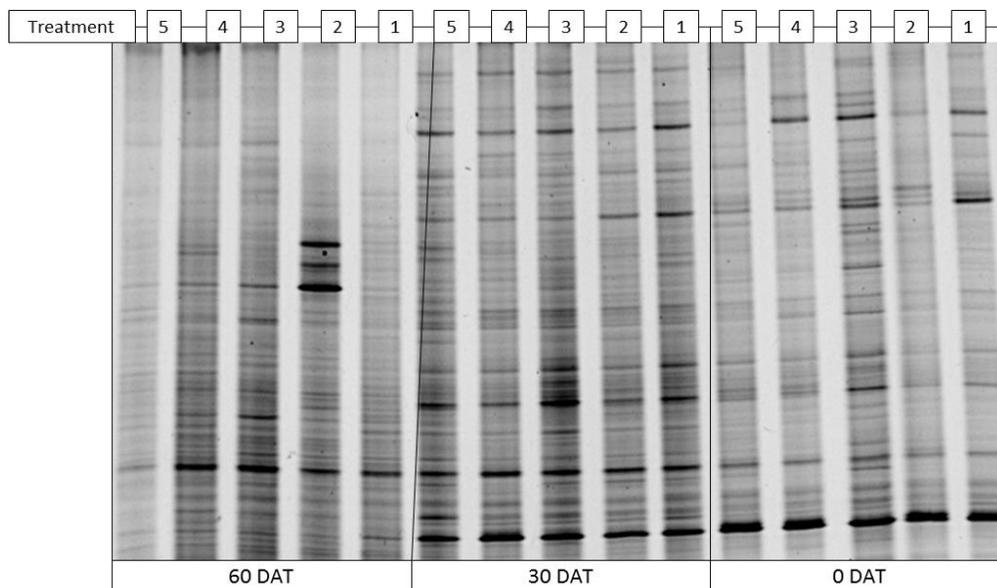
**Graph 33:** intracellular CO<sub>2</sub> concentration (C<sub>i</sub>) at 0 DAT and 60 DAT for applied treatments.

### 5.3.3 SOIL MICROBIAL ANALYSIS

PCR-DGGE analysis was used to examine the effect of different amendments on the soil microbial communities, taking into account that the detection limit of the technique is 1% of the total DNA (Marzorati et al., 2008). Profiles of the bacterial communities are shown in Figure 64 and Figure 65.

