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Improvement of seed quality and control of seed transmitted fungi by beneficial microorganisms

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Abstract

The primary component of the agricultural supply chain is the seed, seen not only as a source for perpetrating the genotype but also as an integral component necessary for food production. For this reason, the quality of the seed plays an essential role in agriculture: characteristics such as germinability, vigour and purity must always be guaranteed. In the past, agrochemical products have been used extensively always to ensure high-quality phytosanitary standards. Nevertheless, the need for sustainable and environmentally friendly agriculture is paving the way for using products based on microorganisms, limiting synthetic pesticides that are undoubtedly harmful to humans and the environment.

The thesis work was based on improving the quality of the seed through the use of beneficial microorganisms that increase its vigour and control any pathogens present and the application of new technologies for the disinfection of the seed, such as ozone.

The first phase of the PhD project was dedicated, to the execution of *in vitro* tests, for the selection of microorganisms that could exert an antagonistic action against some pathogenic fungi such as *Fusarium oxysporum* f. sp. *cepae, F. oxysporum* f. sp. *raphani, Fusarium oxysporum* f. sp. *lactucae, Sclerotinia* sp., *Pythium* sp., *Stemphylium botryosum, Colletotrichum dematium.* Various microorganisms were tested, which showed a more or less pronounced antifungal activity, among these *Streptomyces* sp. *Pseudomonas* sp. *Agrobacterium* sp. The tests allowed to identify a microorganism with a powerful antifungal action belonging to the genus *Streptomyces* sp., the code assigned was DLS1568. Such streptomycete was isolated from rice.

The second phase of the PhD project was dedicated to the evaluation of the effects of *Streptomyces sp*. DLS1568 on germination of the seed of some horticultural crops, onion, rocket, fennel and lettuce, to the method of application of microorganism to the seed and the seed treatment with ozone for its disinfection. From the results obtained, the beneficial streptomycete favoured the germination of the seed and the development of the seedling, in particular in onion and rocket. Various products were tested to develop a helpful formulation for applying *Streptomyces* on the seed, particularly the following products: Bio-friendly-1, xanthan gum, and teal green. Bio-friendly-1 was selected from these tanning products, as it did not compromise the vitality of the microorganism, the seed germination, and the seedling development. For the treatment of the seed with ozone, different treatment times were tested; from the germination tests carried out after the ozonation of the seed, it was found

that treatment of 45 minutes at 20 ppm almost eliminates the microbial load present on the seed and has a stimulating effect on the germination and development of the seedlings.

To confirm the possible internalization of the streptomycete and the consequent colonization of the plant by the microorganism, *Streptomyces sp.* DLS1568 was transformed using the puf275 plasmid, creating mutants *Streptomyces sp.* DLS1568, which produced the fluorescent protein. Therefore, the endophytic colonization of the beneficial microorganism in onion, fennel, rocket, and lettuce was confirmed by observation under a confocal microscope.

Field tests were carried out by direct sowing to evaluate the effectiveness of the seed tanning of onion, fennel and rocket with *Streptomyces* sp. DLS1568. Further field tests were performed with seeds treated with ozone. The results obtained from field tests confirmed the data obtained in tests carried out in a controlled environment: in particular, these treatments positively influenced the germination and development of the treated seed. Furthermore, in the field trials which involved the control of fungal pathogens, it was shown that the streptomycete applied to the onion seed showed a similar effect, sometimes superior, to the synthetic fungicide (Fludioxonil).

Finally, the genome of the DLS1568 strain of *Streptomyces* sp. was entirely sequenced to identify the possible biosynthetic pathways involved both in the antifungal activity and in the stimulation of plant growth.

Sinossi

La componente primaria della filiera agricola è il seme, visto non solo come una fonte per perpetrare il genotipo, ma anche come una componente integrale necessaria per la produzione di cibo. Per questo la qualità del seme gioca un ruolo essenziale in agricoltura: infatti, caratteristiche quali germinabilità, vigore e purezza devono essere sempre garantiti. In passato si è fatto uso massiccio di fitofarmaci per poter garantire sempre standard elevati di qualità fitosanitaria, tuttavia, la necessità di garantire maggiore sostenibilità ai sistemi agricoli e rispetto dell'ambiente sta aprendo la strada all'uso di prodotti a base di microorganismi limitando l'uso di fitofarmaci di sintesi certamente dannosi per l'uomo e l'ambiente.

Il lavoro di tesi si è stato impostato sul miglioramento della qualità del seme attraverso l'utilizzo di microorganismi benefici che ne aumentassero il vigore e controllassero eventuali patogeni presenti e l'applicazione di nuove tecnologie per la disinfezione del seme come l'ozono.

La prima fase del progetto di dottorato è stata dedicata, all'esecuzione di prove *in vitro*, per la selezione di microorganismi che potevano esplicare un'azione antagonista nei confronti di alcuni funghi patogeni quali: *Fusarium oxysporum* f. sp. *cepae, F. oxysporum* f. sp. *raphani, F. oxysporum* f. sp. *lactucae, Sclerotinia* sp*., Pythium* sp*., Stemphylium botryosum, Colletotrichum dematium.* Sono stati saggiati numerosi microorganismi che hanno mostrato una più o meno pronunciata attività antifungina, tra questi *Streptomyces* sp. *Pseudomonas* sp. *Agrobacterium* sp. Le prove eseguite hanno permesso di individuare un microorganismo con una potente azione antifungina appartenente al genere *Streptomyces* sp. al quale è stato assegnato il codice DLS1568 Tale streptomicete è stato isolato da riso.

La seconda fase del progetto di dottorato è stata dedicata alla valutazione degli effetti dello *Streptomyces* sp. DLS1568 sulla germinazione del seme di alcune specie orticole quali cipolla, rucola, finocchio, al metodo di applicazione del microrganismo sul seme e al trattamento del seme con ozono per la sua disinfezione. Dai risultati ottenuti lo streptomicete benefico ha favorito la germinazione del seme e lo sviluppo del semenzale, in particolare in cipolla e rucola. Sono stati saggiati diversi prodotti per sviluppare un formulato utile all'applicazione di *Streptomyces* sul seme; in particolare i seguenti prodotti: gomma di xantano, teal green e Bio-friendly-1. Di tali prodotti concianti è stato selezionato Bio-friendly-1, in quanto non comprometteva la vitalità del microorganismo, la germinazione del seme e lo sviluppo del semenzale. Per il trattamento del seme con ozono, sono state saggiate diverse tempistiche di trattamento; dai test di germinazione effettuati dopo la ozonizzazione del seme è

risultato che un trattamento di 45 minuti a 20ppm abbatte quasi totalmente la carica microbica presente sul seme ed ha un effetto stimolante sulla germinazione e sviluppo dei semenzali.

Per confermare la possibile internalizzazione dello streptomicete e la conseguente colonizzazione della pianta da parte del microorganismo, *Streptomyces sp.* DLS1568 è stata trasformato usando il plasmide puf275, pertanto creando mutanti di *Streptomyces sp.*DLS1568 che producevano la proteina fluorescente. Mediante l'osservazione al microscopio confocale è stata pertanto confermata la colonizzazione endofita del microrganismo benefico in cipolla, finocchio, rucola e lattuga.

Sono state svolte prove in campo, per semina diretta, per valutare l'efficacia della concia con *Streptomyces* sp.DLS1568 del seme di cipolla, finocchio e rucola. Ulteriori prove di campo sono state eseguite con, semi trattati con ozono. I risultati ottenuti dalle prove in campo hanno confermato i dati ottenuti in prove eseguite in ambiente controllato: in particolare, tali trattamenti hanno influito positivamente sulla germinazione e sviluppo del seme trattato. Nelle prove di campo che hanno avuto oggetto il controllo di patogeni fungini, si è evidenziato che lo streptomicete applicato al seme di cipolla ha mostrato un effetto simile, a volte superiore, al fungicida di sintesi (Fludioxonil).

Infine, il genoma del ceppo DLS1568 di *Streptomyces* sp. è stato interamente sequenziato, al fine di individuare le possibili vie biosintetiche coinvolte sia nell'attività antifungina, sia nello stimolo alla crescita delle piante.

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1. Introduction

Agriculture currently occupies more than 1/3 of Earth's surface land (FAOSTAT, 2016). The primary component of a productive system in agriculture is the seed, not only as a source for the perpetuation of a genotype, but also as an integral component of food. In the past, the production and use of seeds was almost exclusively for domestic needs: the farmer selected the seeds to be sown the following year and to exchange them with other farmers. Over the years, due to the introduction of new technologies, the intensive cultivation of crops has been made possible, but without a steady supply of high-quality seeds, yields and crop quality would be significantly lower. Therefore, seed quality plays an essential role in the production of horticultural crops. The seed characteristics, such as trueness to type, germination percentage, purity, vigour, and appearance are essential to farmers. Achieving and maintaining high seed quality is the goal of every professional seed producer. Therefore, seed quality and performance must be guaranteed over time; for this reason, chemicals and other seed treatments are applied to allow production and to minimize losses in any cropping system.

High seed quality is always demanded by farmers and may result in up to a 30% increase in crop yields (Ellis *et al.,* 2004). Sowing high-quality seeds is essential, but their use does not guarantee successful stand establishment. The difference in time between sowing and stand establishment is a crucial period. Seeds and seedlings may be exposed to a wide range of biotic and abiotic stresses resulting in decreased stand performance (Zinsmeister *et al.,* 2020*)* Therefore, judicious use of chemical, biochemical, and biological seed treatments can protect and enhance establishment, growth and potential productivity (Sharma *et al.,* 2015).

In 1866, a technique was developed to improve sowing of cotton seed using a paste of wheat flour to form a kind of pellet (Porter *et al.,* 1979). During the mid-20th century, many coating technologies for an improved agricultural productivity were developed and reviewed by Jeffs (1986). Seed coating technology continued to advance through the 1970s to 1990s, as reviewed by Scott (1989), Taylor and Harman (1990) and Hill (1999).

A commonly used strategy in seed technology is seed treating and coating, which helps to ensure yield and reduce losses due to the impact of seed-associated pathogens (Porter *et al.,* 1979). Thanks to seed coating it is possible to control many plant diseases caused by microorganisms, such as fungi (Afzal *et al.,* 2020). This has made seed coating one of the most used techniques in the treatment of seeds and in the prevention of plant diseases. The success of this technique lies in the fact that it is possible to intervene directly on the pathogens that may be present on the seed surface or inside it; moreover, the protection offered by the treatment carried out on the seed is also effective against soilborne pathogens. The seed coating can perform both a protective function (contact with the surface of the seed) and a systemic function (from the seed into the plant) (Ehsanfar and Modarres-Sanavy, 2005). In particular, the protective treatment of the seed helps to control the pathogens present on its surface, while the systemic nature of other treatments can control the pathogens transmitted by the seeds and residing within the seed itself (Martha *et al.*, 2003).

Chemical pesticides are substances or mixtures of substances that are mainly used in agriculture or in public health protection programs in order to protect plants from pests, weeds or diseases, and humans from vector-borne diseases, such as malaria, or dengue fever. Insecticides, fungicides, herbicides, rodenticides, and plant growth regulators are typical examples (World Health Organization, 1990).

However, intensive use of chemicals has caused environmental problems, such as soil depletion and groundwater pollution: therefore, it is necessary to re-examine many existing seeds treatments.

Nonetheless, the unsustainability of the current conventional agricultural practices and future climate scenario urges for alternatives that can increase agricultural production and bring environmental and economic sustainability, thus ultimately improving human well-being (Reganold and Watcher, 2016).

For this reason, the use of sustainable approaches, such as the use beneficial plant microorganisms and innovative technologies, such as physical treatment (*e.g.*, ozone) for seed sanitation and priming, is crucial (Ivanovich, 2011). Nowadays, various commercial formulations are available that are based on microorganisms (*e.g.*, antagonistic fungi and bacteria) or secondary metabolites used as bio-pesticides (Glare *et al.*, 2012). The use of antagonistic microorganisms for seed coating in agriculture, in general, has the following advantages: reduce chemical pollution, support the microbial communities in soil, and guarantee the safety of operators (Rocha *et al.*, 2019). An alternative for seed sanitation and priming is the use of ozone as an antimicrobial agent (Kang *et al.*, 2015).

Ozone is an inorganic molecule, allotropic form of oxygen (trivalent oxygen), with the chemical formula O_3 it is characterized by a pungent odour. Ozone has a high oxidizing power; thanks to this characteristic it is used in various sectors of industry and in research. The ozone molecule is highly unstable and decomposes into oxygen and its reactive species. In fact, ozone at room temperature and pressure has a short half-life. In nature it is found in the stratosphere and in the troposphere, thanks to the photo dissociation operated by the highly energetic rays of the sun, the molecular oxygen (O_2) splits into active oxygen atoms which, recombining with the other active oxygen molecules, form trivalent oxygen. (O3). The natural formation of ozone on the earth's surface is operated by lightning which with their energy is able to split the oxygen molecules which for a short period of time recombine in groups of three thus forming ozone (Oyama, 2000). Therefore, an ozone generator consists in passing oxygen between two electrodes that emit high-energy discharges such as to break the bonds between the oxygen molecules so that the latter can recombine into trivalent oxygen (Fig. 1).

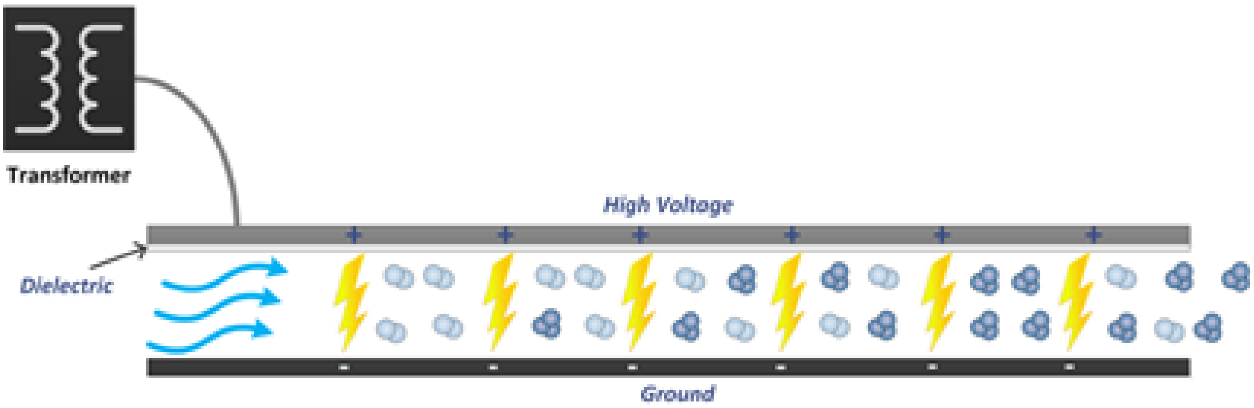

Figure 1. *Representation of an ozone generator: ozone is formed via an electrical discharge that is diffused over an area using a dielectric to create a corona discharge. Oxygen passed through this corona discharged is converted to ozone (www.oxidationtech.com)*

In general, thanks to its highly oxidizing power, ozone is used as a sanitizing agent. In fact, the ozone molecules interact with cellular components such as phenolic rings, sulphide groups, membrane phospholipids and various cellular enzymes causing an oxidation reaction with consequent cell damage, growth inhibition and death of microorganisms (Pandiselvam *et al.*, 2020).

One of the first applications of ozone occurred in Europe in the first decade of the 1900s for water treatment (Wojtowicz, 2000).

Over the years, the use of ozone has also extended to the agri-food chain, it is used as a sanitizing agent during the transformation of food both as water treatment and in the form of gas, to obtain a safer product, with an extended shelf-life and healthier, as it has the potential to reduce pesticide residues on food. (O'Donnell *et al.*, 2012).

The use of ozone on the seed is being developed both as a sanitizing agent (given the action it has on the microbes) and as a stimulating agent for germination: in fact, thanks to the oxidation of O3, it induces in seed the process of cell signalling that initiates germination, at the same time the dioxides and peroxides that are formed cause an increase in ethylene synthesis and a decrease in abscisic acid (ABA) in seedlings (Pandiselvam *et al.,* 2020).

1.1 Biological control

The term biological control is used in several areas of life sciences. For instance, in entomology it has been used to describe predatory insects (and parasitoids) able to reduce or suppress populations of insect pests. In plant pathology, particularly, this term applies to the use of microbial antagonists against plant pathogens (*e.g.,* fungi and bacteria). In both cases, the organism or the microorganisms able to suppress the pest or pathogen is the biological control agent (BCA) (Pal and McSpadden Gardener, 2006).

So-called biocontrol organisms are widely found in nature and include bacteria, fungi, viruses, yeasts, and protozoans and can control plant diseases either directly or indirectly (Köhl *et al.,* 2019). The former implies a direct antagonistic effect of the biocontrol organism on the pathogen, which can be achieved by parasitism, antibiosis, or competition for nutrients or infection sites. In the indirect way of disease control, the biocontrol organism induces plant-mediated responses allowing the plant to react faster and more efficiently upon subsequent pathogen attack (Vos *et al.,* 2015). In a broader definition, biocontrol has been suggested to also include the use of non-living agents of biological origin (Timmusk *et al.,* 2017; Vinale *et al.,* 2008), a definition which is also supported by the International Biocontrol Manufacturers Association (IBMA, 2019). In this introduction, an even more extended definition of biocontrol is applied, thereby covering the use of biological agents (*e.g.,* organisms or their secreted compounds) and compounds that trigger the plant's own defence, and term them all as biocontrol agents (BCAs). The high potential of BCAs as plant protection tools in IPM is currently limited by different factors. Research on BCAs, including both their identification and further unravelling of their mode of action, has mainly focused on a single crop - disease/pest system in a bottom-up approach, rather than using a more holistic top-down one (Barratt *et al.,* 2018; Mathys *et al.,* 2012). Another hurdle is the stringent registration procedure (including environmental risk assessments), characterized by high costs (reported over $250 million per case) and long application procedures (often more than ten years), restricting the commercial implementation of newly identified BCAs (Holtappels *et al.,* 2019). Therefore, improvement of the entire process of identification, development and, implementation of new BCA-based products is necessary (Barratt *et al.,* 2018; Berg, 2009; Lamichhane *et al.,* 2017; Le Mire *et al.,* 2016; Regnault-Roger, 2012; Robin and Marchand, 2019).

The action of BCAs can be divided into direct effects, when the pathogen is directly inhibited or killed by the antagonist and indirect effects, when the antagonist induces a physiological response in the plant, making the plant itself able to counteract the pathogen attack **(**Köhl *et al.,* 2019).

The main mechanisms of action of biocontrol agents that underlies a direct antagonistic interaction include antibiosis and competitive exclusion.

- Antibiosis is expressed through the production by the antagonistic microorganisms of secondary metabolites capable of inhibiting the growth and development of pathogens. Their lethal activity is carried out, however, only when they penetrate and accumulate in the tissues (inhibition due to toxicity). Often, these metabolites are characterized by a low molecular weight, and are produced by the antagonistic microflora in conditions of reduced availability of nutrients (Lewis *et al.,* 1991). Among the most studied antibiotic-producing agents there are several species of phenazine-producing pseudomonads, the first described antibiosis clearly involved in biocontrol. Actinomycetes, particularly the abundant *Streptomyces* species as filamentous spore-forming bacteria with superior biocontrol and nutrient-cycling activity, are among the most promising PGPR to increase overall soil health and boost agricultural productivity (Vurukonda *et al.*, 2018). In fact, *Streptomyces* spp. have the capacity to produce cellulolytic enzymes and various secondary metabolites, which directly act on herbivorous insects and show toxic activity on phytopathogens and/or insect pests (Book *et al.,* 2014; Copping *et al.,* 2000). A set of different molecules from *Streptomyces* spp. that act against insect pests have been found and characterized; these are, for instance, flavensomycin (Craveri *et al.*, 1957), antimycin A (Kido *et al.*, 1950), piericidins (Takahaski *et al.,* 1968), macrotetralides (Oishi *et al.*, 1970) and prasinons (Box *et al.,* 1973). Among the antagonistic fungi producing antibiotics, the case of *Trichoderma* spp. should be mentioned, from which many antibiotics have been obtained and characterized, both from a chemical and biological point of view (Zin and Badaluddin, 2020*)*.

- Competitive exclusion (or simply competition) is a biocontrol mechanism that can be described as the struggle for space, nutrients, or other factors indispensable for the activity and development of the pathogen. An important example of competition for nutrients is provided by some bacterial biocontrol agents, able to hinder the pathogens in soils with acidic pH, through sequestering iron and reducing its availability for pathogens (Agrios, 2005); indeed, such a nutrient is normally available to microorganisms only in low concentrations and at acidic pH (Duijiff *et al.*, 1994). For that reason, these bacteria and some fungi have developed iron assimilation systems that are highly effective, based on the use of siderophores (proteins with a selective binding domain for iron) and internal transport of the cell-mediated by a specific protein (carrier). There is no specificity in the use of siderophores, since there are other microorganisms that the plants themselves can use. The strategy developed by the antagonist consists, therefore, in excluding the pathogen from its usual ecological niche, exposing it to environmental stresses and nutritional and / or interrupting the reproductive cycle. However, it does not imply direct contact between the two and it is often used in the antagonisms between different pathogens or even between the same biocontrol agents. Another way, in which we can explain the antagonism against pathogens, is represented by competition for the sites of infection: as an example, we may observe a reduction in the colonization of roots by *F. oxysporum* in the presence of non-pathogenic strains of the same species (Schneider, 1984; Mandeel and Baker, 1991; Eparvier and Alabouvette, 1994).

- Parasitism is a phenomenon that consists of a specific interaction between an antagonistic organism and a pathogen, during which the antagonist establishes an intimate association with the pathogen, from which it takes part and / or all of its nutrients, without offering any advantage. The interaction involves a phase of physical contact with the host and, in many ways, typically resembles the typical plant-pathogen interaction (Parratt *et al.*, 2016*)*. Before the contact phase, there is a phase of recognition of the remote host, a chemotactic growth towards this, the establishment of a compatible physical interaction between the two organisms, the formation of specialized structures (such as appressors), the penetration and then the colonization of the host followed by the colonisation phase. A double level of parasitism can occur and, in this case, we refer to hyperparasitism, while a specific interaction between antagonist and the fungal pathogen is called mycoparasitism. In this process, lytic-type enzymes (chitinase, glucanase, protease) actively participate, capable of degrading cell walls and thus allowing the colonization of host tissues. Mycoparasitism culminates in the lysis of the host mycelium, its conidia, chlamydospores, sclerotia and other storage structures (Köhl *et al.*, 2019).

In other plant-microbe interactions, the antagonistic microorganisms can interact with the plant at a physiological-biochemical level, as they can increase the growth of the plant, improving its general state of health and productivity and activate the production of molecules that are involved in the defence reaction of the plant from diseases: therefore, we speak of indirect control mechanisms. The interaction with the plant is linked to colonization phenomena by the organism employed in biocontrol or to induced resistance phenomena. In the first case, the colonization of the biocontrol agent on the plant prevents pathogens from attacking the plant. The colonization capacity is often not directly correlated with biocontrol. It is influenced by external factors, such as the characteristics of the soil and roots, the accumulation of secondary metabolites, osmotic tolerance, and the ability to use exudates and produce degradative enzymes (Handelsman *et al.,* 1996). In some cases, and here we refer to the phenomenon of induced resistance, the biocontrol agents induce a substantial change in the ability of the plant to defend itself against diseases, often activating the same genes used for protection against pathogenic microorganisms (activation of the hypersensitive response and acquired systemic resistance - SAR).

Many microorganisms can increase plant growth and productivity (Whipps, 2001). Rhizobacteria are known as plant growth promoters and, for this reason, called PGPR "Plant Growth Promoting Rhizobacteria". In addition, to containing the disease, rhizobacteria stimulate plant growth and development, which does not occur if pesticides are used (Beneduzi *et al.,* 2012).

Van Loon *et al*. (1998) defined the induction of resistance as a physiological state of the plant with "increased defensive capacity" elicited by specific environmental stimuli that enhance the plant's innate defences against subsequent biotic attacks. Throughout their coevolution, plants and pathogenic organisms have developed complex relationships resulting from a constant exchange of molecular information.

Pathogenic organisms have developed a whole series of offensive strategies to parasitize plants. Consequently, plants have developed defensive mechanisms that include both pre-formed physical and biochemical barriers (pre-infection or passive resistance) and induced defence mechanisms (post-infection or active resistance); such defensive mechanisms include the strengthening of cell walls and the production of defence-related molecules that resemble the immune mechanisms of animals (Benhamou and Picard, 1999).

Induced defence is the mechanism most involved in applying antagonistic microorganisms, such as bacteria colonizing the rhizosphere. These microorganisms establish a relationship with the plant that is very similar, from a biochemical point of view, to what occurs following the attack by a pathogen. Many metabolic events are the same.

Post-infection biochemical defences include the activation by the plant of mechanisms such as the hypersensitive response, the local or systemic resistance and the secretion of substances (absent or present in small quantities before infection) showing a marked antimicrobial activity. These substances are described as proteins related to pathogenesis (PR or pathogenesis-related proteins), phytoalexins, phenolic compounds (active in the form oxidized by phenolases, phenoloxidases, peroxidases and polyphenol oxidase) and ROS compounds (reactive oxygen species) (Andersen *et al.,* 2018). The latter compounds may accumulate during incompatible reactions, can destroy cell membranes, produce lipid peroxides and, at the same time, promote reactions that strengthen cell walls. They play a direct role as antimicrobial and signal molecules to activate (or induce) systemic resistance and hypersensitivity reaction.

Finally, the interaction of the organism used for biocontrol with the microbial community associated to crop plants is particularly complex, but equally fundamental for understanding the mechanisms of action and for the correct application of the antagonists. This type of interaction assumes that the use of biocontrol agents can alter the balance between existing species in favour of non-pathogenic species; for example, thanks to this mechanism, a population of soil microbes is established on the roots instead of those typical of the rhizosphere, which competes with pathogens (Philippot *et al.,* 2013). Fungi and antagonistic bacteria, in addition to the control of plant diseases, can also have beneficial effects on plants.

1.2 Plant beneficial microbes

In the long biological history of the planet, microorganisms have adapted to derive nourishment from a wide variety of habitats. During this evolution, the competition with others for sources of nourishment and space was the cause that resulted in the development of those mechanisms of antagonism or suppression of their competitors (Hibbing *et al.,* 2010). The source of nutrition of a microorganism which it competes with may be a biological or abiotic entity that is directly or indirectly harmful to plants. The plant beneficial microbes (PBMs) can help the plant to overcome biotic and abiotic stresses and increase productivity, regulating the nutritional and hormonal balance of the plants solubilizing nutrients and inducing resistance against plant pathogens. In addition, these microbes also show synergistic and antagonistic interactions with other microbes in the soil environment (Nadeem *et al.,* 2016).