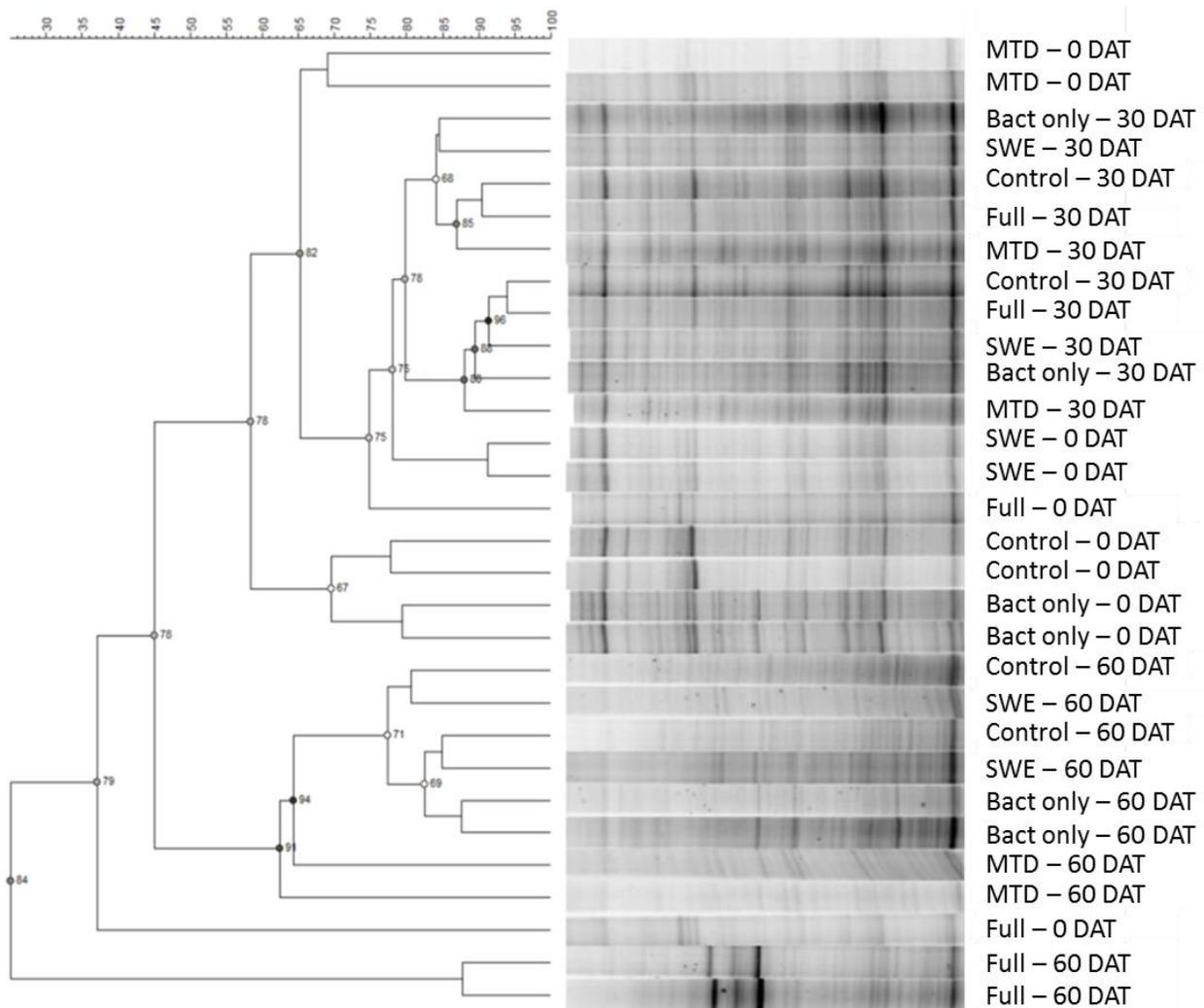


**Figure 64:** PCR-DGGE analysis. Comparison of DGGE patterns of 16S rDNA fragments amplified from BULK SOIL DNA 1 at three different sampling time (0 DAT, 30 DAT and 60 DAT). Treatment: 1 (Control), 2 (Full), 3 (Bact only), 4 (SWE), 5 (MTD).



**Figure 65:** PCR-DGGE analysis. Comparison of DGGE patterns of 16S rDNA fragments amplified from BULK SOIL DNA 2 at three different sampling time. Lanes 1 and 17: ladder; lanes 2 to 6: TRT 1, 2, 3, 4, 5 -05; lanes 7 to 11: TRT 1, 2, 3, 4, 5 -06; lanes 12 to 16: TRT 1, 2, 3, 4, 5 -07.

Overall, DGGE of 16S rDNA fragments from soil DNA revealed high similarity of the DGGE patterns obtained from each of the two replicates per treatment and time sampling (similarity value more than 80%), suggesting a low degree of variability caused by the sampling, DNA extraction, PCR amplification, and DGGE analysis. At all sampling times, the bulk soil patterns consisted of some stronger bands detectable, particularly, in 0 DAT and 30 DAT samples and a large number of less intense bands, indicating that in bulk soil samples the 16S rDNA fragments of only one or two populations dominated, while many populations which were less prevalent seemed to be equally abundant. At 60 DAT, in all treatments most of the abundant bands detected at 0 DAT and 30 DAT lose their intensity. In the dendrogram, two main groups could be observed related to June and July samples with a similarity value around 79.8% for soil sampled in June and over 90% in July. Full treatment at 60 DAT (both replicates) has a unique behavior and is separated in the dendrogram (Graph 34) with a similarity of 87.8%.

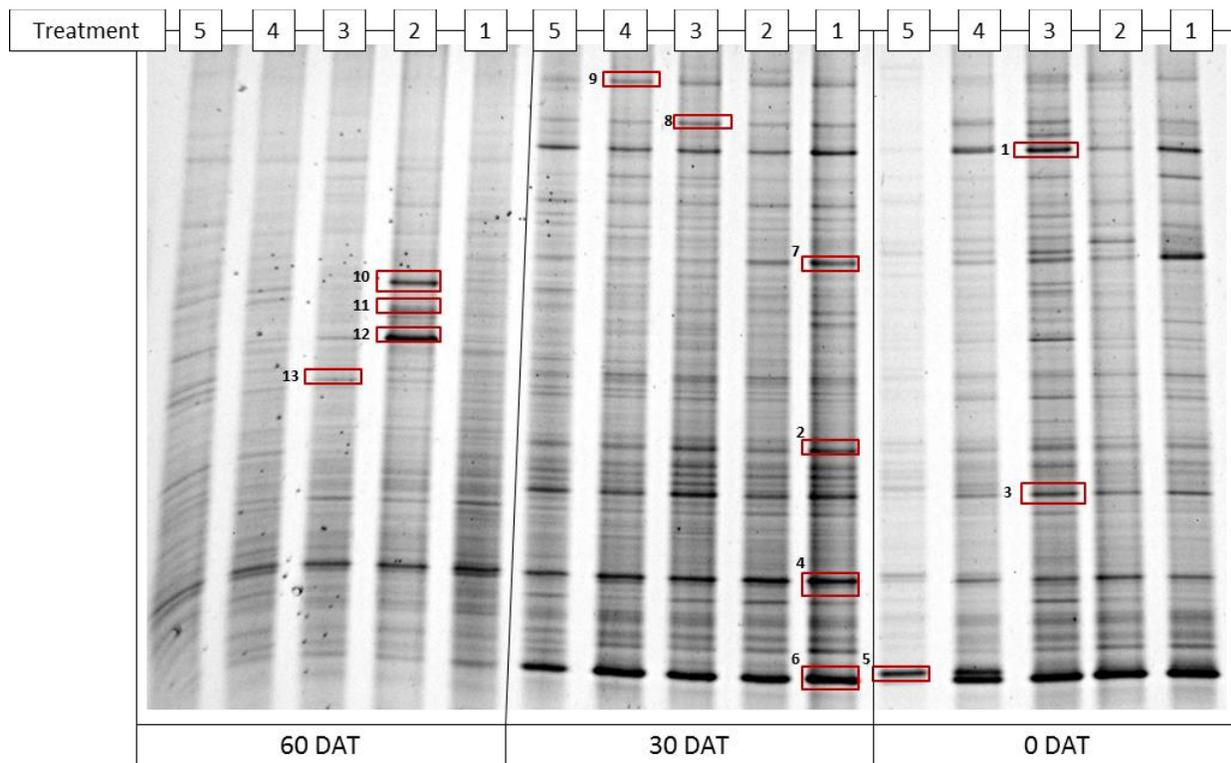


**Graph 34:** Dendrogram of similarity between bands obtained from the different treatments.

On the other hand, different these collected before the beginning of the experiments (0 DAT) don't cluster always together, although each duplicate, except for MTD (Maltodextrine) that has a high similarity value. This probably indicates a more heterogeneity of the samples considering that they all refer to virgin soil which has still not received any treatment. Interestingly, in Full thesis the presence of three strong bands, two of them peculiar to this treatment.

### Sequence analysis

Prominent bands were excised from both gels and sequenced to get further information about the dominating bacterial populations in the different treatments from the DGGE gel of the three sampling time (Figure 66).



**Figure 66:** PCR-DGGE analysis from BULK 1. Arrows indicated the most relevant bands excised (Band number in Table X). Treatment: 1 (Control), 2 (Full), 3 (Bact only), 4 (SWE), 5 (MTD).

The results of the partial sequence analysis of these bands and their tentative phylogenetic affiliation are shown in Graph 34. Sequencing results obtained from bands excised in Bulk 1 were similar to those obtained from Bulk 2. The sequence of the majority of bands (band 2,3,5,6) could be assigned to *Arthrobacter* spp. with a similarity score from 1.00 to 0.828. These bands are very strong in all treatments referred to 0 DAT and 30 DAT, whereas

in 60 DAT they substantially disappeared. The sequence of the strong band 1, clearly detectable in 0 DAT and 30 DAT, showed 0.979 similarity score to *Pseudomonas* spp., while in 60 DAT any detection is possible. *Arthrobacter* and *Pseudomonas* are common bacterial genera frequently associated with soil and their absence in 60 DAT samples could be due to seasonal changing.