1.2.1 Plant Growth Promoting Rhizobacteria (PGPR)

Plant Growth Promoting Rhizobacteria (often referred as PGPR), refers to a diverse group of bacteria that invade and support plant growth in the rhizosphere. PGPRs are classified into two categories:

1) Growth promoting bacteria that establish symbiosis with plants.

2) Bacteria capable of promoting growth without establishing symbiosis with plants.

PGPRs can promote plant development through direct or indirect ways. Direct stimulation includes nitrogen fixing, the production of phytohormones, the solubilization of minerals such as phosphorus, while indirect stimulation involves the production of antibiotics, of siderophores (molecules that chelate iron) and the synthesis of extracellular enzymes to hydrolyse the fungal cell wall (Van Loon, 2007). The indirect effects are related to the biocontrol activity of deleterious microorganisms. Experimental results have shown that the stimulation of plant growth is the result of multiple mechanisms that can be activated simultaneously (Martinez-Viveros *et al.,* 2010). To be effective, PGPRs must maintain a high population density over a long period of time. These microorganisms can also increase tolerance to environmental stresses such as floods (Grichko and Glick, 2001), salt stress and water shortage (Mayak *et al.*, 2004). The use of PGPRs in agriculture is constantly increasing, as they offer an alternative to the use of chemical fertilizers, pesticides and other substances.

Based on their activities we may classify PGPRs in different, functional categories:

1) Biofertilizers: they increase the availability of nutrients for plants;
2) Phytostimulators: they promote plant growth through the production of phytohormones;
3) Rhizomediators: they degrade organic pollutants (bioremediation activity);
4) Biopesticides: they control plant pathogens by producing antibiotics and antifungal metabolites.

In addition to PGPR, the microbial community associated to the roots may also contain bacteria that have a negative influence on plant development, called deleterious rhizobacteria (DRB). DRBs can inhibit plant growth through the production of phytotoxins, including hydrogen cyanide, HCN, phytotoxic metabolites and phytohormones, especially IAA. They act by inhibiting the function of mycorrhizal fungi, diazotrophs and PGPRs or by developing a competition with the plant for nutrients such as phosphate and iron (Nehl *et al.,* 1997; Barazani and Friedman, 1999). The positive or negative effect of rhizobacteria on plants will vary depending on the environmental conditions, the genotype of the host and the mycorrhizal state. Signal molecules exchanged between plants and microorganisms able to favour the beneficial colonization of the plant have been identified (Giovannetti *et al.,* 2006). A root growth promoting organism should be first and foremost a good root colonizer.

1.2.1.1 Plant growth promotion mechanisms

Many of the processes related to biogeochemical cycles are located in the rhizosphere, as it represents an exchange area between plants and microorganisms. The main mechanisms are those that involves the solubilization of phosphorus, nitrogen fixation, the production of phytohormones and siderophores, which involve an influx of nutrients towards the roots and greater stimulation of growth by means of phytohormones (auxins) (Ahkami *et al.,* 2017).

1.2.1.2 Solubilization and mineralization of phosphorus

Phosphorus-solubilizing bacteria are very abundant and ubiquitous in soils, accounting for 40% of the cultivable population (Richardson, 2001). Phosphorus is present in soil in two forms: inorganic phosphates, such as calcium phosphates, a soil mineral component, and in the form of organic phosphates, essentially represented by phytates (Shen *et al.,* 2011). The availability of this nutrient is limited by the poor solubility of most of these forms of phosphate, while plants can only absorb phosphorus in the two soluble forms of monobasic ($H_2PO_4^-$) and dibasic (HPO_4^{2-}) ions. The main mechanism underlying this process is due to the release of organic acids by the roots which, by acidifying the surrounding pH, favours the release of phosphate ions in solution (Sharma *et al.*, 2013). The mineralization of organic phosphates in soil is mediated by enzymatic reactions catalyzed by acid phosphatases and phytases (Azeem *et al.,* 2015). However, rhizobacteria consume organic acids in root exudates and, indirectly, can moderate the solubilization of phosphate and other elements, such as Fe and Mn (Hakim *et al.,* 2021). Rhizobacteria are able to produce a wide range of organic acids with P-solubilizing activity and, among these, gluconic acid and 2-ketogluconic acid appear to be the most active and important (Kalayu, 2019). Some Gram negative rhizobacteria have membrane bound enzymes that allow the extracellular conversion of glucose to gluconic acid (through the action of glucose dehydrogenase) and then to 2-ketogluconic acid (Kumar *et al.,* 2018). Plants benefit from the association with microorganisms capable of solubilizing phosphates, as they provide access to sources of phosphorus otherwise not usable by the plant alone (Etesami and Jeong, 2021).

1.2.1.3 Production of siderophores

Although iron is very abundant in soil, its concentration available for the microflora is very low. The abundance of available iron in soil and its solubility depends on soil pH. In most environments, iron deficiency is not linked to low concentrations, but to low bioavailability (Kraemer, 2004). In aerobic conditions, free ferrous iron, Fe (II), is oxidized to ferric iron Fe (III), thus forming oxides and hydroxides that are not easily soluble (Neilands, 1995). Iron is an indispensable element for the life of most living organisms. Most of the microorganisms have developed a mechanism for the acquisition of iron based on the production of compounds called siderophores. The word siderophore comes from the Greek *“σιδεροφόρου”* and stands for "iron bearer". They are secondary metabolites with a molecular weight below 2000 Da, capable of chelating iron and with a strong affinity for Fe (III) ions (Budzikiewicz, 2010). Since not all siderophores have the same affinity for Fe (III), the iron available in the environment is mainly used by microorganisms that produce siderophores with a high affinity for iron. Siderophores form complexes with iron by hydroxamate, α-hydroxy-carboxylated or catecholate groups, called ferric siderophore complexes. The process is carried out thanks to three components:

1) the siderophore, which binds with high affinity to the ferric ion;
2) a membrane receptor that allows the ferric siderophore complex to be transported across the microbial membrane. An extremely specific receptor corresponds to each type of siderophore;
3) an enzymatic system, present inside the cell, which releases the ion bound to the siderophore: ferric-chelate-reductase.

The production of siderophores gives a competitive advantage to many organisms in biotic and abiotic ecosystems. All aerobic fungi and bacteria produce siderophores (Neilands and Leong, 1986). Generally, the bacterial siderophores, even if they differ in their ability to sequester iron, deprive the pathogenic fungi of this essential element, since the fungal siderophores have a lower affinity for Fe (III); this constitutes one of the main biocontrol mechanisms towards phytopathogenic fungi (Loper and Henkels, 1999). In soil, at neutral pH, the concentration of Fe (III) in equilibrium with ferric oxides is about 10-17 M (Budzikiewicz, 2010). Microorganisms require higher concentrations of Fe (III) and when cells detect concentrations below this threshold, they begin to produce siderophores (Miethke and Marahiel, 2007). Organic acids such as lactate, succinate, fumarate, malate, acetic acid, of the radical exudates of iron-deficient plants, can contribute to the solubilization of Fe (III) and influence the microbial acquisition of iron (Jones, 1998).

In most environmental systems, siderophores exist in a complex form and in the rhizosphere, we find the highest concentrations. Production of siderophores by PGPB has been demonstrated (Maheshwari, 2013).

1.2.1.4 Nitrogen cycle

The importance of bacteria in the nitrogen cycle can be found in both the rhizosphere and afar from roots (Delwiche, 1970; Rosswall, 1983). The carbon substrates present in the rhizosphere provide much of the energy required for this process. The nitrogen cycle consists of four phases: fixation, ammonification, nitrification and denitrification. These phases allow the assimilation of ammonium and nitrate by the plant and the organisms present in the soil. The molecular nitrogen present in the atmosphere (N_2) is reduced by the fixation process operated by the bacteria, to ammonium ion (NH_4^+), which can become part of the organic compounds. Bacterial ammonification is important for the return of large quantities of ammonia to the soil, through the mineralization of organic nitrogen compounds. The possibility of denitrification allows many bacteria to use nitrate, rather than oxygen, as the final acceptor of electrons in respiration. The presence of water in the soil limits the presence of oxygen, as water acts as a barrier to its diffusion. Furthermore, the combined respiration of the rhizobacteria and the plant, results in a reduction in the availability of oxygen in the rhizosphere. Nitrogen-fixing (or diazotrophic) microorganisms can be divided based on their behaviour, into free or symbiotic nitrogen-fixing microorganisms. Free nitrogen fixers are found both at the rhizosphere and not, while symbiotics fix the atmospheric nitrogen only after interaction with the roots of the plant (Ahemad *et al.,* 2014*).*

The process by which the rhizobia penetrate inside the roots of legumes and form radical nodules, in which they fix the atmospheric nitrogen, is called "nodulation" (Oldroyd *et al.,* 2008*).* These associations show a high degree of adaptation between the plant and diazotrophic rhizobacteria (Bhattacharyya *et al.,* 2012).

1.2.1.5 Production of phytohormones

PGPRs can produce various phytohormones, such as IAA, gibberellins and cytokines, in addition to ethylene (Maheshwari *et al.*,2015).

In plants, phytohormones contribute to the coordination of various physiological processes such as quiescence regulation and seed germination, root formation or flowering. They increase resistance to environmental stress factors, act as inducers or suppressors of gene expression and the synthesis of metabolites, pigments or enzymes (Wong *et al.*, 2015*)*.

Ethylene, unlike phytohormones, is a growth inhibitor: it slows cell growth and extension while accelerating fruit ripening and aging. It is produced during stressful conditions and, being a gas, it is easily transported by diffusion (Iqbal *et al.*, 2017).

Among the auxins, indole-3-acetic acid (IAA) is the most important molecule and shows the greatest biological activity. In plants, auxins are responsible for the division, extension and differentiation of cells and plant tissues (Maheshwari *et al.*, 2015). IAA is formed from tryptophan, which is deaminated by oxidation (through the formation of indole-3-pyruvic acid) or decarboxylated (through the formation of tryptamine) (Tahir *et al.*, 2013).

Rhizosphere bacteria are the most efficient producers of IAA, together with rhizosphere fungi, particularly present in root exudates, where tryptophan, precursor of the IAA, is found (Oleńska *et al.*, 2020).

The high concentration of IAA in the rhizosphere, given by the sum of that produced by plants and that synthesized by bacteria, stimulates the elongation, differentiation and proliferation of roots, thus improving the absorption of nutrients and water and allowing the plant to better overcome stresses (Maheshwari *et al.*, 2015).

An excess of IAA can cause negative effects, as it can induce the synthesis of the key enzyme for the biosynthesis of 1 aminocyclopropane-1-carboxylate (ACC), precursor of ethylene.

Among the gibberellins, gibberellic acid is the phytohormone with the greatest biological activity, regulates cell division and elongation and stimulates flowering (Binenbaum *et al.*, 2018).

Cytokines are substituted derivatives of adenine, which, in plants, act as activators of RNA and protein synthesis, stimulate the division of plant cells, activate seed germination, stabilize the photosynthetic apparatus in conditions of water stress and increase resistance of plant cells under various adverse environmental conditions (Osugi and Sakakibara, 2015).

The ability of bacteria to synthesize plant hormones is exploited in agriculture. Microorganisms, compared to expensive synthetic phytohormones, have several advantages, such as the wide spectrum of action and the presence of other substances that perform a beneficial action such as vitamins (Hakim *et al.,* 2021).

1.3 Streptomycetes

Streptomyces spp. are Gram-positive bacteria containing a high G + C ratio in their genome. They belong to the class of *Actinobacteria*. *Streptomyces* are one of the most abundant sources of bioactive compounds known and, consequently, they are extensively investigated (Viaene *et al.,* 2016). Several bioactive compounds are produced in the form of secondary metabolites, such as antibiotics and extracellular enzymes, not to mention anticancer and agro-active chemicals that aid in the degradation of cellulose and chitin (Tyc *et al.,* 2017). The majority of *Streptomyces* spp. are capable to colonize the rhizosphere and rhizoplane. Additionally, they may be endophytes that colonize the internal tissues of host plants. These characteristics might be attributable to *quorum sensing* controlled gene expression, rapid multiplication, antibiotics, siderophores, cellulases, phytohormones, amino acid synthesis, chitinase, lipase, and 1,3-glucanase production (Olanrewaju *et al.,* 2019).

The ability of *Streptomyces* spp. to control plant pathogens is well known (Vurukonda *et al.,* 2018) and is based on the following characteristics, such as synthesis of plant growth regulators (Goudjal *et al.,* 2013), production of siderophores (Vijayabharathi *et al*., 2018), secretion of volatile compounds (Jones and Elliot, 2017) and nutrient competition.

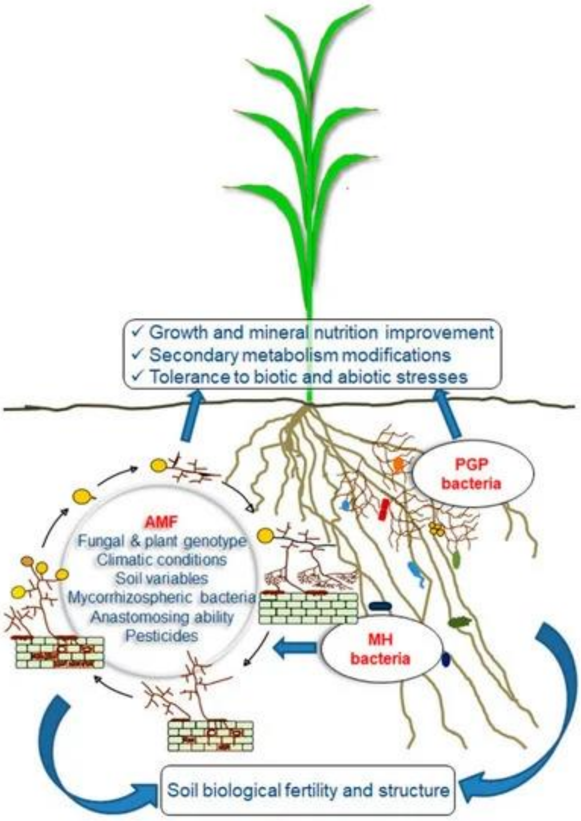

Figure 2 *Schematic representation of possible interactions between beneficial microorganisms and plants (Giovannini et al., 2020).*

1.4 Seed coating with beneficial microbes

Plant beneficial microorganisms (PBMs) are viewed as a natural alternative for mitigating the environmental pressures caused by traditional farming. These bacteria can assist plants in maintaining or increasing productivity while minimizing pesticide input, restoring soil fertility, and overcoming issues associated with abiotic and biotic stressors (Nadeem *et al.*, 2014).

Seed inoculation has been proposed as a precise and cost-effective approach for delivering microbial inoculants on a broad scale (Ehsanfar and Modarres-Sanavy, 2005). Seed coating is a process in which an active substance (*e.g.*, the microbial inoculant) is applied to the seed's surface using a binder and, in some situations, a filler that acts as a carrier. Seed coating has been recommended as a promising strategy for inoculating a variety of crop seeds, due to its ability to apply small amounts of inocula precisely (Rocha *et al.*, 2019). The three main types of seed coatings are seed dressing, film coating, and pelleting, each of which can be used differently, depending on the application and the type of seed or microbes (Rocha *et al.*, 2019).

1.4.1 Advantages and disadvantages of the various seed inoculation methods

Each technique of inoculation has advantages and drawbacks, depending on the quantity of inoculants used, the availability of equipment, the kind of seed (*e.g.,* size, shape, and fragility), the presence of inhibitory chemicals in the seed (*e.g.*, fungicides, micronutrients, and PBM), and the cost (Bashan *et al.,* 2014).

Direct soil inoculation is a technique for introducing a large volume of microbial inoculant into the soil prior to sowing without causing damage to delicate seeds or protecting the inoculant from inhibitory chemicals applied to or generated by the seed (*e.g.*, fungicides and antimicrobial compounds). Nevertheless, due to the vast volume of microbial inoculum necessary for wide-scale applications, direct soil inoculation is not economically and/or environmental acceptable (Vosátka *et al.,* 2012).

While root dipping and foliar inoculation are currently employed to inoculate plants, these approaches need substantial quantities of inoculant and plant nursery preparation in the case of root dipping. On the other hand, seed inoculation can be an economically viable method of delivering microorganisms in large-scale field applications (O'Callaghan, 2016).

In general, microbial seed coating is used in agriculture to increase plant protection and crop yield. Seed coating with PBM has been effectively applied to various seeds with various sizes, shapes, textures, and germination types (Ma, 2019). The most extensively studied crops in seed coating inoculation include cereals, such as wheat and maize, and fruit/vegetable, crops such as tomato, cucumber and sugar beet. In addition, soybean, chickpea, and pea are three of the most frequently mentioned oil and seed pulse crops. Additionally, research on PBM seed coatings has been conducted on fibre crops such as cotton and feed crops such as alfalfa (Rocha *et al.,* 2019).

The carrier is a critical component of the inoculants because it enables the delivery of an optimum amount of PGPM under physiologically favourable circumstances (Pacheco Aguirre *et al.,* 2017). The carrier ingredients in seed coating formulations are frequently polymeric and hygroscopic in nature (Pedrini *et al.,* 2017). Carriers can be generally classified according to their role as nutrients (*e.g.,* P, K, Cu, Mn, and Zn) or fillers (*e.g.,* cellulose, chitosan, peat, talc, lime, charcoal, and vermiculite) (Padhi and Pattanayak, 2018). Fillers are used to enhance the form, size, and weight of the seeds. The majority of carriers are organic, inorganic, or synthetic. Cost and safety are the primary determinants of carrier selection. The carriers employed for microbial seed coating should cling easily to seeds and ensure seed germination and seedling growth, as well as the survival of PGPM on the seeds. Silica and charcoal are the most often used carriers for microbial coating (Ma *et al.,* 2019).

Microbial survival is critical throughout the storage period of biological products and after they are introduced into the soil, where the inoculants fight for resources with indigenous soil microorganisms (Malusá *et al.,* 2012). Therefore, shelf life is a crucial factor that needs to be considered. Microorganisms' survival on seeds is conditional on the trade-off between variables that favour large initial cell loads and those that promote long-term stability (Deaker *et al.,* 2004). Microbe survival and viable cell populations often fall during storage on seeds under a variety of environmental circumstances. The rate of desiccation is dependent on the relative humidity in the air during seed inoculation and storage. Although the optimal long-term storage conditions for microbial survival on coated seed are unknown, various variables have been implicated in microbial viability over time, including temperature, moisture status, coating materials, and pollutants (Deaker *et al.,* 2012). Coated seeds that are maintained at a low temperature and moisture content retain a high level of microbiological viability and vigour (Ma, 2019).

Additionally, polymers may increase the likelihood of microorganisms surviving on seeds by protecting them from a variety of environmental challenges (Deaker *et al.*, 2007). On the other hand, pollutants present in inoculant carriers may impair microbial survival and colonization of the rhizosphere and plant tissues since they usually restrict microbial growth during inoculant coating development (Deaker *et al.*, 2012). Using dilution plate counts, the microbial viability and quality of inoculated seed are assessed regularly (*e.g.,* bacterial colony count).

1.5 Ozone: applications for seed sanitation

The seed physiologically supports the developing seedling until the latter emerges as an independent life form. Therefore, it is necessary to find effective methods to inactivate any pathogens on seeds before germination (Bari *et al.*, 2011). Some plant diseases, caused by pathogenic microorganisms, can be transmitted through seeds, and the seed can be a primary source of inoculum. Therefore, the inactivation of pathogenic microorganisms possibly presents in or on seeds, with physical or chemical methods, is essential to reduce the risk of disease outbreaks. (Buck *et al.*, 2003). Physical treatments include exposing the seeds to high temperatures, since heat can kill the pathogens transmitted by the seeds (Beuchat and Scouten, 2002). Nevertheless, these methods are difficult to be implemented commercially, because of the range for the temperature and exposure time for the different types of seeds, despite their effectiveness (Buck *et al.*, 2003). Chemical methods for sanitising seeds are generally effective, but they can interact with organic matter, compromising environmental and food safety. Therefore, according to Richardson *et al.* (1998), methods of sanitation are needed that can reduce the environmental impact and are safe for food production. A suitable method for sanitation should preserve the vitality, germination and vigour of seeds and should be safe for environment. Moreover, treatment efficiency is seed-type dependent, because of the different seed morphology and composition that can influence the efficacy of these treatments. A possible strategy for seed sanitation is the application of ozone. The primary advantages in ozone treatment include its fast decomposition in water to oxygen, and the absence of residues or by-products (Wang *et al.,* 2004); another advantage is that ozone reacts instantly and is an efficient sanitation tool for different groups of microorganisms.

Ozone is both safe and economically convenient to use, since it can be reliably generated on-site as needed, avoiding handling and costs associated with transportation and storage. Ozone treatments leave no chemical residues, unlike other chemical sanitization procedures, and in the processing premises, it reverts back to oxygen naturally.

According to Glowacz *et al.* (2015), an ozone treatment is considered as a practical, economically viable and eco-accommodating sustainable innovation. Currently, ozone technology is applied as a disinfectant to control the growth of undesirable microorganisms in food products, as well as in cleansing the types of equipment used in the food industries (Pandiselvamet *et al.,* 2018a), either in the form of gas or aqueous solution. According to Rodrigues *et al.* (2019) ozone could be used for a multitude of applications, such as an antifungal or bactericide on seeds.

1.7.1 Ozone as a seed priming agent

In addition to the sanitising and disinfectant properties, ozone acts on seed germination, with additional various effects on plant growth (Abeli *et al.,* 2016; Landesmann *et al.,* 2013; Mohammad *et al.,* 2019; Sudhakar *et al.,* 2011; Vazquez-Ybarra *et al.,* 2015). Sudhakar *et al.* (2008) investigated the effect of ozone on the growth of tomato seedlings. In this study, the exposure of seeds to 0.2 ppm of ozone for 2 min/day for ten days showed a positive effect on the growth parameters of tomato seedlings, including increased leaf area and enlarged root length. It was concluded that 0.2 ppm of ozone treatment provides 33% enhanced dry weight and stringent biomass allocation among the root, shoot, and leaves. The speed of seed germination could be due to the ozone-inducing oxidation of compounds in the seed mantle, thus inducing the cell signalling process that initiates germination. On the other hand, dioxides and peroxides cause an increase in ethylene synthesis and decrease in abscisic acid (ABA) in seedlings (Sudhakar *et al.*, 2008). According to Violleauet et al. (2008), high root growth in ozonated seedlings could be attributed to the increased production of jasmonic acid.

2. Aim of the present study

Seed coating is currently performed using chemical fungicides that may impact on environmental safety and on the development of resistant pathogens, especially fungi. Additionally, the market is more and more prone to request organic seeds for organic vegetable production.

Recently, the European Parliament resolution of 6 October 2021 on Commission Implementing Regulation (EU) 2021/1449 of 3 September 2021 amending Implementing Regulation (EU) No 540/2011 as regards the extension of the approval periods of several active substances (EU, 2021), include a few fungicides, as fludioxonil (However, because to worries about its environmental fate and influence on human health and non-target creatures, it is very likely to be taken off the market in the European Union (EU) after the end of 2021 (Commission Implementing Regulation (EU) 2021/1449), the most popular seeds coating pesticide, states that the approval of those active substances are likely to expire before a decision has been taken on their renewal. Thus, there is the possibility that very soon no fungicide will be available for seed coating.

Therefore, the aim of this study, financed by a private Italian seed company, was to improve the seed quality of selected vegetables by using beneficial microorganisms as seed coating agents suitable for industrial purposes. Our goal was to identify new potential biological control agents and to develop and implement a protocol for industrial application to seeds; this to replace fungicides that are currently used as control methods, in order to avoid their extensive and routinely use, which often lead to resistance in plant pathogens.

Particularly, we aimed to discover, select and study streptomycetes, as prospective biocontrol agents and plant growth promoters. Once selected and characterised, the most prospective streptomycetes will be used as seed coating agents in experiments under controlled conditions and in the open field, under relevant pathogen pressure. As crops, we selected those that are quite important along the Adriatic areas of Italy: onion, fennel, rocket salad and lettuce.

Finally, studies on the activity of the prospective biocontrol agents, were completed by comparing the efficiency of at least one streptomycete as a biocontrol agent with other commercial agents and their formulations, together with the registered chemical active substance indicated for seed treatment (*i.e.*, Fludioxonil).

Additionally, we aimed to sequence and study the draft genome of at least one strain in order to discover the possible presence of putative metabolic pathways promoting plant growth, together with additional properties that may indicate this strain as a prospective agent for future biocontrol applications *in planta*.

3. Materials and methods

3.1 Microbial cultures

The bacterial strains used in the present study belong to the collection of the Plant Pathology Laboratory, Department of Life Sciences, University of Modena and Reggio Emilia (Tab.1). In previous experiments such listed bacteria demonstrated antifungal activity and the ability to stimulate plant growth (Vurukonda *et al.*, 2018).

Table 1 *List of antagonistic bacteria used in the present study. Strain DLS 1568 was isolated and characterized during our study*.

Bacterial antagonist	***Strain number***	***Isolated from***	***Geographical origin***
***Streptomyces* sp.**	DLS 1568	Rice seeds	Italy
***Streptomyces* sp.**	SA 51	Olive roots	Italy
***Pseudomonas* sp.**	PT 65	Tomato plants	Italy
***Agrobacterium* sp.**	AR 39	Hazelnut roots	Italy

The phytopathogenic fungi employed in this study represent the most common agents of soil-borne diseases affecting horticultural crops. The fungal strains are present in the collection of the Plant Pathology Laboratory, Department of Life Sciences, University of Modena and Reggio Emilia (Tab. 2).

Table 2 *List of phytopathogenic fungi used in the present study*

Phytopathogenic fungi	***Strain number***	***Isolated from***	***Geographical origin***
Fusarium oxysporum* f. sp. *cepae	1585	Onion	Italy
Fusarium oxysporum* f. sp. *raphani	Fus Ruc 9A	Rocket salad	Italy (Courtesy of Dr. Gilardi, University of Turin, Italy)
***Sclerotinia* sp.**	1584		Italy (Courtesy of Dr. Prodi, University of Bologna, Italy)
Stemphylium sp.	1773	Onion	Italy (Courtesy of Verdelab, Rimini, Italy)
Colletotrichum dematium	1772	Spinach	Italy (Courtesy of Verdelab, Rimini, Italy)
Pythium sp.	1583		Italy (Courtesy of Dr. Prodi, University of Bologna, Italy)

3.2 Plant material

Four different plant species were used during our research (Table 3). Cora Seeds s.r.l. provided seed batches for the experiments and, additionally, made experimental and commercial fields available for the research activity in the open.