Band 4, which is present in all samples and in all sampling time showed an high similarity score (0.989) with *Bradyrhizobium* spp. which are slow-growing bacteria capable of nitrogen fixation and nodule formation on leguminous plants (Jordan, 1982).

Bands 10, 11 and 12 are all assigned to *Bacillus* spp. (high similarity score with *B. thuringiensis* and *B. cereus*) (Table 31) and are visible only in Full thesis which correspond to the parcel treated with bacteria and other biostimulants. As already mentioned, these bands appear after two months inoculation of the related soil parcels with a microorganisms-based product where presence of species of *Bacillus* spp. is certified. These bacteria remain probably into the soil producing the desired positive effects.

**Table 11:** Assignment of band sequences from PCR-DGGE profiles. ND: non determined.

Band number	Closest Identity	Similarity Score
1	<i>Pseudomonas</i> spp.	0.979
2	<i>Arthrobacter</i> spp.	0.828
3	<i>Arthrobacter</i> spp.	1.00
4	<i>Bradyrhizobium</i> spp.	0.989
5	Uncultured soil bacterium <i>Arthrobacter</i> sp.	1.00
6	Uncultured soil bacterium <i>Arthrobacter</i> spp.	1.00
7	ND	
8	ND	
9	ND	
10	<i>Bacillus</i> spp.	0.992
11	<i>Bacillus</i> spp.	0.993
12	<i>Bacillus</i> spp.	0.993
13	ND	

## 5.4 DISCUSSION

In the third and final step of this experimental thesis the microbial biostimulants were once again applied on an *Agrostis stolonifera* putting green. Differently at the experimental trial conducted in the Modena Golf, the scheduled managing (chemical applications and agronomical maintenances) were maintained for the entire duration of the trial. Therefore this conclusive trial aimed to test the effectiveness of the treatments based on microbial mixes in a real managing context during the summer period. Unfortunately during this trial was not possible to repeat the test with DiSTA and D-PLUS microbial solutions because a patent procedure was initiated in order to protect the intellectual property rights. For this reason similar products commercialized on the U.S. market were utilized.

Concerning the morphological parameters, leaves, thatch and roots growth were analyzed. The production of leaves did not show significant differences for all the treatments applied. This data was in accordance with the balanced mineral input across the different thesis. Therefore biostimulants and PGPR (principally *Bacillus* spp.) did not determine a further stimulus to the foliar growth in accordance with a preponderant incentive to rooting. This not significant effect on the biomass production was a positive data because an eventual increment of the leaf growth determines higher costs of mowing (Johnson, 1994) and management operations due to a higher thatch accumulation. The dry/fresh ratio of leaves biomass did not show significant effects in plots treated with microorganisms, differently by the Modena's trial where this kind of treatments resulted effective.

The thatch biomass did not show a reduction for all the treatments applied, including the bacterial only and Full theses. This result was attributable to the microbial mixes utilized in this last experiment, that were principally composed by *Bacillus* spp., which had PGPR properties largely described by Podile and Kishore (2007) and Singh et al. (2011), unlike the DiSTA and D-PLUS microbial solution that were composed by bacteria able to metabolize lignin and cellulose of thatch, as previously described.

The growth of roots showed the highest values (comparing to the control) for Full and SWE treatments, which both contain the *Ascophyllum nodosum* extract, which cytokines-like activity has been proved on creeping bentgrass in several works (Zhang et al., 2003; Crouch and Van Staden, 1993; Senn, 1987). Even though the Full treatment included different *Bacillus* spp. and leonardite (HA) these did not determine a further deepening of the root apparatus in comparison to the other treatments.

The visual assessment of the green surface was based on the 1-9 ratings scale considering the color and quality parameters. The analysis of the surface showed a better color rating for the Full treatment while SWE and Bact only have led to lower values but still significantly higher than the control. These data confirmed the positive effects obtained with bio-stimulants on the color quality of the leaves as previously demonstrated in the Modena's trial. In the other hand the quality of the turf surface (a combination of color, density, uniformity,

texture and diseases) was not significantly affected by the different applications probably due to the high standard quality of the turf surface of the experimental plots.