Table 3 *List of crops used in the present study*

Scientific name	***Common name***	***Variety***	***Additional features***
Allium cepa	Onion	Fundador	Long day genotype
		Elenka	Long day genotype
Foeniculum vulgare	Fennel	Crono	Early cycle (summer) genotype
Diplotaxis tenuifolia	Wild rocket (Santamaria *et al.*, 2002).	CRX 265 (Kristina) CRX 267 (Karina) Adagio	
Lactuca sativa	Lettuce	Summerbel	Batavia type

3.3 Antagonistic assays in vitro

3.3.1 Media

Microbial antagonism was studied using the dual-culture plate method. Each bacterial culture was grown in Nutrient Sucrose Broth (NSB = Nutrient broth 8.0 gr l^{-1}, Sucrose 50 gr l^{-1}) for 24 hours at 27 ± 2°C. One drop (20 µl) of the bacterial suspension was taken from the growing culture and pipetted on one side of a Potato Dextrose Agar (PDA = Potato extract 4 g l^{-1}, Dextrose 20 g l^{-1}, Agar 15 g l^{-1}) plate, 1 cm from the edge, 24 hours prior fungal inoculation. A disk (3 mm) from a five-days-old fungal mycelium grown on PDA was placed at either side (1 cm from the edge) of PDA plates, opposite to test bacterial inoculum. Plates without antagonistic bacteria were used as controls. All plates were incubated at 27 ± 2 °C for seven days.

The fungal growth inhibition (GI) was quantified in percentage, using the average (three technical replicates per strain) of diameter of fungal mycelium that formed around the area of the bacterial colony (Wang *et al.,* 2011):

$$GI\ \% = \frac{C - S}{C} x\ 100$$

(C = diameter of positive control, S = diameter of sample)

3.3.2 Plant growth promotion traits

Plant Growth Promoting Rhizobacteria (PGPR) characteristics, such as siderophore production and phosphate solubilization, were determined following standard procedures, with some modifications.

3.3.2.1 Siderophore production

To determine the production of siderophores, a modified methodology was used (Schwyn and Neilands, 1987). Each *Streptomyces* sp. was grown in a flask containing a liquid medium named “International *Streptomyces* Project-2” (ISP-2, Yeast extract 4.0 g l^{-1} Malt extract 10.0 g l^{-1}, Dextrose 4.0 g l^{-1}) for 48 hours at 26 ± 2° C. The resulting suspension was centrifuged at 6000 r.p.m. for 20 minutes, and the resulting pellet was previously rinsed and then suspended in sterile distilled water; an aliquot of such suspension was inoculated into CAS-LB agar (LB agar medium supplemented with Chrome Azurol S) prepared according to Schwyn and Neilands (1997). Sterile distilled water was used as negative control and, as a positive control, 1 M pyrocatechol solution was used (Lakshmanan *et al.*, 2015). Then, the plates were incubated at 28 ± 2°C. After 5 days of incubation, the development of a yellow-orange halo was measured. Three technical replicates per strain were performed. Statistical analysis of data was conducted as decribed below.

3.3.2.2 Phosphate solubilization

Phosphate solubilisation was investigated using the protocol developed by Lucas *et al.* (2014), with the same modifications. Potato dextrose yeast agar (PDYA = PDA medium supplemented with $CaHPO_4$, [50 ml 10% (w/v) K_2HPO_4 and 100ml (w/v) $CaCl_2$]) (De Freitas *et al.*, 1997) was used to assay *Streptomyces* sp. DLS1568. A drop of bacteria suspension (20µl) was placed in the centre of the plate. Control was performed using a drop of sterile distilled water. The ability to solubilize was given by the presence of a clear zone around the colony after 14 days of cultivation. Data were expressed as a means of the halo diameter and statistically analysed.

3.4 Bacteria transformation

For bacteria transformation, the calcium chloride heat-shock method was used, following the procedure by Chang *et al.* (2017). The wild-type *Streptomyces* sp. DLS1568 was grown in ISP-2 liquid medium for 24 h at 27 ± 2 °C until the optical density at $OD_{600} = 0.6$. The suspension was then centrifuged at 6000 r.p.m. for 10 minutes and the supernatant was discarded. The pellet was washed twice with sterile saline. Subsequently, the pellet was resuspended in 100 ml of ice-cold $CaCl_2$ solution (0.1 M $CaCl_2$), shaking gently until the pellet was dissolved. Cells were recovered by centrifugation at 5000 r.p.m. for 10 minutes; thereafter the pellet was resuspended in 20 ml of ice-cold 0.1 M $CaCl_2$ and incubated on ice for 30 min. Subsequently the suspension was centrifuged at 5000 r.p.m. for 10 minutes. The supernatant was discarded and the pellet was resuspended in 5 ml of 0.1M $CaCl_2$. 200 µl of $CaCl_2$ treated cells were transferred in tubes, and tagged with the *gfp* marker gene. The tube was stored in ice for 30 min and then placed in a water bath at 42°C for 90 seconds. One ml of LB was then added in the tube. Finally, 100 µl of transformed cells were spread onto the LB agar plates supplemented with kanamycin. The identification and selection of clones carrying the *gfp* gene were carried out under UV light.

3.4.1 Internalisation of a GFP-tagged streptomycete in plant tissues

Seeds (onion, fennel, wild rocket and lettuce) were used for microbial coating with *Streptomyces* sp. DLS1568 GFP mutants. Seed samples (n = 1 gr of seeds) were surface-disinfected according to Fatmi et al. (1991). Briefly, seeds were incubated in 2% sodium hypochlorite (NaOCl) for 20 minutes at 25°C on a rotary shaker at 250 rpm (Innova, New Brunswick Scientific, Edison, NJ) and then rinsed under running tap water for three minutes.

The bacterial suspension was routinely grown on ISP-2 liquid medium for 24 hours at 27 ± 2°C. The suspension was centrifuged at 6000 r.p.m. for 20 minutes, the supernatant was then discarded and the pellet resuspended in 20 ml of sterile distilled water. The concentration of cells was determined using a spectrophotometer (Spectronic 20; Bausch and Lomb, Rochester, NY) (optical density at 600 nm = 0.3; approximately 1×10^8 CFU ml^{-1}) and adjusted to a final concentration of 10^8 CFU ml^{-1}.

The seed samples were coated with sterile distilled water (used as negative control) and with the bacterial suspension described above, respectively, according to Kaufman (1991). Briefly, each seed sample was incubated for 20 minutes at 25°C on a rotary shaker at 250 rpm (Innova, New

Brunswick Scientific, Edison, NJ) and then air-dried for 24 hours at room temperature and stored at 4°C until sowing.

The evaluation of the internalization of such *Streptomyces* sp. was performed 20 days after sowing on plant seedlings. For each sample, roots, stems and leaves were carefully dissected (approximately 10 mm) with a sterile scalpel and placed on the slide for observation through the use of a Leica TCS SP8 Confocal Laser Scanning Microscope (CLSM) (Leica Microsystems, Mannheim, Germany) equipped with solid state lasers for excitation was used to unravel root, stem and leaf colonization patterns by the EGFP-*Streptomyces* sp. DLS1568 strain, whose wild-type strain showed promising biocontrol potential, at CIGS (Centro Interdipartimentale Grandi Strumenti), University of Modena and Reggio Emilia, Italy.

3.5 Comparison of different seed coating strategies for industrial application

During the current experiment, three different formulations for the microbial inoculant application were tested for their efficacy for bioagent (*Streptomyces* sp.) coating to onion var. Elenka, fennel var. Crono, wild rocket var. CRX 267 and lettuce var. Summerbel seeds. Three different biocoating strategies, which can be used as seed treatment in organic agriculture, were considered and assessed in order to identify the most feasible industrial method in terms of ease of application and examined for spore viability, respectively, as a measure of compatibility. Moreover, the most performing formulation was then evaluated for its impact on the microbial survival during the process of product elaboration, storage, and application, and in its efficiency once applied on the target seed and in the economic feasibility of the application (John et al., 2011; Herrmann and Lesueur, 2013). It is worth noting that factors such as incorrect inoculant formulation or limited shelf-life (i.e., inoculant viability on the seed surface) can hamper a wider use of seed coating (O’Callaghan, 2016).

In this experiment, the bacterial inoculant was prepared as follows: the *Streptomyces* sp. DLS 1568 was grown in ISP-2 broth for 48h at 120 r.p.m. by continuous shaking at 27 ± 2° C. The microbial suspension was centrifuged at 6000 r.p.m. for 20 minutes, discarding the supernatant. According to the three strategies described below, 1 kg of seed was surface-disinfected according to Fatmi et al. (1991). Then, each seed coating treatment was carried out at Cora Seeds S.r.l. warehouse (Cesena, IT), using an industrial coating machine (Cimbria Centricoater CC Lab, Stockerau, AU):

1) The pellet was then resuspended in 20 ml of deionized sterile water at a final concentration of 10^9 CFU ml^{-1}. Each seed sample was coated with such microbial suspension according to the industrial practice.

2) The pellet was then resuspended in 20 ml of 0,3% biopolymers xanthan gum (Sigma-aldrich, USA) to the final concentration of 10^9 CFU ml^{-1}. Each seed sample was then coated with Xanthan gum and bacterial suspension according to the industrial practice.

3) The pellet was then resuspended in 20 ml of deionized sterile water at a final concentration of 10^9 CFU ml^{-1} and it was added with Bio Friendly 1 binder (Centor Group, Andijk, NL) in a 1:1 ratio. Each seed sample was then coated with such microbial suspension according to the industrial practice.

After the experimental seed coating strategies described above, each seed sample was dried in the drying machinery according to the Cora Seeds s.r.l protocol. Disinfected and not coated seed was used as negative control for lettuce, onion, wild rocket and fennel crops.

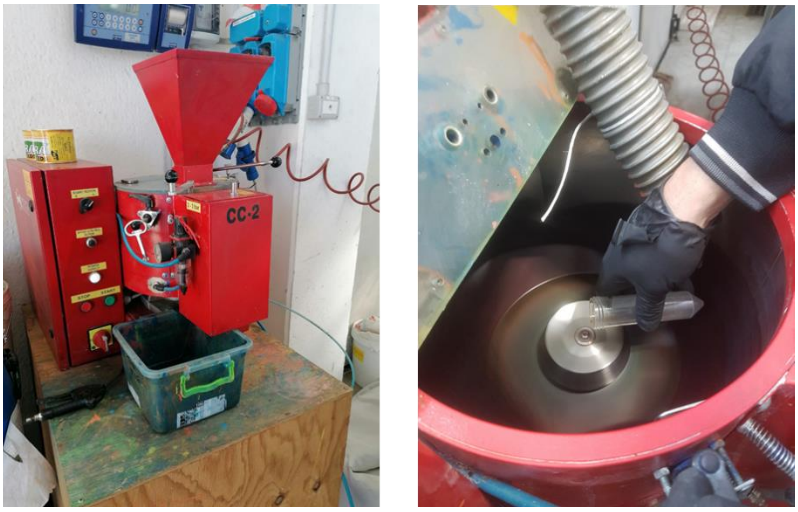

Figure 3 *Seed coating machine used in the experiments*

3.5.1 Assessment of Streptomyces sp. DLS 1568 load on coated seeds

For all the seed coated samples belonging to the crops mentioned above (*i.e.*, onion var. Elenka, fennel var. Crono, wild rocket var. CRX 267 and lettuce var. Summerbel), the method developed by Giovanardi *et al.* (2015) was used to determine the *Streptomyces* sp. DLS 1568 concentration (*i.e.*, CFU per seed), with some modifications.

One gram of previously treated seeds was transferred into 10 ml of sterile saline (NaCl 0.8%) and placed at 4°C for 24 h. Then, 0.5 ml of washing saline was pipetted and transferred into 4.5 ml of 0.8% NaCl for dilutions up to 10^{-4}. All dilutions were plated on ISP-2 medium and incubated at 27 ± 2°C for 48 h. The developing colonies were then counted to determine the number of CFU per plate. The following formula was used to calculate the number of CFU per seed:

$$CFU\ per\ seed = \left(\frac{CFU}{(n°seeds\ /\ gr)}\right) \times 100$$

The n° seeds ⁄ gr is corresponding to [(1/TSW)*1000]. TSW is weight grams of 1000 seeds.

Vitality assessment (shelf-life) of the *Streptomyces* sp. DLS 1568 on the different seed crops over the time was repeated 6, 12 and 16 months after the seed coating treatment.

3.6 Ozone treatment

The experiments were conducted using an ozone generator prototype supplied by Elegen (Scandiano, Reggio Emilia, IT) (Fig.3). The ozone concentration applied was 20 ppm, with a chamber saturation of 80% and a temperature of 25 ± 2° C. For monitoring the ozone concentration, the Kono-meteroz ozone meter (Temper, Granada, ES) was used.

Figure 4 *Vacuum machine and ozone generator*

3.6.1 Ozone seeds treatment

Ten grams of seeds belonging to onion var. Elenka, fennel var. Crono, wild rocket var. CRX 267 and lettuce var. Summerbel were evenly distributed on a glass plate (150 mm in diameter) and then placed into the ozone generator chamber, respectively, according to the protocol described by Jiang et al. (2014) with modifications. Seeds were exposed to ozone at time intervals of 15 minutes as follows: T1 = 15 min.; T2 = 30 min.; T3 = 45 min., and T4 = 60 min., T0 as an untreated control, respectively. After exposure, a germination test on each seed sample for each exposure time was performed, as previously described. In order to evaluate the potential disinfection activities of the different ozone treatments (i.e, T1 = 15 min.; T2 = 30 min.; T3 = 45 min., and T4 = 60 min.), the microbial load on each seed sample was assessed according to Giovanardi *et al.*, (2015). One gram of seeds was placed in a bag with 10 ml of sterile PBS buffer and crushed with a hammer; subsequently, a series of dilutions up to 10 cells ml^{-1} were made, and each dilution was plated onto NSA medium, as described in Lelliott et al. (1966), and incubated at 27 ± 2° C. Colony counting was done after 24 hours.

3.7 Germination test assay

All the onion, fennel, wild rocket and lettuce seed samples belonging to the different treated and untreated samples for biocoating with *Streptomyces* sp. DLS 1568 and ozone treatments, were tested for their germination performance. Each seed sample (n = 100 seeds) was tested in four replicates according to ISTA (International Seed Testing Association, 2018). The seed samples were placed into plastic boxes with germination test paper (Sartorius, Göttingen, DE), and were moistened. The plastic boxes were then placed into germination cabinets, and germination experiments were made. The germination cabinets were set at an average temperature of 21 ± 2 °C, with 16 h hours of daylight. After the incubation period, the number of germinated seeds was counted for each box, and the percentage of germination was calculated. A seed was considered to have germinated when the seedlings with all their essential structures were well developed, complete, in proportion and healthy with cotyledons and radicles, according to the Handbook on Seedling Evaluation (International Seed Testing Association, 2018). The result of the germination test was expressed as percentages by number of normal and abnormal seedlings and hard, fresh and dead seeds. The experiments were repeated four times.

$$Germination\ rate\ \% = \frac{n}{N} \times 100$$

Where **n** represents the number of germinated seeds and **N** represents the number of seeds used for the test.

Onion var. Elenka, fennel var. Crono, wild rocket var. CRX 267 and lettuce var. Summerbel seed samples, generated by seed coating with the *Streptomyces* sp. SA 51 (Vurukonda 2020, PhD thesis) using the Bio Friendly 1 binder (Centor Group, Andijk, NL) through the process previously described, were also included as commercial controls.

3.8 Efficacy of microbial seed coating on wild rocket against *Fusarium oxysporum* f. sp. *raphani in planta* trials

For wild rocket var. Adagio, supplied by Cora Seeds s.r.l as susceptible to FOR (*personal communication by the Company*), an experimental assay was performed to evaluate the antifungal efficiency of *Streptomyces* spp. as seed inoculants against FOR (Fus Ruc 9A strain), according to Gilardi *et al.* (2007), with modifications. Trials were carried out in the greenhouse on wild rocket seed samples, previously treated with *Streptomyces* sp. DLS 1568 and *Streptomyces* sp. SA51, as described above. Untreated seed samples were used as control. The conidia suspension of FOR was prepared by adding 15 ml of sterile distilled water to the seven-day-old PDA plates to rehydrate the conidia. Subsequently, the suspension was filtered using sterile gauze. The concentration of conidia and mycelium fragments was determined by a haemocytometer and adjusted with deionized water to a final concentration of 10^7 CFU ml^{-1}, which was adopted in all trials.

The seeds were sown in polystyrene trays, for a total of 104 seeds per thesis. Roots of 20 day old plants were trimmed to a length of 5 cm, and dipped for 20 min in conidial suspensions of the Fus Ruc 9A strain, prepared as previously described. Inoculated seedlings were transplanted into new containers, and twenty-five seedlings per thesis were transplanted. Each container was considered as a replicate, for a total of 4 replicates per treatment. Values of disease index are reported in Table 3.

Table 4 *Disease index: the values range from 0 for the healthy plant to 100 for the dead plant*

	Disease index
0	Healthy plant
12,5	Plant with slight vascular discolouration
25	Plant with reduced growth vascular discolouration and mild foliar chlorosis
50	Plant with reduced growth of vascular discolouration and foliar chlorosis
75	Plant with strongly reduced growth, extended vascular discolouration, strong foliar chlorosis and necrosis
100	Dead plant

Figure 5 *Disease indices associated with increasing disease severity of rocket plants.*

3.9 Field trials

The field trials were carried out by direct sowing in commercial fields, according to the best agronomic practises, to evaluate the effectiveness of seed coating for onion and fennel with *Streptomyces* spp.

All field experiments were set up in a completely randomized block design, with 2-4 replicates per thesis (according to the experiment's design).

3.9.1 Onion field trials

3.9.1.1 Experimental onion field trials

Field experiments were performed in Italy on long day onion varieties, as listed in Table 3. The experiments started in 2020 and were conducted during two cropping seasons. In March 2020, an experimental field trial was performed by direct sowing in an experimental field located in Cesena (FC); such field was known to have high levels of natural inoculum from the continuous monocropping of onions and previous history of *Fusarium* spp.. To evaluate the effectiveness of seed treatments with both *Streptomyces* spp., the antifungal activity of the *Streptomyces* spp. strains were compared with a commercial fungicide, such as Fludioxonil (Maxim®, Syngenta, CH), used as a control. The seed treatments are listed in Table 4. For this test, two varieties of long-day onion were used: Fundador (F) and Elenka (E). The four treatments were arranged in a randomized complete block design with each treatment replicated twice. Experimental plots were 1.5 m wide × 20 m long and consisted of four rows of onions. The sowing was carried out in four rows for two replicas with plots of 20 metres in length. Fields were planted using a mechanical seed planter (mod. SPX 2015 - Bassi Seminatrici, IT) with 750,000 onion seeds per hectare. The assessment surveys were carried out every 21/30 days from the day of sowing over a length of 15 metres on the inner part of each plot in the central part of the plot, for a total of 5 surveys during the cropping season. Dates and types of assessments are reported in Table 5. No fungicide treatments were applied during the cropping season. Weeds were managed according to Cornell Vegetable Management Guidelines and Recommendations (Reiners *et al.,* 2019). The field test was repeated in March 2021.

Table 5 *List of treatments on onion seed used for the experimental field trials in Cesena (FC)*

	Treatment
1	Control (untreated seed)
2	FLUDIOXONIL [0,3 ml Kg^{-1} seed; according to Cora Seeds s.r.l. industrial practice]
3	*Streptomyces sp.* SA51 [BINDER: 20 gr Kg^{-1} seed + Bacterial suspension: 20 ml * Kg^{-1} seed]
4	*Streptomyces sp.* DLS1568 [BINDER: 20 gr Kg^{-1} seed + Bacterial suspension: 20 ml * Kg^{-1} seed]
5	Ozone (seeds ozonization)

Table 6 *List of onion field trial assessments in Cesena ('Minotti'; FC)*

Assessments on onion field trials 2020

	Date	Types
	16-04-2020	Sowing
1st	04-05-2020	Number of emerged plantlets
2nd	28-05-2020	Number plants and plant height (cm)
3rd	24-06-2020	Number plants and number leaves per plant
4th	22-07-2020	Number plants and bulb diameter (cm)
5th	20-08-2020	Weight of bulbs

Assessments on onion field trials 2021

	Date	Types
	02-03-2021	Sowing
1st	08-04-2021	Number of emerged plantlets
2nd	06-05-2021	Number plants and plant height (cm)
3rd	01-06-2021	Number plants and number leaves per plant
4th	29-07-2021	Number plants and bulb diameter (cm)

***Figure 6** Experimental onion field located in* Cesena (’Minotti’; FC) *during the growing season 2020*

3.9.1.2 Isolation and identification of *Fusarium* spp. from diseased onion plants

Samples of diseased onion plant parts, including onion roots and bulbs, were collected in the field to attempt the *Fusarium* sp. isolation during both cropping seasons 2020 and 2021. The collected onion plant samples were preliminarily thoroughly washed under tap water. Infected tissue from roots and basal plate was separated using a sterile scalpel. The tissue was cut into small segments, and each piece was surface-sterilized by dipping it in 70% ethanol for 30 seconds, followed by immersion in 1% sodium hypochlorite for 1 min and then washing twice in sterile double-distilled water. After drying the tissue on a sterile paper towel in a fume hood, the pieces were plated on PDA supplemented with 2.5 mg ml^{-1} streptomycin. After incubation for 48–72 hours at 28 ± 1°C in the dark, the grown fungi were separated from the plant tissues and transferred into a new plate. In order to obtain a pure colony, the single-spore subcultures method was used. The *Fusarium*-like isolates were PCR tested for the confirmation of their identity . In specific, the total genomic DNA was extracted following the methods of Huang *et al.* (2018). Molecular identification of *Fusarium* cultures was carried out based on conserved ribosomal internal transcribed spacer (ITS) region. universal primer pairs ITS1 (5′ TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used (White *et al.*, 1990). Amplification was performed on a Thermal Cycler (Applied Biosystems 9700) with 25 µl reaction mixtures containing 1X buffer (5X Green GoTaq, Promega); 2.5 mM $MgCl_2$; 2 mM each of dNTP; 25µM primer (each of ITS-1 and ITS-4); 1U of Taq DNA Polymerase; 2 µl DNA.

The DNA amplicons were separated in 1.5% agarose gel, stained with ethidium bromide and observed using BioDoc Analyze (Biometra, Göttingen, Germany) to check for the presence of unspecific fragments. PCR products were then purified using the QIAquick 96 PCR purification kit (QIAGEN, Chatsworth, CA) and sequenced by Bio-Fab research s.r.l. (Roma, IT) using the same primers as those used for amplification. The sequences were aligned and trimmed using SeqManII software (DNAStar, Madison, USA). The partial ITS gene sequences obtained from the isolates were compared by BLAST analysis with sequences in the NCBI nucleotide database (BLAST Nucleotide collection).

3.9.2 Commercial onion field trials

In February 2021, two trials were simultaneously performed in Conselice (RA) and Cesena (FC) commercial fields. For these trials, the onion variety Elenka was used. The seed treatments are listed in Table 5. The four treatments were arranged in a complete randomized block design with each treatment replicated two times. In the Conselice (RA) commercial field, the experimental plots were 1.8 m wide × 25 m long and consisted of five rows of onions. In Cesena (FC) commercial field, the sowing was carried out in four rows for two replicas with plots of 25 metres in length. Fields were planted using a mechanical seed planter (mod. SPX 2015 - Bassi Seminatrici, IT) with 750,000 and 600,000 onion seeds per hectare in Conselice (RA) and Cesena (FC), respectively. The sowing, for a field located in Conselice (RA), was carried out in five rows in two replicates, with plots of 25 metres; while the field located in Cesena was carried out in four rows in two replicates. The assessment surveys were carried out every 28 days starting from the day of sowing over a length of 20 metres on the inner part of each plot, for a total of 5 surveys during the cropping season. The surveys were carried out every 30 days from the day of sowing over a length of 20 metres in the central part of the plot, for a total of 5 surveys. The field was neither sprayed with herbicide nor with fungicide before sowing. No other plant pathogens or insect pests causing any damage to onions were observed over the duration of the experiment in either year. Weeds were managed according to the Cornell Vegetable Management Guidelines and Recommendations (Reiners *et al.*, 2019).

Table 7 List of treatments on onion seed used during the commercial field trials in Cesena (FC) and Conselice (RA)

	Treatment
1	Control (untreated seed)
2	FLUDIOXONIL [0,3 ml Kg^{-1} seed; according to seed company practice]
3	IRF360 [according to seed company practice]
4	*Streptomyces sp.* DLS1568 [BINDER: 20 gr Kg^{-1} seed + Bacterial suspension: 20 ml * Kg^{-1} seed]
5	Ozone (seeds ozonisation)

*[Notes: *microorganism suspension concentration: [1 × 10^9 CFU ml^{-1}]*

Table 8 List of onion field trial assessments in Cesena (FC) and Conselice (RA)

Assessments on onion field trials Cesena 2021

	Date	Types
	28-02-2021	Sowing
1st	29-03-2021	Number of emerged plantlets
2nd	29-04-2021	Number plants and plant height (cm)
3rd	29-05-2021	Number plants and number leaves per plant
4th	29-06-2021	Number plants and bulb diameter (cm)
5th	20-08-2021	Weight of bulbs

Assessments on onion field trials Conselice 2021

	Date	Types
	19-02-2021	Sowing
1st	22-03-2021	Number of emerged plantlets
2nd	19-04-2021	Number plants and plant height (cm)
3rd	19-05-2021	Number plants and number leaves per plant
4th	17-07-2021	Number plants and bulb diameter (cm)
5th	17-08-2021	Weight of bulbs

3.9.3 Commercial fennel field trials

Field experiments were conducted during 2020 and 2021 in the municipality of Avezzano (Abruzzo, IT) in commercial fields to study the effect of microorganisms used as seed coatings on the growth, development and yield of fennel. The tests were carried out by direct sowing. The three treatments were arranged in a randomized complete block design with each treatment replicated two times.

The 2020 trial was performed using the treatments listed in Table 6. Each experimental plot was 1.5 m wide × 100 m long and consisted of four rows (Fig. 2). The assessments were carried out 20 days from the day of sowing over the whole length of each plot, for a total of 3 surveys. Dates and types of assessments are reported in Table 6.

Based on the phenological development of the seedling, uniformity and vigour (Tab. 7 and Tab. 8) have been assessed by assigning values from 1 to 4, as shown in Figure 3.

Table 9 *List of treatments out on fennel seed used during the commercial field trials in Avezzano (AQ)*

	Treatment
1	Control (untreated seed)
2	FLUDIOXONIL [0,3 ml Kg^{-1} seed; according to seed company practices]
3	*Streptomyces sp.* DLS1568 [BINDER: 20 gr Kg^{-1} seed + Bacterial suspension: 20 ml * Kg^{-1} seed]

*[Notes: *microorganism suspension concentration: [1 × 10^9 CFU ml^{-1}]*

Table 10 *List of fennel commercial field trials assessments in in Avezzano (AQ)*

Assessments on fennel field trials 2020		
	Date	Types
	13-06-2020	Sowing
1st	03-07-2020	Number of emerged plantlets
2nd	15-07-2020	Number plants and vigour
3rd	13-08-2020	Number plants and vigour

Table 11 *Vigour assessment index for the fennel commercial field trials*

	Vigour assessment index
1	Initial development of the first true leaves stage
2	Development of first leaves stage
3	Completely developed first true leaves stage
4	Initial development of the second leaves stage

Figure 7 *Commercial field trials located at Avezzano (AQ) in 2020*

Figure 8 *Vigour index based on the phenological fennel seedling development*

The second experimental field trial conducted in 2021, was performed using the three treatments as mentioned above. Experimental plots were 1.5 m wide × 50 m long and consisted of four rows of fennels (Fig. 5). The assessments were carried out approximately 20 days from sowing over the whole length of each plot, for a total of 4 surveys. In comparison to 2020, a final assessment on fennel harvest was additionally conducted in order to assess the possible influence on fennel yield of the different treatments. Dates and types of assessments are reported in Table 7.

Figure 9 *Commercial field trial located in the municipality of Avezzano (AQ) where the experiments were conducted in 2021*

Table 12 *List of commercial fennel field trials assessments in Avezzano (AQ)*

Assessments on fennel field trials 2021		
	Date	Types
	18-06-2021	Sowing
1st	02-07-2021	Number of emerged plantlets
2nd	14-07-2021	Number plants and vigour
3rd	12-08-2021	Number plants and vigour
4th	14-09-2021	Bulb Weight (Kg)

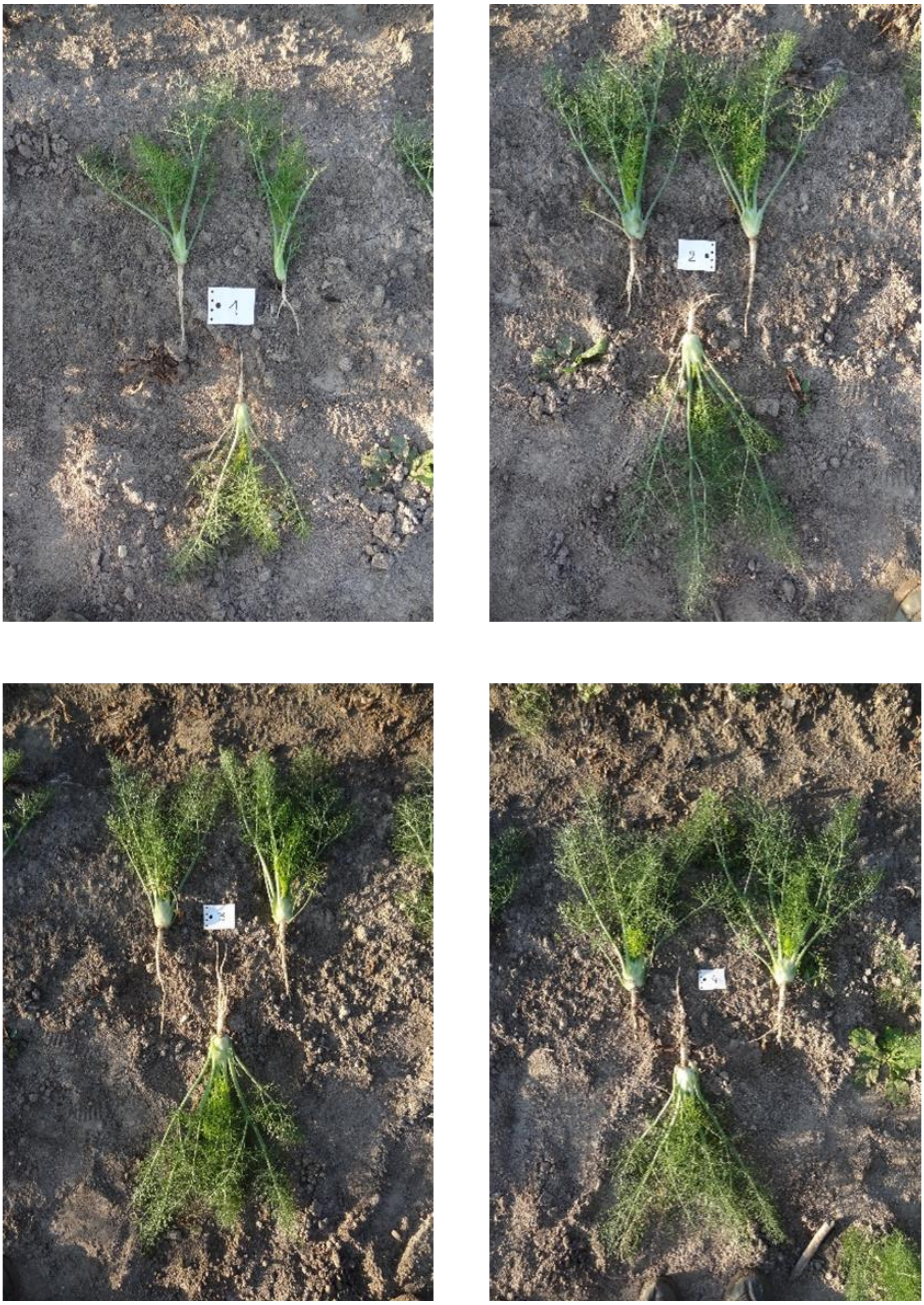

Figure 10 *Vigour index based on the phenological fennel seedling development during the 3rd assessment*

Figure *11 Commercial fennel field during the 4th assessment*

Figure 12 *Fennel bulbs during the 4th assessment at harvest time*

3.10 Detection of *Streptomyces* sp. DLS 1568 in plant tissues

During the field trials conducted for onion and fennel crops, plant samples belonging to germinated seeds treated with *Streptomyces* spp. DLS 1568 were collected at different phenological stages (*i.e*, seedlings, plantlets and plant) to detect and to confirm the presence of this microorganism inside the plant tissues. Plants germinated from untreated seeds were used as a control. Plant material was collected and subject to DNA extraction using the DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany), according to manufacturers' instructions. A 2 µl aliquot of DNA was then subjected to PCR assay using specific primers targeting the 16S rRNA gene of *Streptomycetes:* StrepB (forward) (5'-ACAAGCCCTGGAAACGGGT-3') and StrepF (reverse) (5'-ACGTGTGCAGCCCAAGACA-3') according to Rintala *et al.* (2001). The PCR was performed on a Thermal Cycler (Applied Biosystems 9700) with 25 µl reaction mixtures. After amplification, the samples were then run into a 2 % agarose gel, stained with ethidium bromide for 30 min and images were captured by BioDoc Analyze (Biometra, Göttingen, Germany).

3.11 Draft Genome Sequence of *Streptomyces* sp. DLS1568

The presence of putative metabolic pathways promoting plant growth and additional properties have been characterised for the *Streptomyces* sp. strain DLS1568 according to Vurukonda *et al.,* (2020). In detail, the *Streptomyces* sp. strain DLS1568 was subcultured three times on ISP-2 agar prior to DNA extraction. For genomic DNA extraction, single colonies of *Streptomyces* sp. strain DLS1568 were grown in tryptic soy broth (TSB) for 3 days at 28° C. Genomic DNA was extracted and purified using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) and its quantity and quality was checked using the NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific,Waltham, USA). DNA sequencing was performed using an Illumina HiSeq2000 sequencer. High-quality Illumina sequence libraries were prepared using the Nextera DNA Flex Library Prep Kit. Genome from pair-ended sequence reads has been done using the default parameters of the assembler module available in the Geneious prime software v 2021.2 (www.geneious.com) that includes quality control, trimming and assembly steps using default parameters. In order to identify genes involved in plant growth promotion, we constructed the *Steptomyces* DLS 1568 metabolic profile using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kaneisa *et al.,* 2000), thus providing evidence for the presence of genes involved in the pathway for indole alkaloid biosynthesis and in iron transport and metabolism, together with genes coding for proteins acting in the regulation of iron homeostasis.

3.12 Statistical analysis

All experiments were performed in a fully randomized block design. Data were subjected to one-way ANOVA, and the mean differences were determined by the Tukey/Tukey-Kramer test at a significance level of $p < 0.05$. All statistical analyses were performed using the software MaxStat Pro 3.6. Data from the experiment were expressed as mean ± SD (Standard Deviation).

4. Results

4.1 Antagonistic assay *in vitro*

The antagonistic activity of the following four strains: *Streptomyces* sp. DLS1568, *Streptomyces* sp. SA51, *Agrobacterium* sp. AR39, *Pseudomonas* sp. PT65, was evaluated through *in vitro* assays against seven pathogenic fungi: *F. oxysporum* f. sp. *cepae*, *F. oxysporum* f. sp. *raphani*, *Sclerotinia* sp., *Stemphylium* sp., *Colletotrichum dematium*, *Pythium* sp. (Fig.13).

The values of growth inhibition (GI in %) obtained from each microorganism is reported in the histograms represented in Figure 14.

All four microorganisms showed inhibitory action against the growth of phytopathogenic fungi. Nevertheless, the *Streptomyces* sp. DLS1568 was particularly active, giving an GI (%) higher than 55% during all *in vitro* assays for all seven pathogenic fungi (Fig.14). *Streptomyces* sp. DLS1568 highlighted the higher inhibitory activity against both *F. oxysporum* f. sp. *cepae*, *F. oxysporum* f. sp. *raphani* strains with an GI (%) up to 60% and 65%, respectively.

Pseudomonas sp. PT 65 and *Agrobacterium* sp. AR 39 showed a lower inhibitory activity (with an IG lower than 50%) against the seven pathogenic fungi mentioned above. *Streptomyces* sp. DLS1568 and *Streptomyces* sp. SA51 strains, which showed an GI higher than 50% against all the seven pathogenic fungi, were therefore further investigated for their plant growth promotion traits.

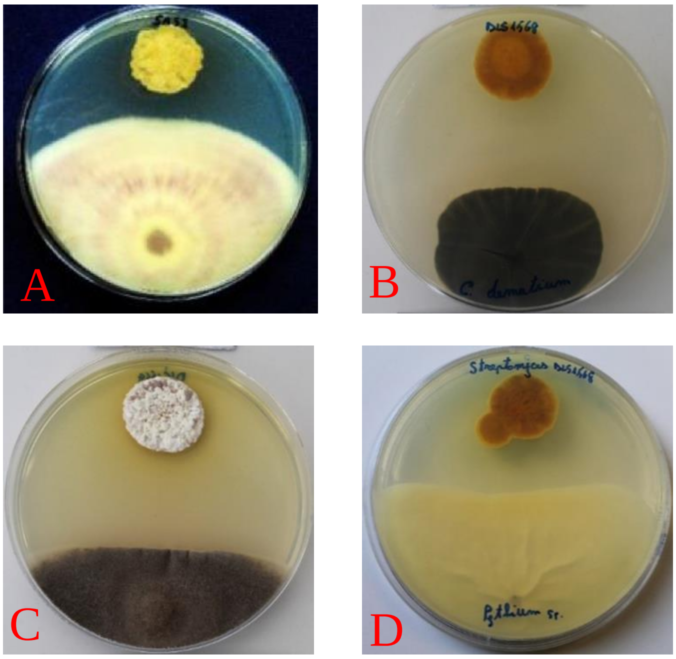

Figure 13 *In vitro antagonist activity of Streptomyces sp. DLS1568 against F. oxysporum f. sp. cepae (A), Stemphylium sp. (B), Colletotrichum dematium (C), Pythium sp. (D).*

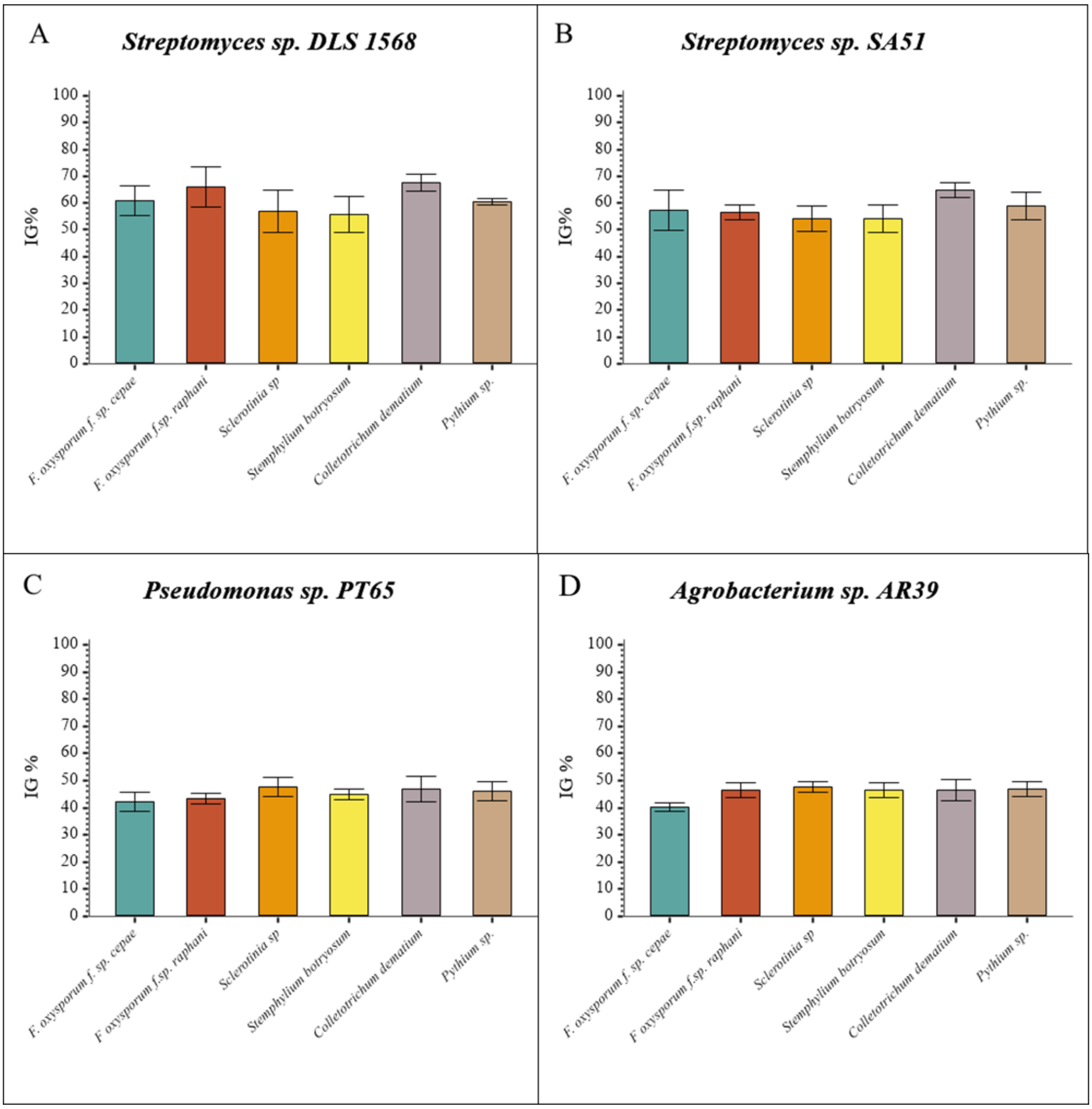

Figure 14 *Percentage of fungal inhibition (GI%) given by: A) Streptomyces sp. DLS1568; B) Streptomyces sp. SA51; C) Pseudomonas sp. PT 65 and D) Agrobacterium sp. AR 39, against phytopathogenic fungi on Potato Dextrose Agar. Bars indicate standard deviation of means (±SD).*

4.2 Plant growth promotion traits

Plant growth promotion traits by *Streptomyces* sp. DLS 1568 and *Streptomyces* sp. SA51 strains were both tested *in vitro* on agar plates for their possible siderophores production and phosphate solubilization activities.

4.2.1 Siderophore production

Microorganisms produce and secrete siderophores to sequester iron. Both *Streptomyces* sp. DLS 1568 and *Streptomyces* sp. SA51 isolates showed siderophore production, as highlighted by the change of colour in the CAS blue medium from bluish-green to orange (Fig.15). The difference in production of siderophores was evaluated by measuring the developed orange halo diameter: the *Streptomyces* sp. DLS 1568 recorded a higher siderophores production, displaying a mean value of 43 mm (SD = 5.00) compared to the mean value of 11 mm (SD = 5.29) of the *Streptomyces* sp. SA51. Tukey test highlighted a significant difference ($p < 0.001$) between the mean yellow halo zone values (mm) for the *Streptomyces* sp. DLS 1568, *Streptomyces* sp. SA51 isolates and the positive control (Fig.16).

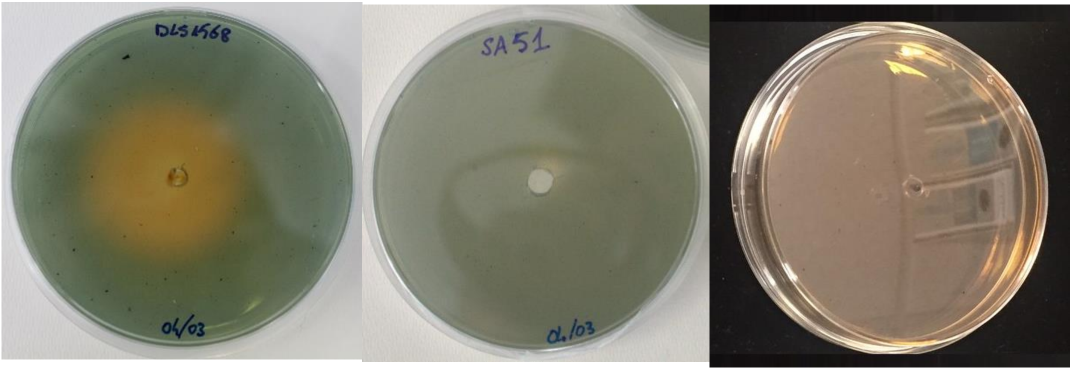
SA51

***Figure 15** Siderophore production for Streptomyces sp. DLS1568 and Streptomyces sp.*

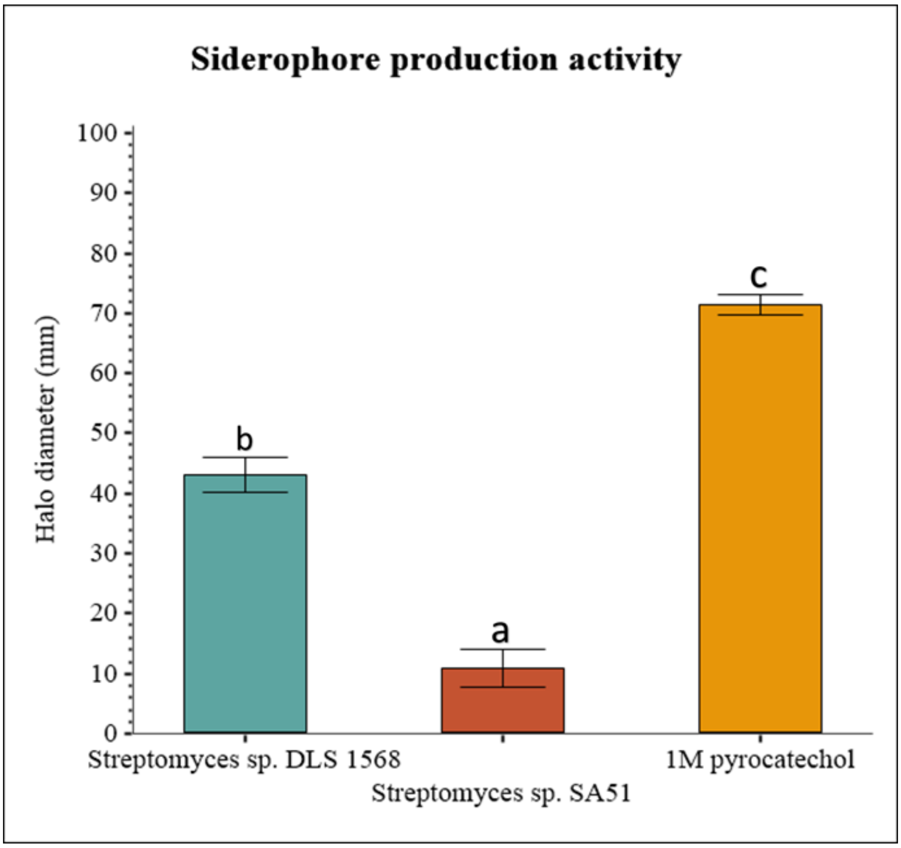

Figure 16 *Siderophore production activity by Streptomyces sp. DLS1568 and Streptomyces SA51 strains, 1 M pyrocatechol as positive control. Means with different letters indicate significant difference between two treatment groups according to One-way ANOVA, Tukey's Test, p < 0.001. Bars indicate standard deviation of means (±SD).*

4.2.2 Solubilization phosphate

We tested *Streptomyces* sp. DLS 1568 and *Streptomyces* sp. SA51 isolates for their phosphate solubilizing ability by detecting extracellular solubilization of precipitated tricalcium phosphate with glucose as sole source of carbon (Fig. 17). *Streptomyces* sp. DLS 1568 isolates showed notable phosphate solubilizing activity. Based on the solubilization zone, the *Streptomyces* sp. DLS 1568 recorded higher solubilization of mineral phosphate, displaying a mean value of 24.33 mm (SD = 3.05). *Streptomyces* sp. SA51 isolate also showed solubilization activity, displaying a mean value of 9.00 mm (SD = 2.00). Tukey test showed significant difference (p < 0.001) between the mean solubilization zone value (mm) belong to *Streptomyces* sp. DLS 1568 than the mean Ct values obtained for the *Streptomyces* sp. SA51 (Fig.18)

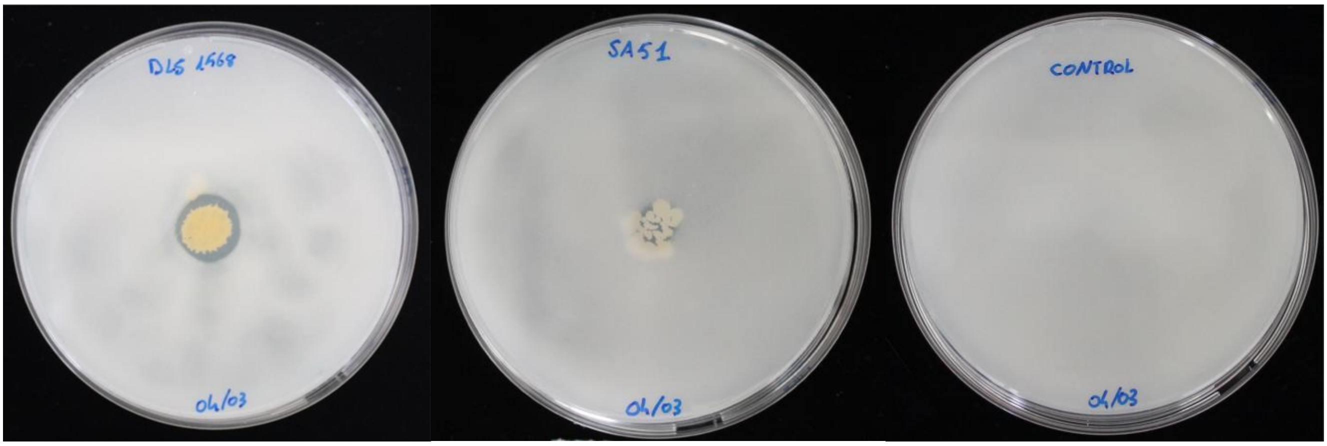

__Figure 17__ Solubilization phosphate activity for Streptomyces sp. DLS1568 and Streptomyces sp. SA51

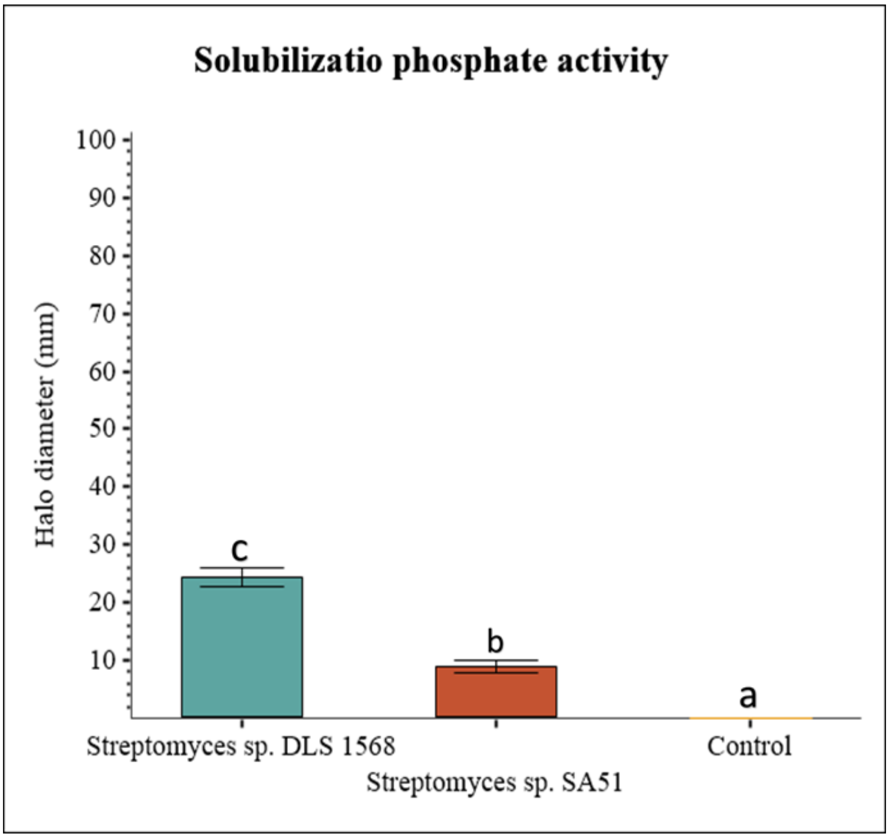

__Figure 18__ Solubilization phosphate activity for Streptomyces sp. DLS1568 and Streptomyces sp. SA51. H_2O was used as control. Means with the same letters are not significantly different (One-way ANOVA, Tukey's Test, $p > 0.05$). Means with different letters indicate significant difference between two treatment group according to One-way ANOVA, Tukey's Test ($p < 0.01$). Bars indicate standard deviation of means (±SD).