The 1-9 visual color rating was confirmed by the measure of the normalized difference vegetative indices (NDVI) obtained with a vehicle-mounted optical sensor. The subjective 1-9 rating and the instrumental NDVI evaluations were highly correlated as reported by Bell (2002) and both confirmed a positive effect given by Full and Bact only treatments. SWE and MD were not significant different by the control plots.

In contrast with the Modena's trial an improved color quality was not correlated with an increasing of total chlorophyll. Indeed the highest color rating obtained with Full treatment, was not followed by an higher Chl $a$ +Chl $b$  content if compared with the other treatments. The content of the photosynthetic pigments seemed to be not influenced by different theses, but given the homogeneity of the response the environmental effects were predominant. The total chlorophyll ( $a+b$ )/carotenoids ( $x+c$ ) ratio is an indicator of the greenness of plants. The ratio  $(a+b)/(x+c)$  showed a diminution from an average value of 4,2 at 0 DAT (common value for sun exposed leaves) to 3,3 at 60 DAT indicating a principle of yellowing/senescence and status of stress due to the environmental condition that normally stress the putting greens during the summer seasons (Lichtenthaler, 2001; Carrow, 1996).

Superoxide dismutase (SOD) activity did not shown significant effect due to different thesis and also the content of two phytohormons abscisic acid (ABA) and indole-3-acetic acid (IAA) was not affected despite the PGPB and bio-stimulants (HA) possess phytohormone's stimulation and precursor characteristics(Arshad and Frankenberger, 1998; Calapp et al., 1998).

The analysis of the proline revealed a significant increased content of this metabolite in plots treated with microbial treatments (Full and Bact only). The higher concentration of proline as result of the inoculums showed positive effects of bacteria to enhance the production of this osmoregulator commonly necessary in stress condition. This result may be associated to an improved turfgrass quality and resistance to environmental stresses such as drought (Zhang, 1997) and salinity (Nabati et al., 1994).

Photosynthetic carbon assimilation was positively influenced from all bio-stimulant treatments, in particular by those containing PGPB. No significant differences in photosynthetic rate ( $A$ ), stomatal conductance ( $g_s$ ), intracellular  $CO_2$  ( $C_i$ ) and leaf transpiration ( $E$ ) were found at anthesis stage. After 60 days of treatments the mean values of the photosynthetic rate were much higher, in contrast with expected values. Generally water deficit and high temperature may determine stress condition through a combination of stomatal and metabolic limitations (Lawlor, 2002; Flexas, 2006). However, it should be considered that creeping bentgrass' plants were grown for two weeks within a growth chamber before to proceed with the gas exchange detection, so the data would be considered in relation to the control because the environmental parameters can be misleading. Indeed, treatments based on microorganisms and biostimulants have led to a further increasing of the photosynthetic rate respect to the control.

In accord with the Modena's trial the combinations of microorganisms and biostimulants determined the higher and significant increasing of the transpiration from the leaves tissues, while bacteria and biostimulants applied separately determined a lower increment but still significantly above to the control.

Concerning the microbial analysis, the first half of the experiment showed a substantial heterogeneity of the results between 0 and 30 DAT and within the different theses. On the contrary all the plots at 60 DAT revealed a similar DGGE profile which differentiates them from the samples of 0 and 30 DAT. In particular the Full thesis at 60 DAT showed a unique behavior and is separated in the dendrogram with the stronger similarity.

The most interesting result obtained from the microbial analysis was obtained for the Full thesis (bacteria and other biostimulants) at 60 DAT, which has evidenced the presence of three strong bands. The subsequent sequence analysis revealed the belonging to *Bacillus* spp. with a high similarity score to *B. thuringiensis* and *B. cereus*. In the Full treatment could be hypothesized that the bi-weekly inoculum would lead to an increase of such microorganisms in the soil bacteria consortium detectable by PCR-DGGE.