4.3 CLSM observations of in planta colonization by EGFP-*Streptomyces* sp. DLS 1568

Streptomyces DLS 1568 internalization was assessed by confocal laser scanning microscopy (CLSM) using different plant tissues such as root and leaf collected from 3-weeks-old onion, fennel, rocket, and lettuce plantlets. Endophytic colonization of plant tissues by EGFP-*Streptomyces* sp. DLS 1568 was observed in 99% of tissue sections from all samples of 3-week-old plantlets. The abundant colonization of young seedlings by EGFP-*Streptomyces* sp. DLS 1568 demonstrated *Streptomyces*' capability to interact with the host from early stages of seed germination and to root and leaf development. EGFP-*Streptomyces* sp. DLS 1568 cells showed an uniform distribution among all the various plant tissues (Fig. 19): these results were an indication that *Streptomyces* sp. DLS1568 has the ability to internalize in the plant, and indicating its potential for long-term interactions with all the four different crops considered in this study.

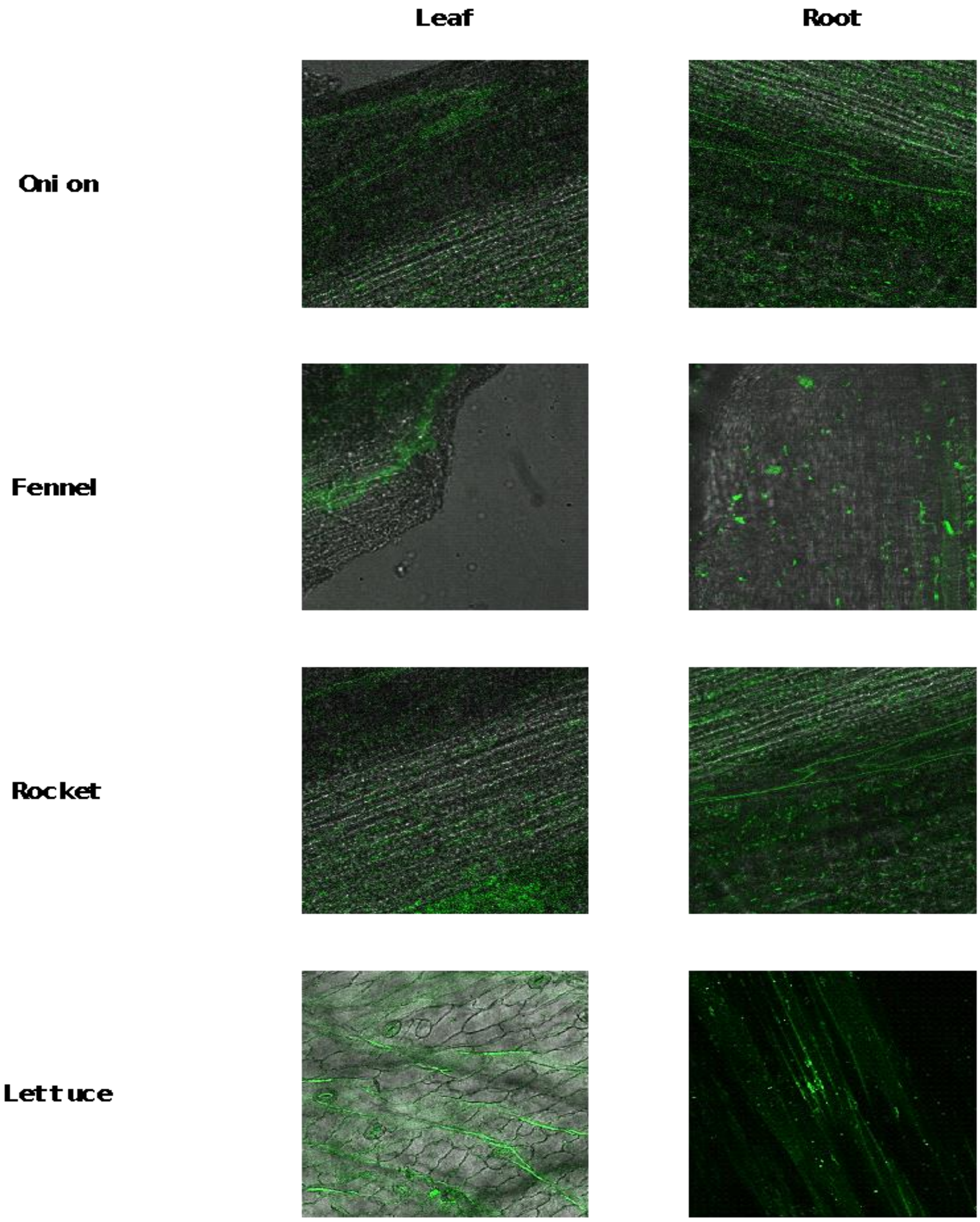

Figure 19 *CLSM observations of* leaf and *root colonization by EGFP-Streptomyces DLS 1568 three weeks after seed sowing for onion, fennel, wild rocket and lettuce plantlets.*

4.4 Assessment of *Streptomyces* sp. DLS 1568 loading on coated seed

Coating efficacy was determined by the CFU seed^{-1} entrapped on the seed surface previously disinfected according to Fatmi et al. (1991). As expected, the specialized polymer Bio Friendly 1 demonstrated high coating efficacy with higher number of bacterial colonies entrapped on seed surface: 2.15×10^3, 4.48×10^3, 1.93×10^2, 1.13×10^3 CFU seed $^{-1}$, for onion, fennel, wild rocket and lettuce seed, respectively, compared to the strategy according to Kaufman (1991) and the Xanthan gum biopolymer (Tab. 13). In particular, the Kaufman (1991) strategy reduced from 10 to 100 times the CFU seed $^{-1}$ concentration of entrapped *Streptomyces* DLS 1568 compared to the polymer Bio Friendly 1 for onion and fennel seed, and lettuce and wild rocket seed, respectively. Coating with 0.3 % Xanthan gum biopolymer, reduced of 1/3, 1/6, 1/2 and 1/3 the CFU seed $^{-1}$ concentration of entrapped *Streptomyces* sp. DLS 1568 compared to the polymer Bio Friendly 1 for onion, fennel, lettuce, and wild rocket seed, respectively. No *Streptomyces* spp. colonies were isolated from disinfected seed samples (control) for lettuce, onion, wild rocket and fennel seed samples, respectively.

Moreover, the treatment with the polymer Bio Friendly by visual inspection resulted in the formation of a smooth and thick layer of coating film covering uniformly the seed surface and requiring less working time with the industrial coating machine and with a short drying time with the drying machinery (Cora Seeds company; personal communication) as well.

Table 13 *Streptomyces DLS 1568 loading on coated seed (CFU seed^{-1}) according to the three different coating strategies.*

	Onion (CFU seed^{-1})	Fennel (CFU seed^{-1})	Wild rocket (CFU seed^{-1})	Lettuce (CFU seed^{-1})
1) Kaufman (1991)	1.66×10^2	2.48×10^2	6.09×10	8.56×10
2) Xanthan gum	5.97×10^2	7.08×10^2	1.05×10^2	4.05×10^2
3) Bio Friendly 1	2.15×10^3	4.48×10^3	1.93×10^2	1.13×10^3

The seed generated by the *Streptomyces* sp. DLS 1568 coating with the Bio Friendly 1 application, which resulted in the higher bacterial colony loading on seed, were stored under *vacuum* according to the Cora Seeds s.r.l. storage practice. After a storage time of 6, 12 and 16 months the bacterial population vitality was assessed. Coating with the Bio Friendly 1 application allowed the recovery of the CFU seed -1 previously reported for lettuce, onion, wild rocket and fennel coated seed samples, reflecting the high number of colonies successfully entrapped on seed surfaces at T0 (day of seed coating treatment). No significant decrease in *Streptomyces* sp. DLS 1568 vitality on seed surface was recorded up to 16 months, as shown in Table 14, for either onion, lettuce, fennel and wild rocket seed samples.

Table 14 *Streptomyces DLS 1568 loading on coated seed (CFU seed^{-1}) with the Bio Friendly 1 binder application at 6, 12 and 16 months after treatment for onion, fennel, lettuce and wild rocket crops*

Crops	***Treatment date***	***Assessment date***	***Time after seed coating treatment***	***CFU seed^{-1}***
Onion	12-09-2019	13-09-2019	T0	2.15×10^3
		09-04-2020	T6	1.46×10^3
		10-02-2021	T12	2.03×10^3
		13-08-2021	T16	1.89×10^3
Fennel	12-09-2019	13-09-2019	T0	4.48×10^3
		09-04-2020	T6	9.87×10^2
		10-02-2021	T12	2.99×10^3
		13-08-2021	T16	3.18×10^3
Lettuce	12-09-2019	13-09-2019	T0	1.13×10^3
		09-04-2020	T6	9.65×10^2
		10-02-2021	T12	2.21×10^3
		13-08-2021	T16	1.96×10^3
Wild Rocket	12-09-2019	13-09-2019	T0	1.93×10^2
		09-04-2020	T6	2.14×10^2
		10-02-2021	T12	3.41×10^2
		13-08-2021	T16	1.77×10^2

4.5 Germination test assay

As a result of the germination experiments performed within the scope of the study, the effects of the applications of seed coating with the *Streptomyces* sp. DLS 1568 and ozone treatment on seed were statistically evaluated, and the results obtained are described below.

4.5.1 Germination data of *Streptomyce*s spp. coated seed

The germination data obtained from treated seed with the *Streptomyces* sp. DLS1568 and *Streptomyces* sp. SA51 showed an improvement of the seed germination performance. The germination of onion seeds was positively influenced by *Streptomyces* spp. treatment (Fig. 19 A); the germination rate assessed for the uncoated seed control (negative control) was 65%, while *Streptomyces* sp. treatment increased the germination rate significantly up to 20% ($p < 0.05$). Similar results were obtained for the fennel germination test. Figure 19 B showed that treatments with *Streptomyces* sp. were increased the germination percentage up to 30% compared to uncoated control. Moreover, the *Streptomyces* sp. DLS1568 showed a higher percentage of germinated seeds compared to the *Streptomyces* sp. SA51, although the difference is not significant ($p > 0.05$).

On the other hand, the germination performances recorded for wild rocket and lettuce coated seed samples (Fig.19 C and D) showed a slight increase in the germination rate. No significant differences ($p>0.05$) between wild rocket and lettuce coated seed with both *Streptomyces* sp. to uncoated controls were highlighted.

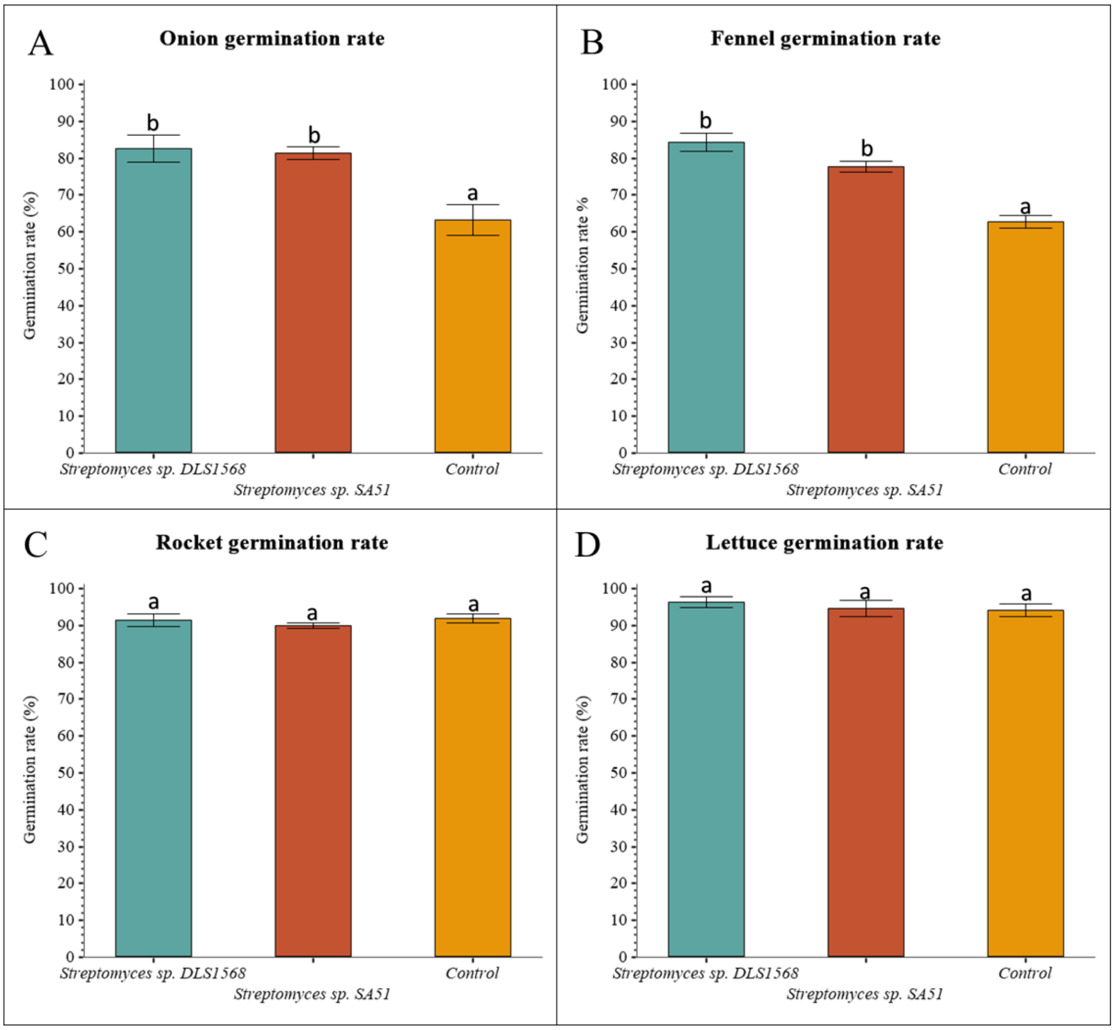

__Figure 19__ Germination results for: A) Onion; B) Fennel; C) Wild rocket and D) Lettuce seed samples. Means with the same letters are not significantly different (One-way ANOVA, Tukey's Test, $p > 0.05$). Means with different letters indicate significant difference between two treatment groups according to One-way ANOVA, Tukey's Test ($p < 0.01$). Bars indicate standard deviation of means (±SD).

4.5.2 Germination data of ozone treated seed

The effect of the 4 ozone treatment timings on seed germination displayed different impacts on the seed germination performances. For onion seed samples, the T3 and T4 displayed a significant increase ($p < 0.05$) in the final germination rate compared to untreated control. No significant differences ($p > 0.05$) were recorded for T1 and T2 ozone treatments compared to untreated onion seed samples (Fig 20 A). By the comparison of fennel seed samples treated with ozone, the T3 and T4 timing treatments showed a significant ($p < 0.05$) increase of 8% and 6% compared to the fennel untreated control (T0), respectively, (Fig 20 B). For wild rocket seed samples, the T2, T3 and T4 timing treatments displayed a significant increase ($p < 0.05$) in the final germination rate of 5.33%, 11.33% and 12%, respectively, compared to untreated control (T0). No significant differences ($p > 0.05$) for the germination performance were recorded for T1 ozone treatment compared to untreated wild rocket seed samples (T0) (Fig.20 C). For lettuce seed samples, the T3 and T4 timing treatments displayed a significant increase ($p < 0.05$) in the final germination rate of 9.33% and 12%, respectively, compared to untreated control (T0) (Fig.20 D). No significant differences ($p > 0.05$) were recorded for T1 ozone treatment compared to untreated wild rocket seed samples.

The differences in the germination performance between untreated and treated onion, fennel, wild rocket and lettuce seed have been recorded as the increasing into the seed samples with seedlings with all their essential structures were well developed, complete, in proportion and healthy with cotyledons and radicles, according to the Handbook on Seedling Evaluation (International Seed Testing Association, 2018) compared to ungerminated or abnormal seedling, which were not registered in the final germination rate.

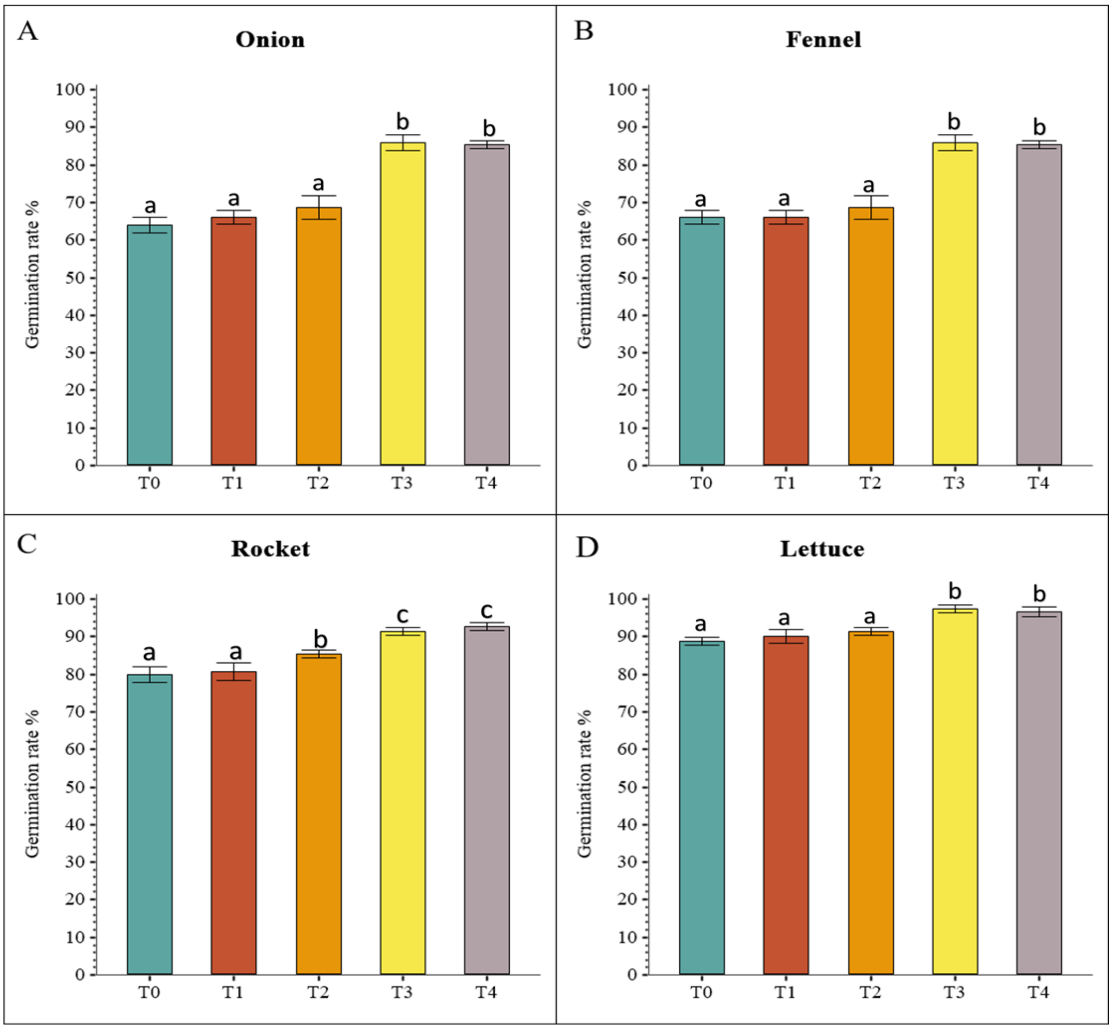

Figure 20 *Effect of ozone seed treatment, at different treatment timing: T0 = Control, T1= 15 min. T2= 30 min., T3= 45 min., T4= 60 min. on germination for A) Onion; B) Fennel; C) Wild rocket and D) Lettuce seed samples. Means with the same letters are not significantly different (One-way ANOVA, Tukey's Test, $p > 0.05$). Means with different letters indicate significant difference between two treatment groups according to One-way ANOVA, Tukey's Test ($p < 0.05$). Bars indicate standard deviation of means (±SD).*

4.5.3 Microbial seed disinfection activity of ozone seed treatment

The effect of the 4 ozone treatment timings on seeds for disinfection showed a different impact on the microbial load of the seed. For onion seed samples, the T3 and T4 treatment, respectively 45 min and 60 min, showed a significant (p <0.05) reduction of the microbial load compared to the control. In particular, T3 showed a significant (p <0.05) reduction of 19% compared to control, but no significant difference with T1 and T3. While T4 showed a significant (p <0.05) reduction of 29%, 26%, and 13% compared to control (T0), T1 and T3 (Fig 21 A). For fennel seed samples, treatment T1, T2 displayed no significant differences (p>0,05) for the reduction of microbial load in seed compared to control. Conversely, T3 and T4 showed a significant (p<0,05) difference compared to control of 22.34% and 26.75% respectively. In addition, T3 and T4 showed no significant (p>0.05) difference between them (Fig. 21 B). For wild rocket seed samples, all treatments T1, T2, T3 and T4 displayed a significant (p<0.05) difference with control. In particular, there was recorded a reduction from 9,5% to 16,40% of microbial load on seed, but no significant (p>0,005) difference was found between T1, T2, T3 and T4 (Fig. 21 C). For lettuce seed samples, the T1, T2, T3 and T4 timing treatments displayed a significant (p < 0.05) reduction of 14,64% for microbial load, compared to control (T0). According to the data reported, the ozone seed treatment T3 was selected for application in all seed treatment in the further experimental field trials.

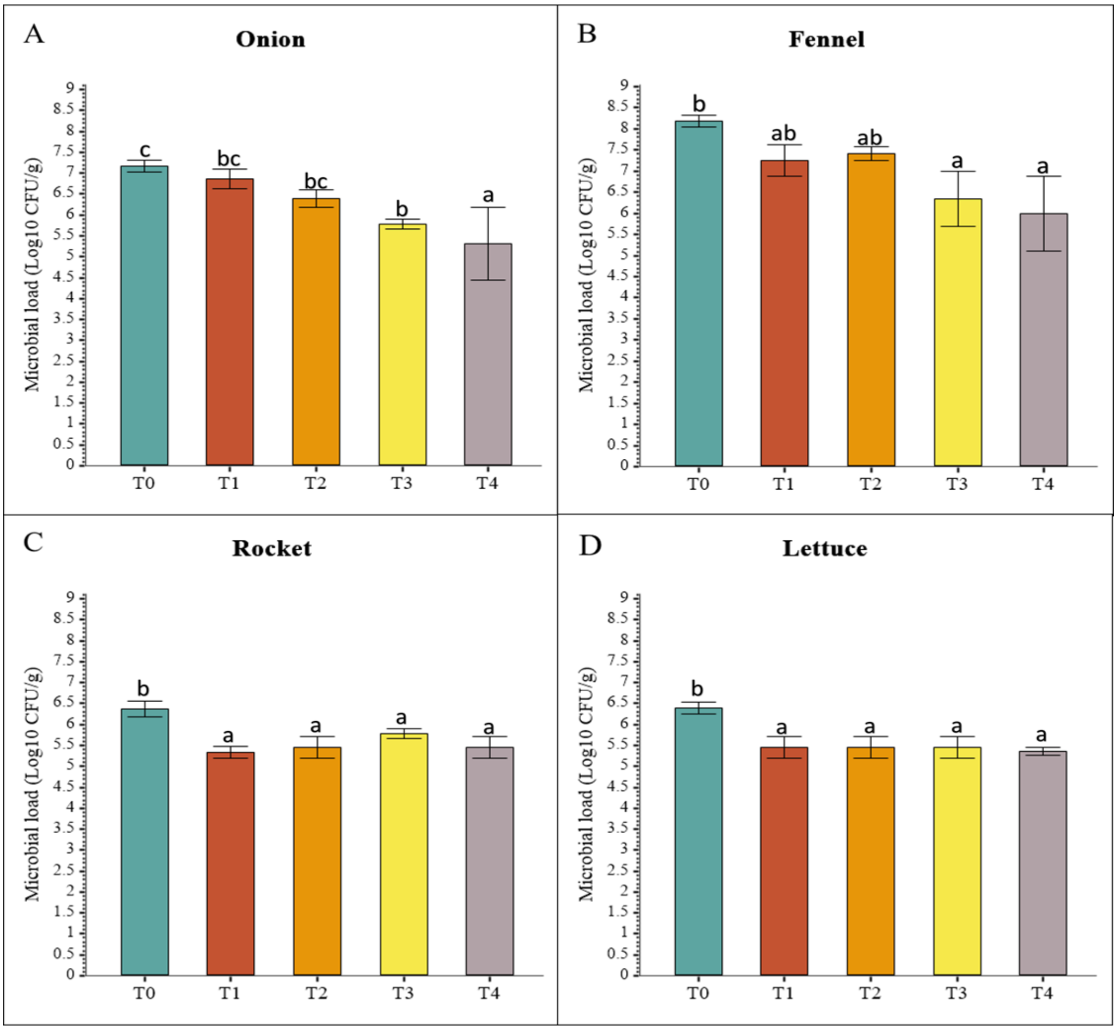

***Figure21** Effect the ozone treatment disinfection on seed surface microbial load for: A) Onion; B) Fennel; C) Rocket and D) Lettuce seed samples for the 4 different exposure timing: T0 = Control, T1= 15 min. T2= 30 min, T3= 45 min, T4= 60 min. Means with the same letters are not significantly different (One-way ANOVA, Tukey's Test, $p > 0.05$). Means with different letters indicate significant difference between two treatment groups according to One-way ANOVA, Tukey's Test ($p < 0.05$). Bars indicate standard deviation of means (±SD).*

4.6 Efficacy of microbial seed coating on wild rocket against *Fusarium oxysporum* f. sp. *raphani* during *in planta* trials

The assay highlighted that the seedlings germinated from seeds coated with both *Streptomyces* spp. significantly ($p < 0.05$) reduced the infection by the pathogenic Fus Ruc 9A strain compared to the uncoated seed sample (Control).

Inoculation of wild rocket seedlings without any biological control treatments(control) showed a relative disease index rating of 36.25%. Different levels of protection against pathogens were observed by treatment with *Streptomyces* sp. strains. Both seed coating treatments with the *Streptomyces* sp. DLS 1568 and *Streptomyces* sp SA 51 strains showed significant protection ($p< 0.05$) against Fusarium wilt. The seed coated with *Streptomyces* sp. DLS 1568 strain exhibited the greatest biocontrol activity/efficacy, showing a relative disease index rating of 16.50% compared to seed coated with *Streptomyces* sp. SA 51, which highlighted a relative disease index rating of 20.75% (Fig. 23).

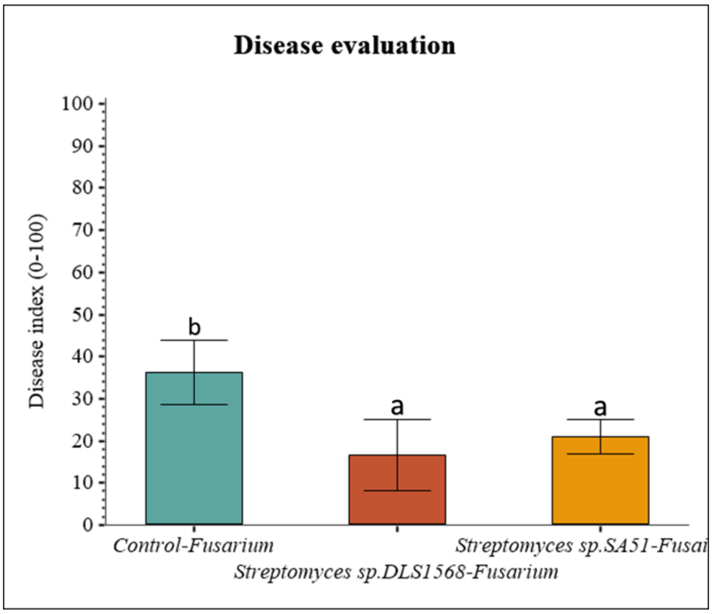

Figure 23 *Disease index of plants emerged by seed coating with Streptomyces sp. DLS1568 and Streptomyces sp. SA51 compared to untreated seed control after 20 day inoculation with the Fus Ruc 9A strain. Data are reported as means ±SD. Means with the same letters are not significantly different (One-way ANOVA, Tukey's Test, $p > 0.05$). Means with different letters indicate significant difference between two treatment group according to One-way ANOVA, Tukey's Test ($p < 0.05$). Bars indicate standard deviation of means (±SD).*

4.7 Field trials

4.7.1 Experimental onion field trials

4.7.1.1 Onion var. Elenka cropping season 2020 in experimental field located in Cesena ('Minotti'; FC)

During the first assessment, the number of emerged seedlings was assessed according to the procedure described in section 3.9.2. Seeds treated with *Streptomyces* sp. DLS 1568 and Ozone (T3) seeds showed an increase in emergence of 15% and 17% compared to the control, respectively. Seeds treated with *Streptomyces* sp. DLS 1568 and Ozone (T3) seeds showed an increase in seedling emergence of 20% and 22% compared to Fludioxonil treatment. Seeds treated with *Streptomyces* sp. DLS 1568 and Ozone (T3) seeds highlighted an increase of 24% and 26% by comparison to *Streptomyces* sp. SA51 seed treatment, respectively. However, statistical analysis with Tukey test did not show a significant difference ($p > 0.05$).

During the second assessment (42 days after sowing), the number of emerged plants from seeds treated with Ozone (207.88; SD=35.62) was significantly higher ($p < 0.05$) by 41% and 51% than those treated with Fludioxonil (147.00; SD = 41.52) and *Streptomyces* sp. SA51(137.71; SD =1 8.25), respectively. However, no significant difference was observed compared to the control (170.00; SD=38.99). On the contrary, the number of plants emerged from seeds treated with *Streptomyces* sp. DLS 1568 (198.13; SD=43.53) was significantly higher by 44% ($p < 0.05$) than emerging plantlets belonging to the treatment with *Streptomyces* sp. SA51. No significant differences ($p>0.05$) were recorded by the comparison of *Streptomyces* sp. DLS 1568 treatment to Control and Fludioxonil thesis, respectively.

During the third assessment (70 days after sowing), a similar behaviour was found in the various treatments mentioned above. Overall, during the fourth assessment (97 days after sowing), the data for the number of plants showed that in both *Streptomyces* sp. DLS 1558 (137.40; SD=16.41) and Ozone (119.17; SD=19.24) treatments the highest number of plants over time were recorded, compared to Fludioxonil (72.75; SD=8.10), *Streptomyces* sp. SA51 (87.17; SD=10.57) treatments, respectively. All data recorded are listed in Table 15.

Table 15 *Number of emerged plants during the cropping season 2020 for onion var. Elenka is an experimental field located in Cesena ('Minotti'; FC). Data are reported as means ±SD. Means with the same letters are not significantly different (One-way ANOVA, Tukey's Test, $p > 0.05$). Means with different letters indicate significant difference between two treatment groups according to One-way ANOVA, Tukey's Test ($p < 0.05$).*

Elenka 2020

Treatments	04/05/2020		28/05/2020		24/06/2020		22/07/2020	
	Avg	SD	Avg	SD	Avg	SD	Avg	SD
Control	182.50	±26.22 ^{a}	170.00	±38.99 ^{abc}	130.67	±38.74 ^{ab}	101.60	±29.48 ^{ab}
Fludioxonil	175.63	±32.70 ^{a}	147.00	±41.52 ^{ab}	119.50	±11.73 ^{ab}	72.75	±8.10 ^{a}
***Streptomyces* sp. SA51**	169.13	±25.82 ^{a}	137.71	±18.25 ^{a}	113.40	±14.21 ^{a}	87.17	±10.57 ^{a}
Streptomyces* sp. *DLS1568	210.75	±36.61 ^{a}	198.13	±43.53 ^{bc}	166.50	±29.03 ^{b}	137.40	±16.41 ^{c}
Ozone	214.63	±53.24 ^{a}	207.88	±35.62 ^{c}	161.00	±17.53 ^{ab}	119.17	±13.24 ^{bc}

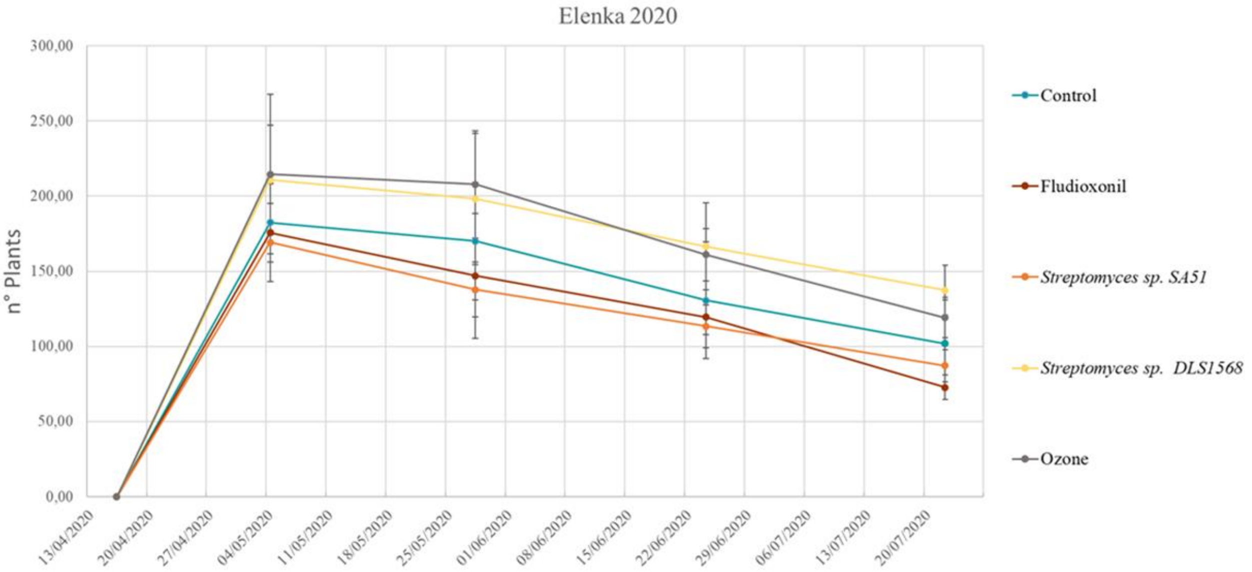

Figure 25 *Number of emerged plants during the cropping season 2020 for onion var. Elenka is an experimental field located in Cesena ('Minotti'; FC).*

4.7.1.2 Effects of different treatments on the onion plant phenological development and bulb weight at harvest time during the cropping season 2020 for onion var. Elenka

The plant height was evaluated during the second assessment. As shown in Figure 26A, only the treatment with *Streptomyces* DLS 1568 displayed a significant increase in the plant height (12.23 cm; SD = 0.9) when compared to Fludioxonil (10.11 cm; SD = 0.7; $p<0.05$). No significant difference was observed between samples previously treated with *Streptomyces* DLS 1568 and Ozone ($p > 0.05$). During the third assessment, the number of leaves per plant was counted. The mean number of leaves per plant developing from the seeds treated with *Streptomyces* DLS 1568 and Ozone was 5.23 (SD = 0.2) and 4.69 (SD = 0.6), respectively (Fig. 26 B). However, by statistical analysis, no significant difference ($p > 0.05$) was displayed between all the 5 treatments.

During the fourth assessment, the onion neck diameter (cm) was measured. Statistical analysis did not highlight significant differences between the different treatments ($p> 0.05$), as shown in Figure 26 C. At this phenological stage, the mean diameter value measured was in between 6-7.5 mm for all the theses. Finally, the weight of the onion bulbs was assessed at harvest time (at 120 days after sowing). Statistical analysis did not show significant differences among all the treatments ($p> 0.05$) for the mean weight of onion bulb (Fig. 26 D).

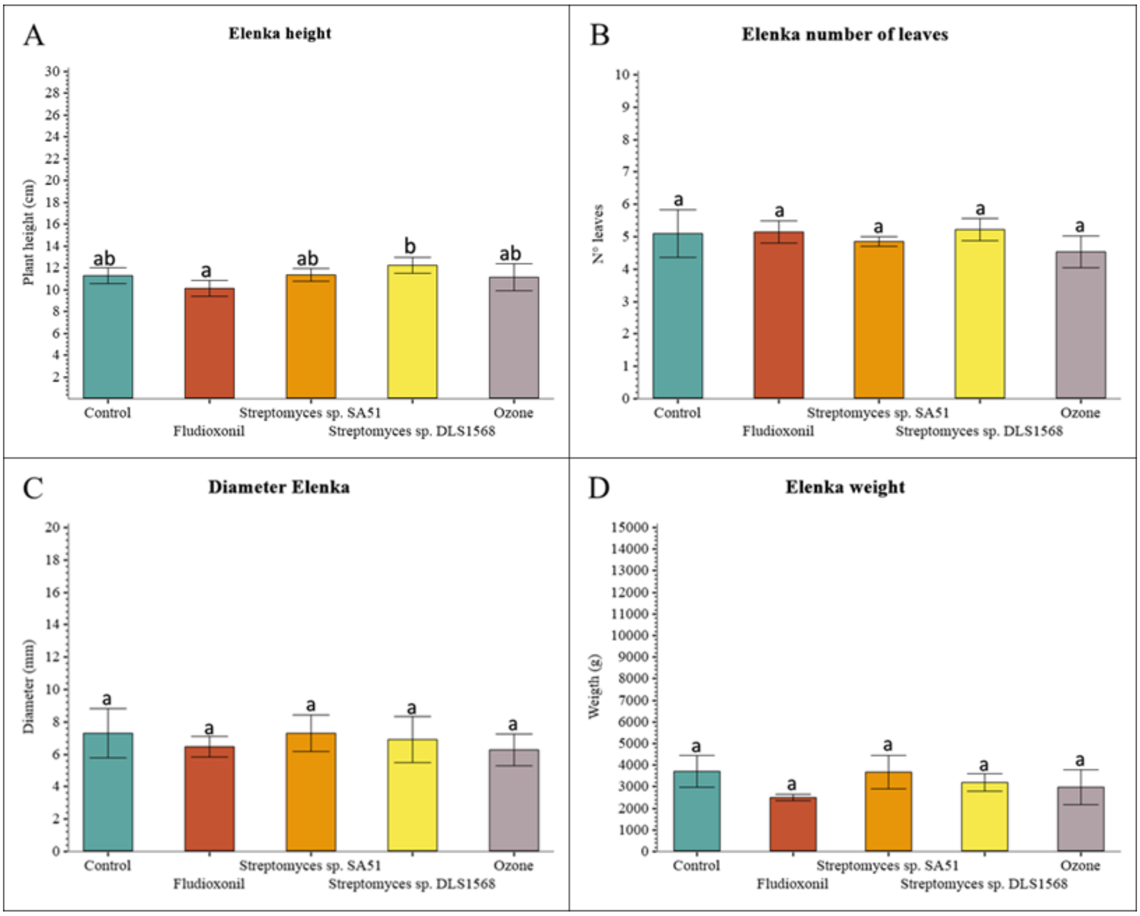

Figure 26 *Different phenological parameters assessed during the cropping season 2020 for onion var. Elenka experimental field located in Cesena ('Minotti'; FC): A) plant height (cm) B) number of leaves C) neck diameter, and D) mean b onion bulb weight. Data are reported as means ±SD. Means with the same letters are not significantly different (One-way ANOVA, Tukey's Test, $p > 0.05$). Means with different letters indicate significant difference between two treatment groups according to One-way ANOVA, Tukey's Test ($p < 0.05$).*

4.7.2 Onion var. Elenka cropping season 2021

During the first assessment, seeds treated with *Streptomyces* sp. DLS 1568 showed no significant difference ($p > 0.05$) in the number of emerged seedlings compared to other treatments. Seeds treated with Ozone displayed a significant increase ($p < 0.05$) in the number of emerged seedlings of 7%, 13%, 20% compared to the Fludioxonil, *Streptomyces* sp. SA51 and *Streptomyces* sp. DLS 1568, respectively (Tab. 16 Fig. 27). During the following 3 assessments, no significant differences were observed ($p > 0.05$) in the number of emerged plants among all treatments.

Table 16 *Number of emerged plants during the cropping season 2021 for onion var. Elenka experimental field located in Cesena ('Minotti'; FC). Data are reported as means ±SD. Means with the same letters are not significantly different (One-way ANOVA, Tukey's Test, $p > 0.05$). Means with different letters indicate significant difference between two treatment group according to One-way ANOVA, Tukey's Test ($p < 0.05$).*

Elenka 2021

Treatments	08/04/2021		06/05/2021		01/06/2021		29/07/2021	
	Avg	SD	Avg	SD	Avg	SD	Avg	SD
Control	180.63	±3.36 ^{bc}	174.00	±8.49 ^{a}	173.38	±2.65 ^{a}	160.75	±14.50 ^{a}
Fludioxonil	178.50	±2.47 ^{b}	168.88	±13.26 ^{a}	170.75	±10.96 ^{a}	176.63	±5.83 ^{a}
***Streptomyces* sp. SA51**	170.00	±1.41 ^{ab}	147.17	±8.24 ^{a}	178.13	±3.36 ^{a}	164.50	±12.73 ^{a}
Streptomyces* sp. *DLS1568	159.25	±4.24 ^{a}	152.50	±0.71 ^{a}	147.88	±11.14 ^{a}	139.88	±10.43 ^{a}
Ozone	191.29	±1.47 ^{c}	169.88	±6.89 ^{a}	174.63	±6.54 ^{a}	162.75	±9.19 ^{a}

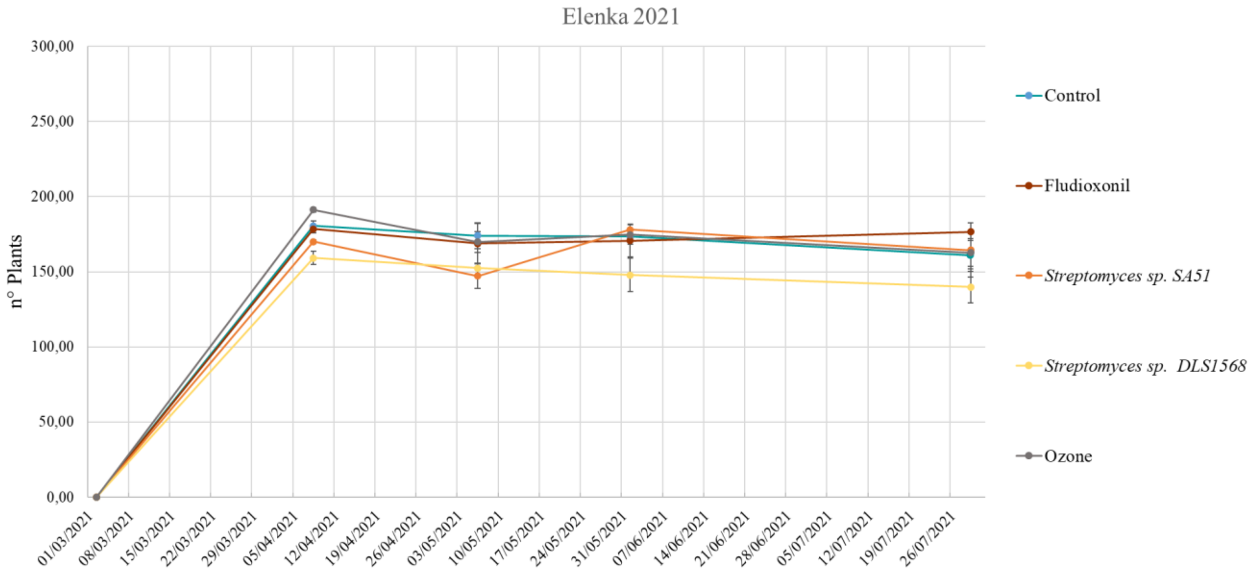

Figure 27 *Number of emerged plants during the cropping season 2021 for onion var. Elenka experimental field located in Cesena ('Minotti'; FC).*

4.7.2.1 Effects of different treatments on the onion plant phenological development during the cropping season 2021 for onion var. Elenka

As shown in Figure 28 A, during the second assessment the plants germinated from seeds treated with Ozone and with *Streptomyces* SA51 displayed a significant increase in the plant height (12.23 cm; SD = 0.9 and 12) compared to *Streptomyces* sp. DLS 1568 (10.11 cm; SD = 0.7; p<0.05). During the third and fourth assessment, the mean number of leaves counted in plants emerged from seeds treated with *Streptomyces* DLS 1568 and Ozone showed no significant difference (p> 0.05) by the comparison with the control and the other treatments (Fig. 28 B; Fig. 28 C). During the cropping season 2021 the assessment of the mean bulb weight was not conducted because of the high presence of weeds, and which uncontrolled growing compromised the correct bulb development.

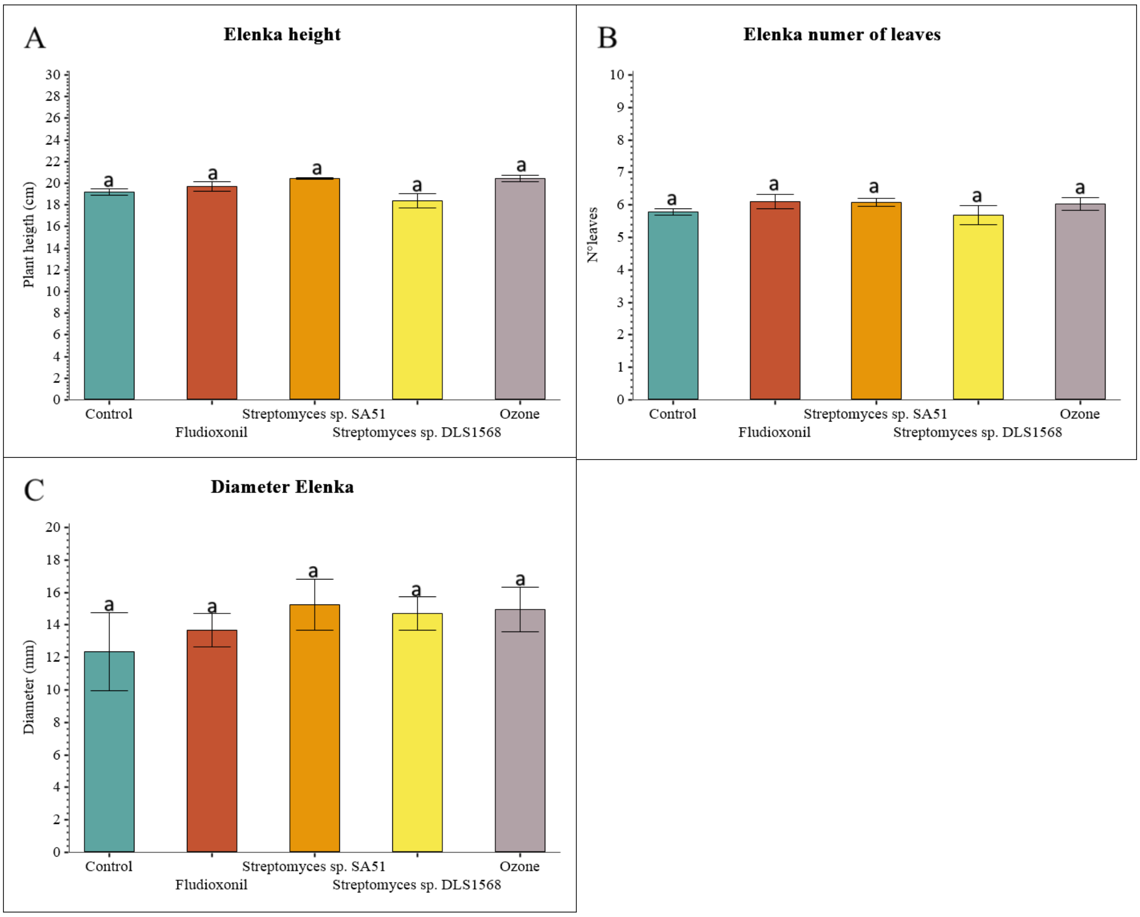

Figure 28 *Different phenological parameters assessed during the cropping season 2021 for onion var. Elenka in an experimental field located in Cesena ('Minotti'; FC): A) plant height (cm) B) number of leaves C) neck diameter. Data are reported as means ±SD. Means with the same letters are not significantly different (One-way ANOVA, Tukey's Test, p > 0.05). Means with different letters indicate significant difference between two treatment groups according to One-way ANOVA, Tukey's Test (p < 0.05).*

4.7.2.2 Onion var. Fundador in the cropping season 2020 in an experimental field located in Cesena (‘Minotti’; FC)

During the first assessment, seeds treated with *Streptomyces* sp. DLS 1568 showed a significant ($p < 0.05$) increase in emergence of 20%, 51% and 15% compared to the Control, *Streptomyces* sp. SA51 and Ozone, respectively. No significant difference ($p > 0.05$) was observed between *Streptomyces* sp. DLS 1568 and Fludioxonil thesis (Tab.17; Fig. 29). During the second and the third assessment, the number of emerged plants germinated from seeds previously treated with Ozone and *Streptomyces* sp. DLS 1568 displayed no significant differences ($p > 0.05$) compared to other treatments. On the contrary, during the fourth assessment, the number of plants emerged from seeds treated with *Streptomyces* sp. DLS 1568 (154.57; SD=16.38) was significantly higher ($p < 0.05$) than those emerging from treatment with *Streptomyces* sp. SA51 (103.50; SD=35.61).

Table 17 *Number of emerged plants during the cropping season 2020 for onion var. Fundador located in Cesena (‘Minotti’; FC). Data are reported as means ±SD. Means with the same letters are not significantly different (One-way ANOVA, Tukey’s Test, $p > 0.05$). Means with different letters indicate significant difference between two treatment group according to One-way ANOVA, Tukey’s Test ($p < 0.05$).*

Fundador 2020

Treatments	04/05/2020		28/05/2020		24/06/2020		22/07/2020	
	Avg	SD	Avg	SD	Avg	SD	Avg	SD
Control	178.12	±30.92 ^{ab}	205.50	±27.63 ^{a}	188.83	±18.28 ^{b}	152.33	±18.44 ^{ab}
Fludioxonil	210.12	±57.65 ^{b}	207.87	±52.91 ^{a}	180.80	±31.97 ^{b}	157.80	±39.48 ^{ab}
***Streptomyces* sp. SA51**	141.12	±35.71 ^{a}	141.75	±52.48 ^{a}	124.50	±30.85 ^{a}	103.50	±35.61 ^{a}
Streptomyces* sp. *DLS1568	212.87	±33.99 ^{b}	202.50	±29.94 ^{a}	168.60	±24.46 ^{ab}	154.57	±26.38 ^{b}
Ozone	185.87	±33.24 ^{ab}	201.12	±50.65 ^{a}	161.20	±22.38 ^{ab}	130.40	±32.35 ^{ab}

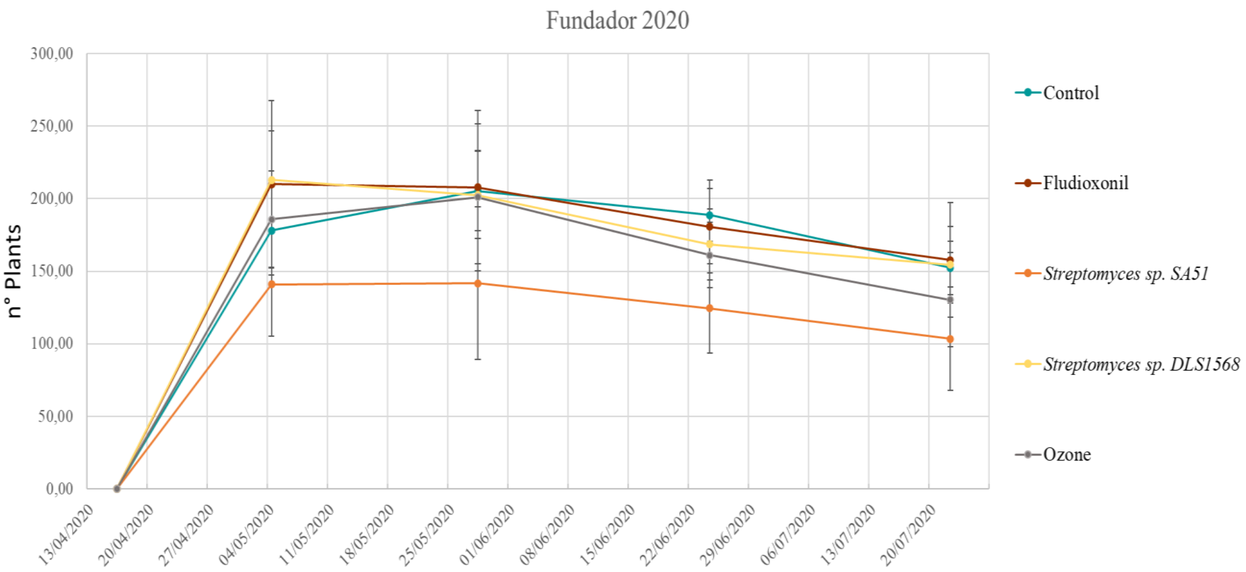

Figure 29 *Number of emerged plants during the cropping season 2020 for onion var. Fundador in experimental field located in Cesena ('Minotti';FC)*

4.7.2.3 Effects of different treatments on the onion plant phenological development and bulb weight at harvest time during the cropping season 2020 for onion var. Fundador

The collected and statistically analysed data by one-way ANOVA with Tukey's test during all the five assessments did not show significant difference ($p > 0.05$) between all the different treatment theses (Fig. 30 A-D).