The second interesting result was the strong diminution of the intensity and some case the loss of DNA bands from 30 DAT and 60 DAT. The significant diminution of the microbial component in the putting green top-soil may have been determined by the application of some chemicals utilized to control the turf diseases during the summer period. Indeed, in previously studies some chemicals such as chlorothalonil, a broad spectrum non-system fungicide commonly utilized to suppress the dollar spot on putting green, have shown a significantly reducing effect on the population of soil microorganisms (Yun long Yu et al., 2006). Chlorothalonil, has been applied in three different dates (20, 30, 40 DAT) during the term of the field trial in order to suppress the propagation of dollar spot disease. The diminution of a large portion of the microorganism detected with the DGGE analysis then could be a direct consequence of the chemicals applications.

## 6. CONCLUSIONS

During this experimental thesis were previously studied the principal morpho-physiological effects that different microbial solutions determine on *L. perenne* L. in a controlled environment condition. The results showed that the inoculation of commercial microorganisms, mainly containing PGPR, promoted the growth of shoots and roots, while effective microorganisms (EM-1) determined principally an increasing of the dry/fresh biomass ratio and a higher resistance to tearing out that is a particularly favorable feature for any essence of sport turfgrass. The PCR-DGGE analysis showed the colonization ability of four species belong to the effective microorganisms that, as evidenced in the literature, have a great potential for the maintenance and recovering of a sport turf for some of their properties such as bio-control against phytopathogens, bio-degraders of organic substances and for the recirculation of nutrients. From the growth chamber analysis was possible to conclude that both effective and commercial microorganisms have a general effectiveness to improving the habitus of the plant in particular the coloration of leaves that were significantly enhance respect to the plants treated with the fertilizer only control.

The tear resistance improved by microbial treatments was confirmed in the test on the golf putting green. This result could be of marginal interest in the golf sector where the damage of the turfgrass is principally determined by the compression impact resulting from the fall of the ball and by the removal of clod portions by strokes. Instead this characteristic may be interesting for future experiments on soccer and football fields where resistance to tearing is a fundamental criterion of judgment.

The two original microbial mixes, DiSTA and D-PLUS were ideated and formulated on the base of the experience gained during the test on *L. perenne* L. in order to meet the needs of an *Agrostis stolonifera* putting green, that is the highest engineered turfgrass system in the sport turf industry. D-PLUS mix, composed by different groups of effective microorganisms, humic acid and arbuscule mycorrhizae determined the highest visual evaluation of the grass surface and a significant reduction of the thatch. The microbial degradation of thatch leads to a parallel diminution of the moisture content and an increased evapotranspiration from the canopy. The decreasing of moisture content and the thinning of the thatch determined an increased oxygenation of the rhizosphere that is a key factor to reduce the insurgence of ROS and to ensure a lower susceptibility of the turfgrass to fungal diseases such as Dollar sport, due to the lack of the optimal humid-substrate for the propagation of this genre of pathogens. Creeping bentgrass treated with D-PLUS confirmed the capacity of VAM to establish endophytic symbiosis by *glomus intraradices*, with the possibility to exploit the positive interaction properties such as the increased resistance to abiotic stresses and wide range of assimilable nutrients.

These interesting results, obtained in a semi-field condition where chemical and mechanical treatments were suspended, could bring to reschedule the agronomic management of the sport turfgrass by the addition of D-PLUS or similar solutions in order to reduce the chemical treatments and the applications of fertilizers because

as demonstrate in this thesis these formulates allow the degradation and recirculation of nutrients and therefore reduce the management costs of the whole golf club.

In the other hand, in a real field condition where chemical and mechanical treatment were applied as scheduled for a sport turf, the treatments based on bacteria and biostimulants showed reduced positive effects.

Anyway, *Bacillus* genre belonging to the plant growth-promoting rhyzobacteria tested in association with humic acid and sea weed extracts confirmed the positive effect on quality and color of the turfgrass and a significant increasing on root's elongation and proline content as general response of an improved physiologic condition if compared to the fertilizer only application.

In conclusion is possible to confirm that the addition of microorganisms and organic biostimulants on a sport turf grass system, especially on a golf putting green, can improve the physiological conditions of the grass essences and the agronomical aspects of the whole area of playing.

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