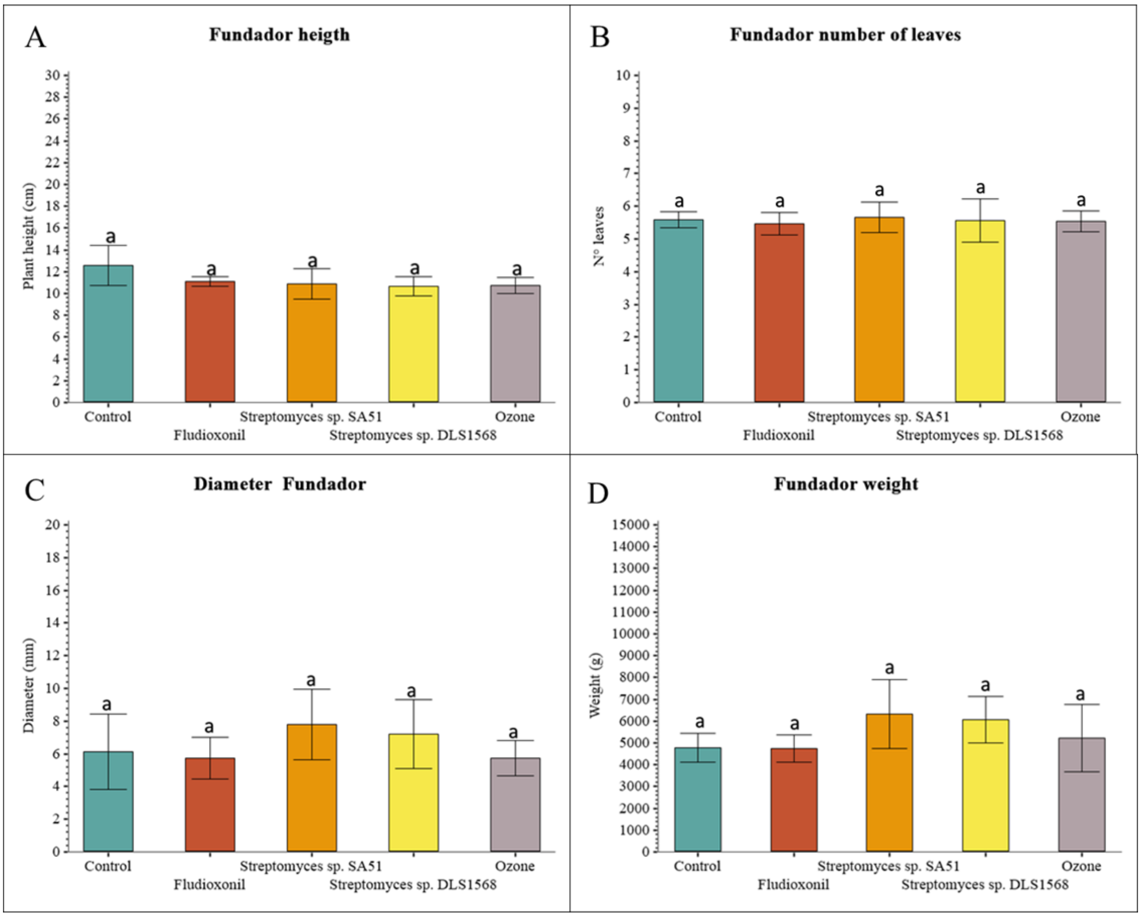

__Figure 30__ Different phenological parameters assessed during the cropping season 2020 for onion var. Fundador in an experimental field located in Cesena ('Minotti'; FC): A) plant height (cm) B) number of leaves C) neck diameter. Data are reported as means ±SD. Means with the same letters are not significantly different (One-way ANOVA, Tukey's Test, $p > 0.05$). Means with different letters indicate significant difference between two treatment groups according to One-way ANOVA, Tukey's Test ($p < 0.05$).

4.7.2.4 Onion var. Fundador cropping season 2021 in an experimental field located in Cesena ('Minotti'; FC)

During the second assessment, the collected and statistically data analysed by one-way ANOVA with Tukey's test showed significant difference ($P < 0.05$) between seeds treated with *Streptomyces* sp. DLS 1568 and Ozone treated seeds with an emergence increase of 16% and 20% compared to the Control, respectively (Tab. 17). No significant difference ($p > 0.05$) with *Streptomyces* sp. SA 51 and Fludioxonil treatments. During the third and fourth assessment, the collected and statistically analysed data by one-way ANOVA with Tukey's test did not show significant difference ($P > 0.05$) between all seed treatments, as shown in Table 17 and in Figure 31.

Table 17 *Number of emerged plants during the cropping season 2021 for onion var. Fundador. Data are reported as means ±SD. Means with the same letters are not significantly different (One-way ANOVA, Tukey's Test, $p > 0.05$). Means with different letters indicate significant difference between two treatment groups according to One-way ANOVA, Tukey's Test ($p < 0.05$).*

Fundador 2021

Treatments	08/04/2021		06/05/2021		01/06/2021		29/07/2021	
	Avg	SD	Avg	SD	Avg	SD	Avg	SD
Control	189.88	±8.66 ^{a}	185.38	±7.95 ^{a}	182.38	±10.78 ^{a}	189.50	±14.14 ^{a}
Fludioxonil	197.38	±3.36 ^{ab}	184.88	±3.01 ^{a}	181.75	±5.30 ^{a}	199.00	±17.68 ^{a}
***Streptomyces* sp. SA51**	215.75	±2.12 ^{bc}	162.88	±1.94 ^{a}	162.25	±3.54 ^{a}	160.63	±2.65 ^{a}
Streptomyces* sp. *DLS1568	219.38	±6.89 ^{bc}	210.00	±12.73 ^{a}	168.63	±14.67 ^{a}	177.88	±1.24 ^{a}
Ozone	228.25	±4.24 ^{c}	186.50	±22.27 ^{a}	183.50	±5.66 ^{a}	196.25	±18.38 ^{a}

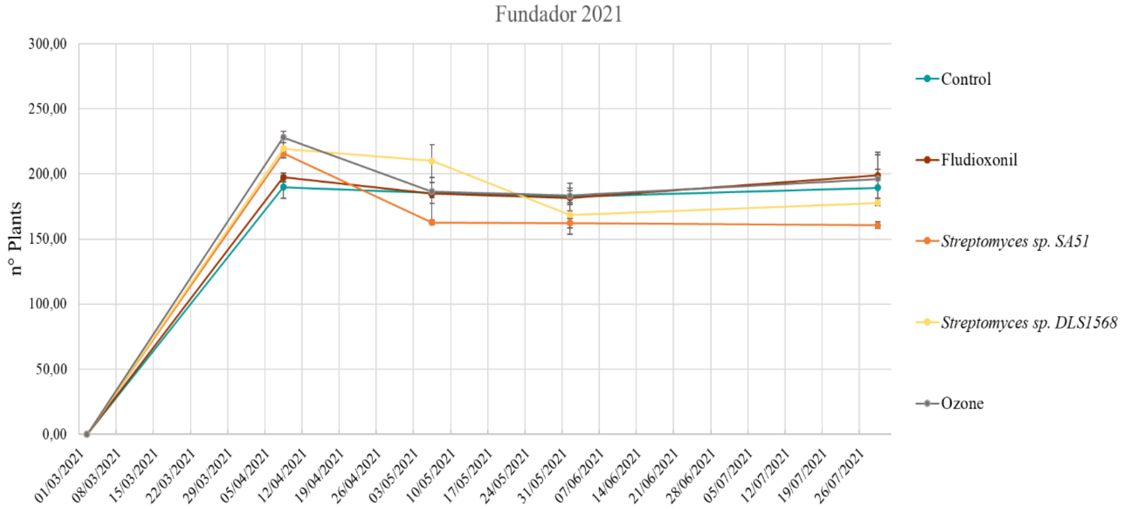

***Figure 31** Number of emerged plants during the cropping season 2021 for onion var. Fundador in experimental field located in Cesena (FC)*

4.7.2.5 Effects of different treatments on the onion plant phenological development during the cropping season 2021 for onion var. Fundador

The collected and statistically analysed data by one-way ANOVA with Tukey’s test during the second (mean plant hight), the third (mean number of leaves) and the fourth assessment (mean onion bulb diameter) did not show significant difference (*P* > 0.05) between all seed treatments (Fig. 32B and Fig. 32 C). During the cropping season 2021 the assessment of the mean bulb weight was not conducted because of the high presence of weeds, whose uncontrolled growing compromised the correct bulb development.

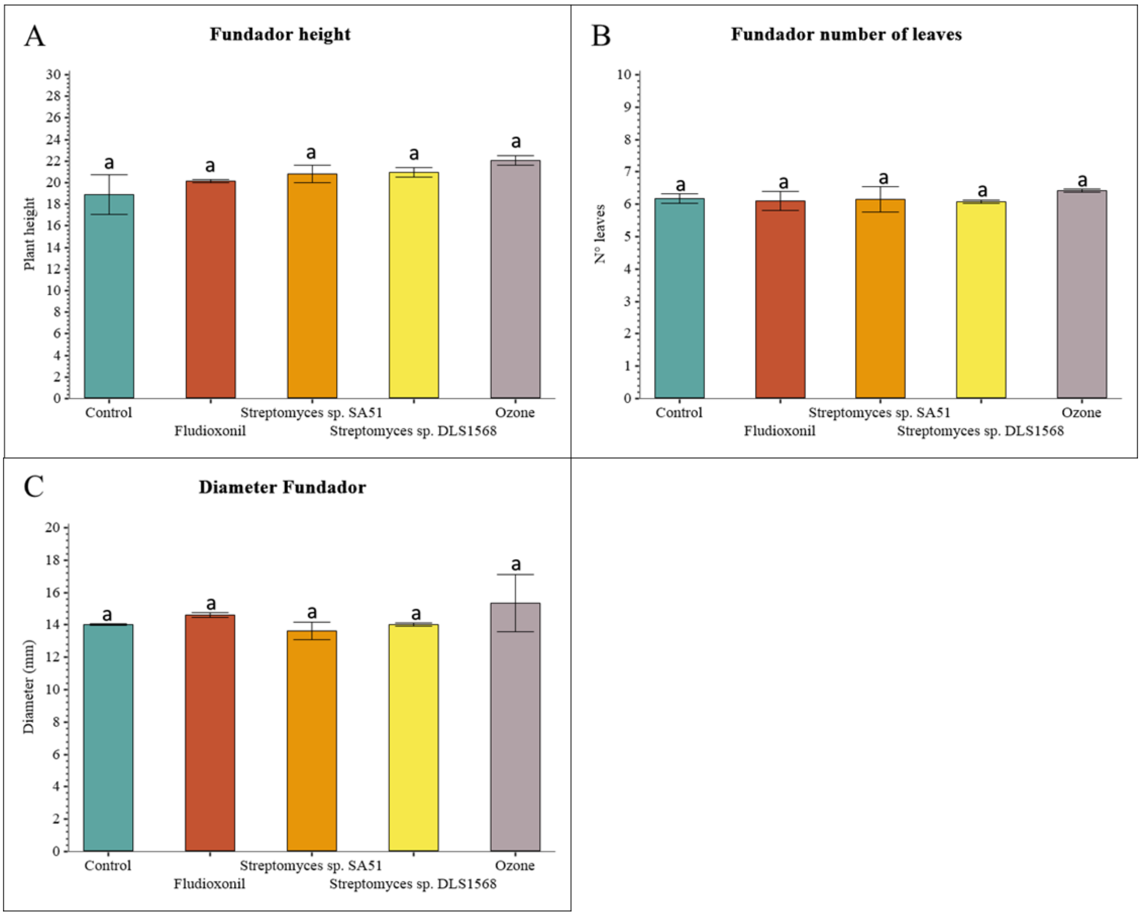

Figure 32 *. Different phenological parameters assessed during the cropping season 2021 for onion var. Fundador in an experimental field located in Cesena ('Minotti'; FC): A) plant height (cm) B) number of leaves C) neck diameter. Data are reported as means ±SD. Means with the same letters are not significantly different (One-way ANOVA, Tukey's Test, $p > 0.05$).*

4.7.3 Isolation and identification of Fusarium spp. from diseased onion plants

Among the diseased onion plant samples collected during the experimental field trials 2020 and 2021, four different *Fusarium* spp. morphotypes have been identified by morphological examination of the fungal isolates (colony characters and microscopically observation of fruit bodies, phialide, absence of chlamydospores *etc.*) (data not shown).

For all four different *Fusarium* spp. isolates (Sample N° 1, 2, 3 and 4), the sequences generated by amplification of the conserved ribosomal ITS region were compared to sequences from the National Center for Biotechnology Information (NCBI) database using BLAST (//www.ncbi.nlm.nih.gov/BLAST), respectively. The sample N° 1, 2, 3 and 4 were then identified as *F. oxysporum* (Tab. 18), confirming the presence of *Fusarium* spp. natural inoculum in diseased onion plant tissues.

Table 18 *List of the 4 different Fusarium oxysporum. isolated from symptomatic onion bulbs. Taxonomical characterization by ITS sequencing*

Sample	**Sequence**	**bp**	**%identity**	**Query coverage**	**Name**	**Accession n°**
N° 1	GGATCGGGGGTTCTACTGACCGAGGTCAACATTCAGAAGTTGGGGTTTAACGGCGTGGCCGCGACGATTACCAGTAACGAGGGTTTTACTACTACGCTATGGAAGCTCGACGTGACCGCCAATCAATTTGAGGAACGCGAATTAACGCGAGTCCCAACACCAAGCTGTGCTTGAGGGTTGAAATGACGCTCGAACAGGCATGCCCGCCAGAATACTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTTGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTGATTTATTTATGGTTTTACTCAGAAGTTACATATAGAAACAGAGTTTAGGGGTCCTCTGGCGGGCCGTCCCGTTTTACCGGGAGCGGGCTGATCCGCCGAGGCAACAAGTGGTATGTTCACAGGGGTTTGGGAGTTGTAAACTCGGTAATGATCCCTCCGCTGGTTCACCAACGGAGACCTTGTTACGATTTTTACTTCCA	540	99.26%	99%	*Fusarium oxysporum*	MK966308.1

N° 2	TTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCTTTAACGGCGTGGCCGCGACGATTACCAGTAACGAGGGTTTTACTACTACGCTATGGAAGCTCGACGTGACCGCCAATCAATTTGAGGAACGCGAATTAACGCGAGTCCCAACACCAAGCTGTGCTTGAGGGTTGAAATGACGCTCGAACAGGCATGCCCGCCAGAATACTGGCGGGCGCAATGTGCGATTTTGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGACAATCGGGCAGCTACCTGATCCGAGGTCAACATTCAGAAGTTGGGGAAGTTTTGATTTATTTATGGTTTTACTCAGAAGTTACATATAGAAACAGAGTTTAGGGGTCCTCTGGCGGGCCGTCCCGTTTTACCGGGAGCGGGCTGATCCGCCGAGGCAACAAGTGGTATGTTCACAGGGGTTTGGGAGTTGTAAACTCGGTAATGATCCCTCCGCTGGTTCACCAACGGAGACCTTGTTACGACTTTTTACTTCCA	542	100%	97%	*Fusarium oxysporum*	MN626452.1

Sample	**Sequence**	**bp**	**% identity**	**Query coverage**	**Name**	**Accession n°**
N° 3	TCCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTCGGTTGGGGACGCGGAGGTCATTACCGAGTTTACAACTCCCAAACCCCTGTGAACATACCACTTGTTGCCTCGGCGGATCAGCCCGCTCCGGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTCTGTTTCTATATGTAACTTCTGAGTAAAACCATAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCTCATTTCAACCCTCAAGCACAGCTTGGTGTTGGGACTCGCGTTAATTCGCGTTCCTCAAATTGATTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAGTAAAACCCTCGTTACTGGTAATCGTCGCGGCCACGCCGTAAAGAACTTAAGCATATCAATAAGCGGAGGAA	537	98,50%	99%	*Fusarium oxysporum*	KJ562370.1
N° 4	GGACGCTCGAACAGGCATGCCCGCCAGAATACTGGCGGGCGCAATATCGGGGGTTCTACTGACCGAGGTCAACATTCAGAAGTTGGGGTTTAACGGCGTGGCCGCGACGATTACCAGTAACGAGGGTTTTACTACTACG	504	99,64%	97%	*Fusarium oxysporum*	OM084964.1

CTATGGAAGCTCGACGTGACCGCCAATCA
ATTTGAGGAACGCGAATTAACGCGAGTCC
CAACACCAAGCTGTGCTTGAGGGTTGAAA
TGGTGCGTTCAAAGATTCGATGATTCACT
GAATTCTGCAATTCACATTACTTATCGCAT
TTTGCTGCGTTCTTCATCGATGCCAGAACC
AAGAGATCCGTTGTTGAAAGTTTTGATTT
ATTTATGGTTTTACTCAGAAGTTACATATA
GAAACAGAGTTTAGGGGTCCTCTGGCGGG
CCGTCCCGTTTTACCGGGAGCGGGCTGAT
CCGCCGAGGCAACAAGTGGTATGTTCACA
GGGGTTTGGGAGTTGTAAACTCGGTAATG
ATCCCTCCGCTGGT

4.7.4 Commercial field Onion

4.7.4.1 Onion var. Elenka cropping season 2021 in Cesena (FC)

During the first assessment (30 days after sowing), the collected data statistically analysed by One-way ANOVA with Tukey's test did not show significant differences ($p > 0.05$) in the number of emerging seedlings between all treatment theses. Similarly, the number of plants were monitored during the following 3 assessments, no significant differences ($p > 0.05$) for the number of emerging plants among all the different treatments were not shown (Tab.19 and Fig. 33).

Table 19 *Number of emerged plants during the cropping season 2021 for onion var. Elenka is a commercial field located in Cesena (FC). Data are reported as means ±SD. Means with the same letters are not significantly different (One-way ANOVA, Tukey's Test, $p > 0.05$)*

Elenka Cesena 2021

	29/03/2021		29/04/2021		29/05/2021		29/06/2021	
Treatments	Avg	SD	Avg	SD	Avg	SD	Avg	SD
Control	150.92	±44.67 ^{a}	141.08	±9.07 ^{a}	153.67	±7.54 ^{a}	160.83	±12.96 ^{a}
Fludioxonil	159.00	±25.46 ^{a}	146.83	±15.32 ^{a}	147.17	±12.49 ^{a}	158.50	±19.80 ^{a}
***Streptomyces* sp. SA51**	127.67	±3.30 ^{a}	124.38	±13.26 ^{a}	130.25	±15.91 ^{a}	133.25	±7.07 ^{a}
Streptomyces* sp. *DLS1568	143.25	±28.64 ^{a}	148.13	±32.70 ^{a}	152.42	±22.75 ^{a}	152.71	±21.27 ^{a}
Ozone	160.63	±30.58 ^{a}	157.88	±28.81 ^{a}	164.88	±7.25 ^{a}	168.63	±3.71 ^{a}

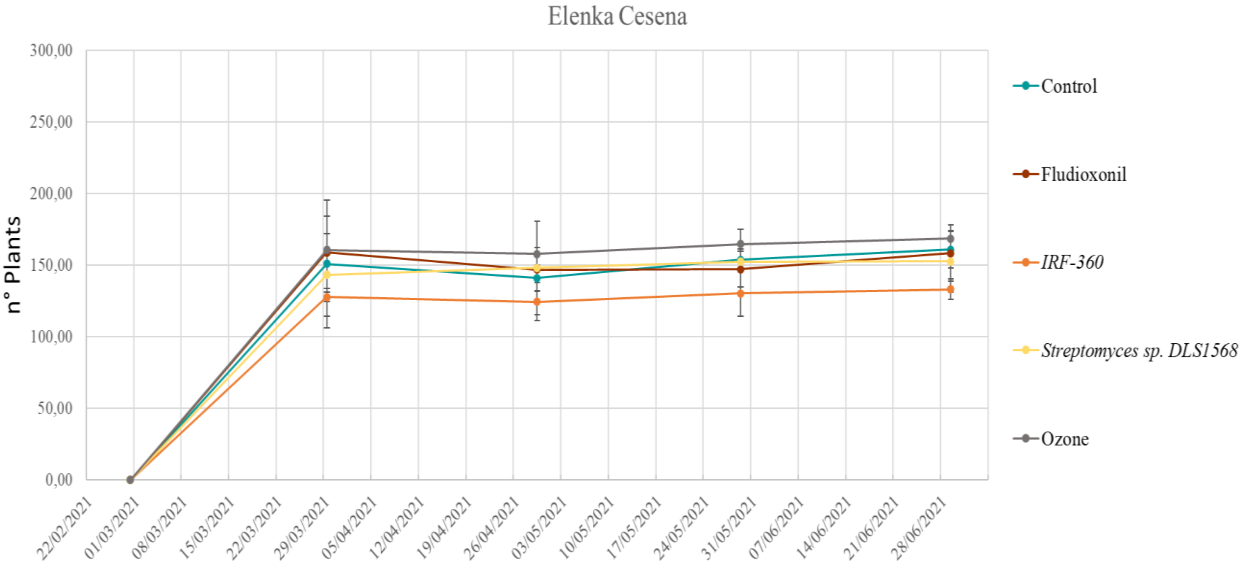

Figure 33 *Number of emerged plants during the cropping season 2021 for onion var. Elenka in commercial field located in Cesena (FC).*

4.7.4.2 Effects of different treatments on the onion plant phenological development and bulb weight at harvest time during the cropping season 2021 for onion var. Elenka is a commercial field located in Cesena (FC)

The collected data statistically analysed by one-way ANOVA with Tukey’s test during the second (mean plant hight), the third (mean number of leaves), the fourth assessment (mean onion bulb diameter) and the fifth assessment (mean onion bulb wight) did not show significant difference ($p > 0.05$) between all treatment theses (Fig. 34B; Fig. 34 C and Fig. 34 D).

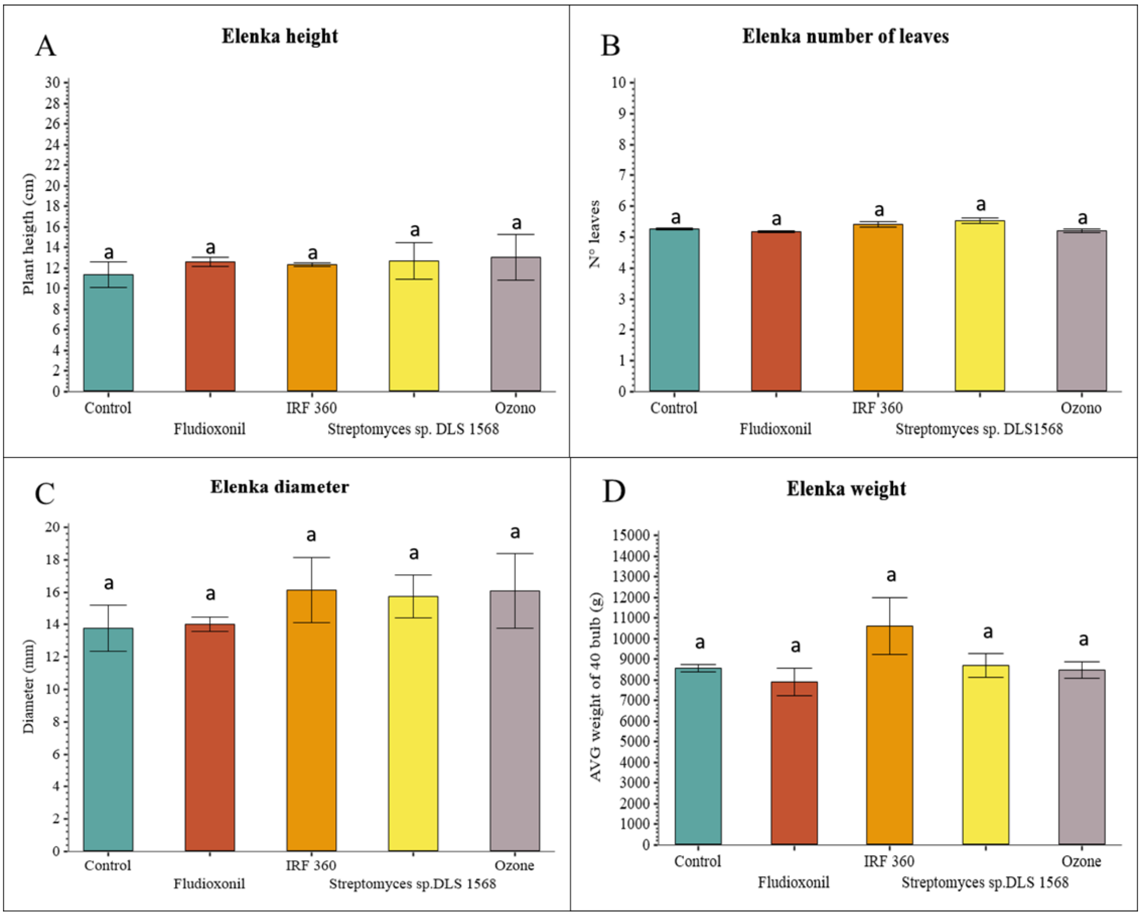

__Figure 34__ Different phenological parameters assessed during the cropping season 2021 for onion var. Elenka in a commercial field located in Cesena (FC): A) plant height (cm) B) number of leaves C) neck diameter. Data are reported as means ±SD. Means with the same letters are not significantly different (One-way ANOVA, Tukey’s Test, p > 0.05).

4.7.4.3 Onion var. Elenka cropping season 2021 in commercial field located in Conselice (RA)

During the first assessment (30 days after sowing), the collected statistically analysed data by one-way ANOVA with Tukey's test did not show significant difference ($p > 0.05$) in the number of emerged seedlings between all treatment theses. During the second assessment, the collected data statistically analysed by one-way ANOVA with Tukey test showed significant difference ($p < 0.05$) in the number of emerged seedlings between *Streptomyces* sp. DLS1568 and treatment IRF-360 ($p<0.05$). No significant differences were highlighted between the other treatment theses. Similarly, the number of plants counted during the last assessment was not shown by one-way ANOVA with Tukey test significant differences ($p > 0.05$) for the number of emerged plants among all the different treatments (Tab.20 and Fig. 35).

Table 20 *Number of emerged plants during the cropping season 2021 for onion var. Elenka in a commercial field located in Conselice (RA). Data are reported as means ±SD. Means with the same letters are not significantly different (One-way ANOVA, Tukey's Test, $p > 0.05$). Means with different letters indicate significant difference between two treatment groups according to One-way ANOVA, Tukey's Test ($p < 0.05$).*

Elenka Conselice 2021

Treatments	22/03/2021		19/04/2021		19/05/2021		19/06/2021	
	Avg	SD	Avg	SD	Avg	SD	Avg	SD
Control	226.30	±19.94 ^{a}	217.30	±6.08 ^{a}	219.20	±2.26 ^{a}	243.03	±1.93 ^{a}
Fludioxonil	228.20	±56.57 ^{a}	210.70	±6.65 ^{a}	214.20	±3.11 ^{a}	221.20	±10.47 ^{a}
***Streptomyces* sp. SA51**	182.70	±41.44 ^{a}	195.33	±4.24 ^{a}	193.33	±0.42 ^{b}	221.00	±7.35 ^{a}
Streptomyces* sp. *DLS1568	173.13	±33.76 ^{a}	195.30	±2.97 ^{a}	212.50	±1.56 ^{a}	207.68	±19.91 ^{a}
Ozone	226.30	±19.94 ^{a}	217.30	±6.08 ^{a}	219.20	±2.26 ^{a}	243.03	±1.93 ^{a}

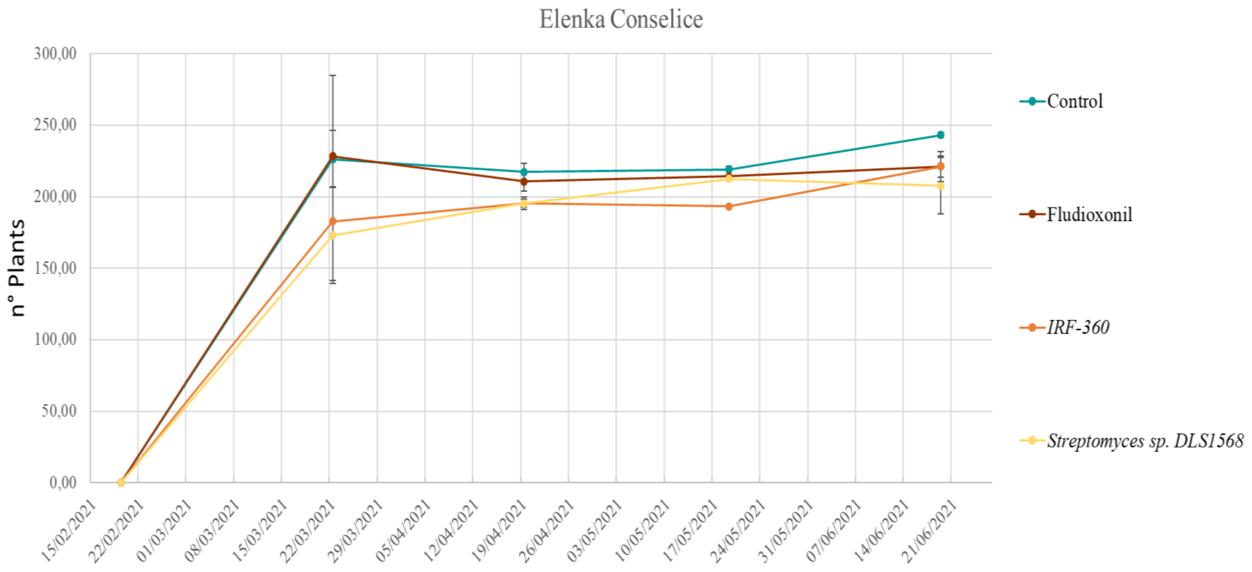

***Figure 35** Number of emerged plants during the cropping season 2021 for onion var. Elenka in commercial field located in Conselice (RA)*

4.7.4.4 Effects of different treatments on the onion plant phenological development and bulb weight at harvest time during the cropping season 2021 for onion var. Elenka in commercial field located in Conselice (RA)

The collected data statistically analysed by one-way ANOVA with Tukey's test during the second (mean plant hight) and the third (mean number of leaves) and the fourth assessment (mean onion bulb diameter) did not show significant difference ($p > 0.05$) between all treatment theses (Fig. 36 A; Fig. 36 B and Fig. 36 C). During the third assessment for the mean onion bulb diameter, the mean value for the *Streptomyces* sp. DLS 1568 displayed a higher but not significant ($p > 0.05$) mean value of 15,73 mm (SD = 1.60) compared to Control, Fludioxonil and IRF 360 treatments, which displayed a mean value of 13.53 mm (SD = 0.24), 14.04 mm (SD = 0.14) and 14.35 (SD = 0.83), respectively (Fig. 36 C). Meanwhile, during the fifth assessment on the mean weight of the onion bulbs, the collected data statistically analysed by one-way ANOVA with Tukey's test showed a significant increase ($p < 0.05$) in the mean bulb weight for the plants germinated by seeds treated with *Streptomyces* sp. DLS 1568 displaying a mean value of 5240 gr (SD = 250) compared to the IRF 360, Fludioxonil and control, which displayed a mean value of 4560 gr (SD = 450), 3980 gr (SD = 650) and 4000 gr (SD = 500), respectively (Fig. 36 D).

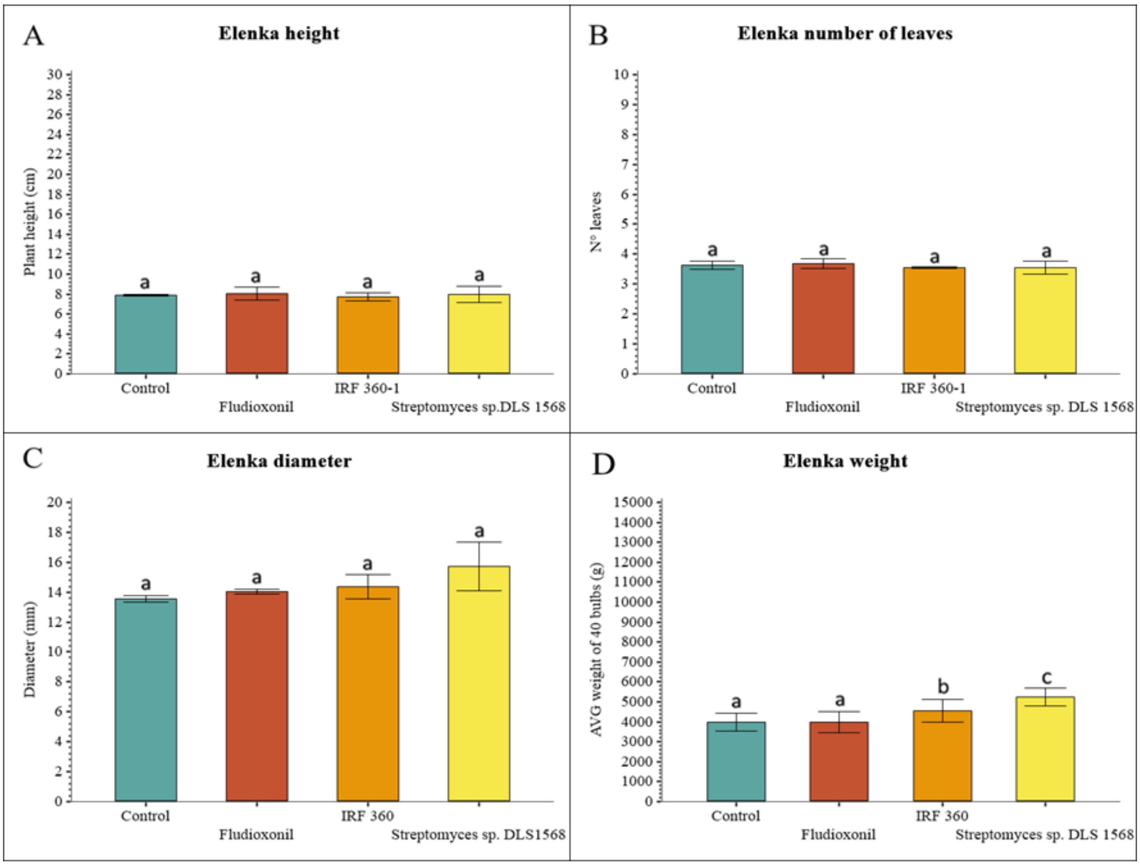

Figure 36 *Different phenological parameters assessed during the cropping season 2021 for onion var. Elenka in a commercial field located in Conselice (RA): A) plant height (cm) B) number of leaves C) neck diameter. Data are reported as means ±SD. Means with the same letters are not significantly different (One-way ANOVA, Tukey's Test, $p > 0.05$). Means with different letters indicate significant difference between two treatment groups according to One-way ANOVA, Tukey's Test ($p < 0.05$).*

4.7.5 Fennel

4.7.5.1 Fennel var. Crono cropping season 2020 in commercial field in Avezzano (AQ)

Emergence results from the treatment with *Streptomyces* sp. DLS1568 showed an overall benefit to promote seed germination and the development of healthy seedlings compared to control and Fludioxonil treatments (Tab. 20). During the first assessment for the number of emerged plants, the mean value for the *Streptomyces* sp. DLS 1568 displayed a higher but not significant ($p > 0.05$) mean value of 365.25 (SD = 26.79) compared to Control and Fludioxonil treatments, which displayed a mean value of 339.50 (SD = 9.33) and 339.50 (SD = 45.35), respectively. During the second assessment (after the manual removal of weak / abnormal plantlets by the farm operators as common agricultural practise) the collected and statistically analyzed data by one-way ANOVA with Tukey's test showed a significant increase ($p < 0.05$) in the number of plantlets of seeds treated with *Streptomyces* sp. DLS 1568 displaying a mean value of 227.94 (SD = 8.31) compared to the control with a mean value of 194.40 (SD = 14.85). No significant differences between *Streptomyces* sp. DLS 1568 and Fludioxonil, which displayed and 217.30 (SD = 3.89), were highlighted. During the third assessment, the collected and statistically analyzed data by one-way ANOVA with Tukey's test showed a significant increase ($p < 0.05$) in the number of plants belong to the treatment with *Streptomyces* sp. DLS 1568 displaying a mean value of 212.50 (SD = 16.26) compared to the control and Fludioxonil thesis which displayed a mean value of 187.00 (SD = 7.07) and 198.62 (SD = 0.88), respectively (Fig. 36 D).

Concerning both vigour assessments conducted during the cropping season, the collected and statistically analyzed data by one-way ANOVA with Tukey's test did no show a significant differences ($p > 0.05$) among the *Streptomyces* sp. DLS1568, Fludioxonil and Control theses.

Table 20 *Number of emerged plants during the cropping season 2020 for fennel var. Crono in commercial field located in Avezzano (AQ). Data are reported as means ±SD. Means with the same letters are not significantly different (One-way ANOVA, Tukey's Test, $p > 0.05$). Means with different letters indicate significant difference between two treatment group according to One-way ANOVA, Tukey's Test ($p < 0.05$).*

Fennel 2020						
Treatments	03/07/2020		15/07/2020		13/08/2020	
	Avg	SD	Avg	SD	Avg	SD
Control	339.50	±9.33 ^{a}	194.40	±14.85^{a}	187.00	±7.07 ^{a}
Fludioxonil	339.50	±45.35 ^{a}	217.30	±3.89^{ab}	198.62	±0.88^{ab}
Streptomyces sp. DLS1568	365.25	±26.79 ^{a}	227.94	±8.31^{b}	212.50	±16.26^{b}

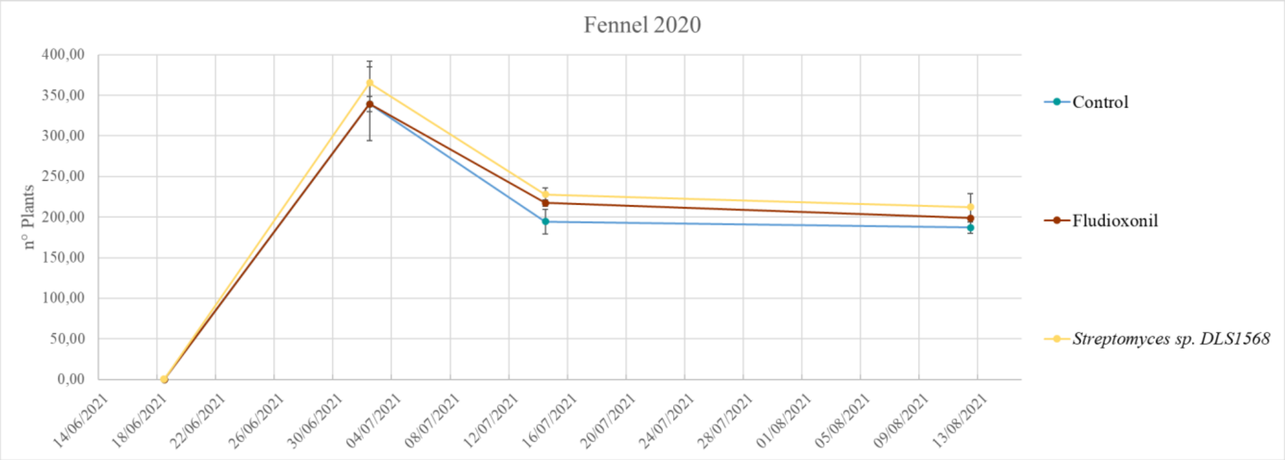

Figure 37 *Number of emerged plants during the cropping season 2020 for fennel var. Crono commercial field located in Avezzano (AQ).*

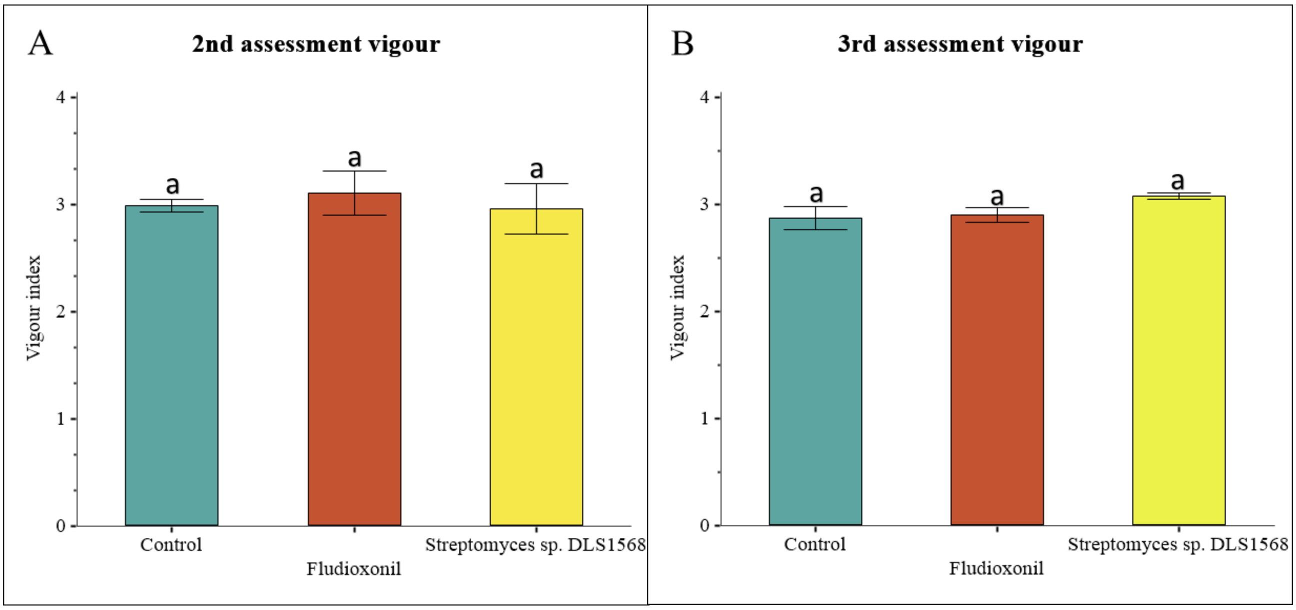

Figure 38 *Vigour mean values recorded of the A) 2nd assessment and B) 3rd assessment during the cropping season 2020 for fennel var. Crono commercial field located in Avezzano (AQ). Data are reported as means ±SD. Means with the same letters are not significantly different (One-way ANOVA, Tukey's Test, $p > 0.05$).*

4.7.5.2 Fennel var. Crono cropping season 2021 in commercial field in Avezzano (AQ)

Emergence results from the treatment with *Streptomyces* sp. DLS1568 showed an overall benefit to promote seed germination and the development of healthy seedlings compared to control and Fludioxonil treatments (Tab. 21). During the first assessment for the number of emerged plants, the mean value for the *Streptomyces* sp. DLS 1568 displayed a higher but not significant ($p > 0.05$) mean value of 335.13 (SD = 6.89) compared to Control and Fludioxonil treatments, which displayed a mean value of 313.00 (SD = 4.60) and 323.88 (SD = 6.19), respectively. During the second assessment (after the manual removal of weak / abnormal plantlets by the farm operators as common agricultural practise) the collected and statistically analyzed data by one-way ANOVA with Tukey's test showed a significant increase ($p < 0.05$) in the number of plantlets of seeds treated with *Streptomyces* sp. DLS 1568 displaying a mean value of 209.13 (SD = 8.31) compared to the control with a mean value of 180.75 (SD = 14.85). No significant differences between *Streptomyces* sp. DLS 1568 and Fludioxonil, who displayed a mean value of 205.00 (SD = 3.89), were highlighted. During the third assessment, the collected and statistically analyzed data by one-way ANOVA with Tukey's test showed a significant increase ($p < 0.05$) in the number of plants belong to the treatment with *Streptomyces* sp. DLS 1568 displaying a mean value of 194.50 (SD = 12.24) compared to the control and Fludioxonil thesis which displayed a mean value of 173.00 (SD = 8.05) and 186.63 (SD = 1.98), respectively (Fig. 36 D).

Table 21 Number of emerged plants during the cropping season 2021 for fennel var. Crono in commercial field located in Avezzano (AQ). Data are reported as means ±SD. Means with the same letters are not significantly different (One-way ANOVA, Tukey's Test, $p > 0.05$). Means with different letters indicate significant difference between two treatment group according to One-way ANOVA, Tukey's Test ($p < 0.05$).

Fennel 2021						
	02/07/2021		14/07/2021		12/08/2021	
Treatments	Avg	SD	Avg	SD	Avg	SD
Control	313.00	±4.60 ^{a}	180.75	±14.85 ^{a}	173.00	±8.05 ^{a}
Fludioxonil	323.88	±6.19 ^{a}	205.00	±3.89 ^{b}	186.63	±1.98 ^{ab}
Streptomyces sp. DLS1568	335.13	±6.89 ^{a}	209.13	±8.31 ^{b}	194.50	±12.24 ^{b}

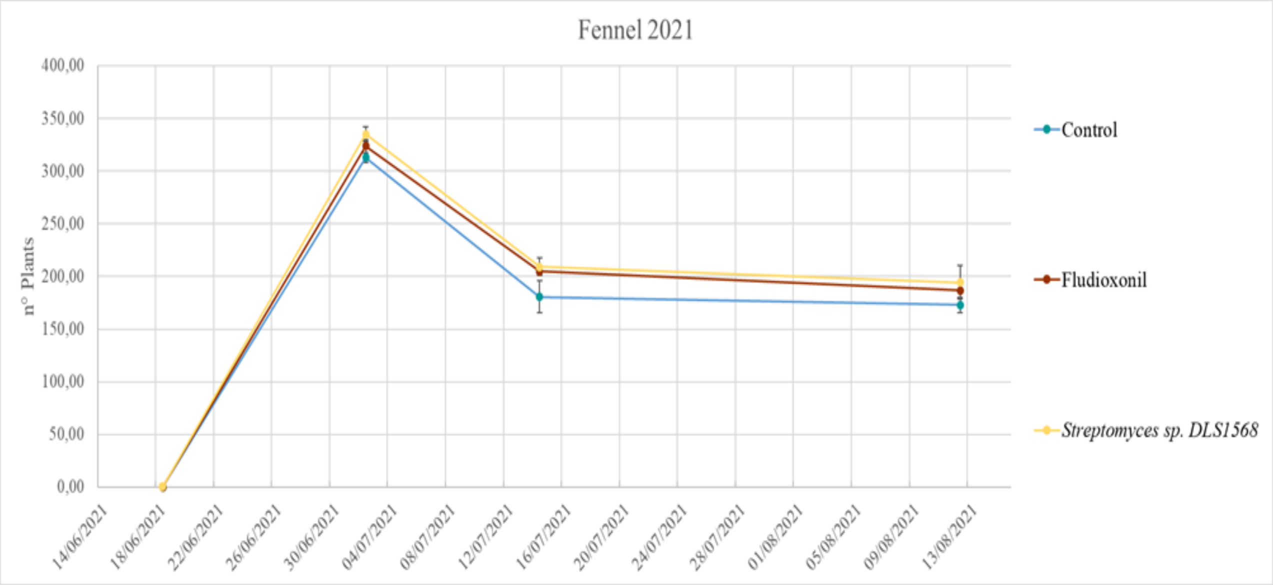

Figure 40 Number of emerged plants during the cropping season 2021 for fennel var. Crono commercial field located in Avezzano (AQ).

During the 2nd vigour assessments, the collected data statistically analysed by one-way ANOVA with Tukey's test showed a significant increase ($p < 0.05$) in the mean vigour index for the plants germinated by seeds treated with *Streptomyces* sp. DLS 1568 displaying a mean value of 2.69 (SD = 0.20) compared to the Fludioxonil and control, that displayed a mean value of 2.49 (SD = 0.27), and 2.34 (SD = 0.22), respectively (Fig. 41 A). Again, during the 3rd vigour assessments, the collected data statistically analysed by one-way ANOVA with Tukey's test showed a significant increase ($p < 0.05$) in the mean vigour index for the plants germinated by seeds treated with *Streptomyces* sp. DLS 1568 displaying a mean value of 3.09 (SD = 0.21) compared to the control, that displayed a mean value of 2.71 (SD = 0.19) (Fig. 41 B). No significant difference was highlighted between Streptomyces sp. DLS 1568 and Fludioxonil treatments, that displayed a mean value of 3.01 (SD = 0.15).

During the assessment of the mean weight of the fennel bulbs, the collected data statistically analysed by one-way ANOVA with Tukey's test did not show significant difference among the 3 thesis ($p > 0.05$) (Fig. 41 C D).

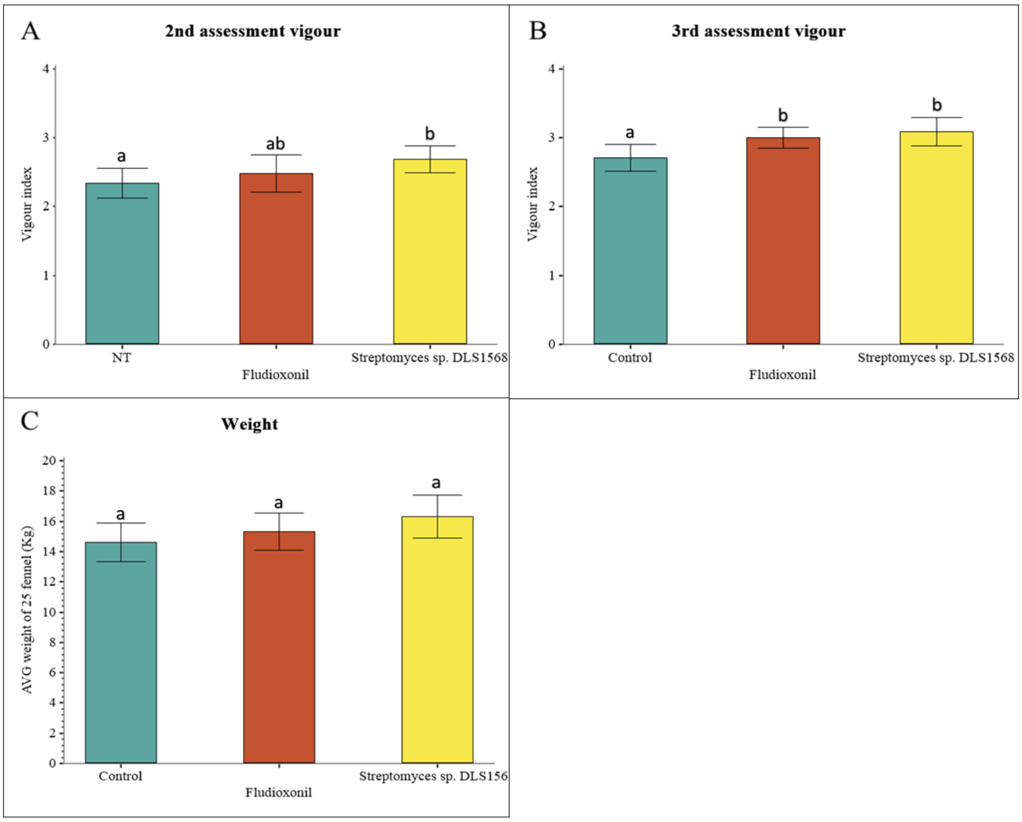

Figure 41 *Vigour mean values recorded of the A) 2nd assessment and B) 3rd assessment and for mean bulb fennel weight during the cropping season 2021 for fennel var. Crono commercial field located in Avezzano (AQ). Data are reported as means ±SD. Means with the same letters are not significantly different (One-way ANOVA, Tukey's Test, $p > 0.05$).*

4.7.6 *Streptomyces* sp. DLS 1568 detection in plant

During both onion and fennel experimental and commercial field trials, the plants germinated from seeds coated with *Streptomyces* sp. DLS 1568 and plant generated from untreated seed (control) were collected.

The 93% and the 89% of onion and fennel plant samples, respectively, tested positive to the specific PCR amplification with the 16S rRNA gene primer pairs StrepB/StrepF generating the specific fragment of 507 bp. Thus confirmed the ability of the *Streptomyces* sp. DLS1568 to colonise both onion and fennel plant tissues by seed coating. No amplification was recorded for any untreated controls.

4.8 WGS results of *Streptomyces* sp. DLS1568

KEGG (Kyoto Encyclopedia of Genes and Genomes) was used for the systematic analysis of gene functions, such as metabolism, membrane transport, signal transduction and cell cycle. Furthermore, the assembled genome was annotated using Rapid Annotation using Subsystem Technology (RAST). The analysis from RAST revealed 5162 coding sequences, along with a total of 67 RNA genes (Tab. 22). Based on RAST system, most of the protein-coding genes were involved in amino acids metabolism, followed by carbohydrate metabolism and production of cofactors, vitamins, prosthetic groups, pigments, various nitrogen metabolisms, pathways involved in copper homeostasis, resistance to cobalt, cadmium and arsenic, riboflavin metabolism. Moreover, there are genes involved in detoxification such as uptake of selenate and selenite (Tab. 23).

Further analysis on antibiotics and Secondary Metabolite Analysis SHell (an-tiSMASH) predicted more than 100 biosynthetic gene clusters in *Streptomyces* sp. DLS1568 genome.

Table 22 *Genome feature of strains DLS1568*

Streptomyces sp. DLS1568	
Genome size	5.935.868 bp
Contings L50	117 bp
Contins N50	16113 bp
G+C contente	72%
Number of Contigs (with PEGs)	685
Number of Subsystems	398
Number of Coding Sequences	5162
Number of RNAs	67

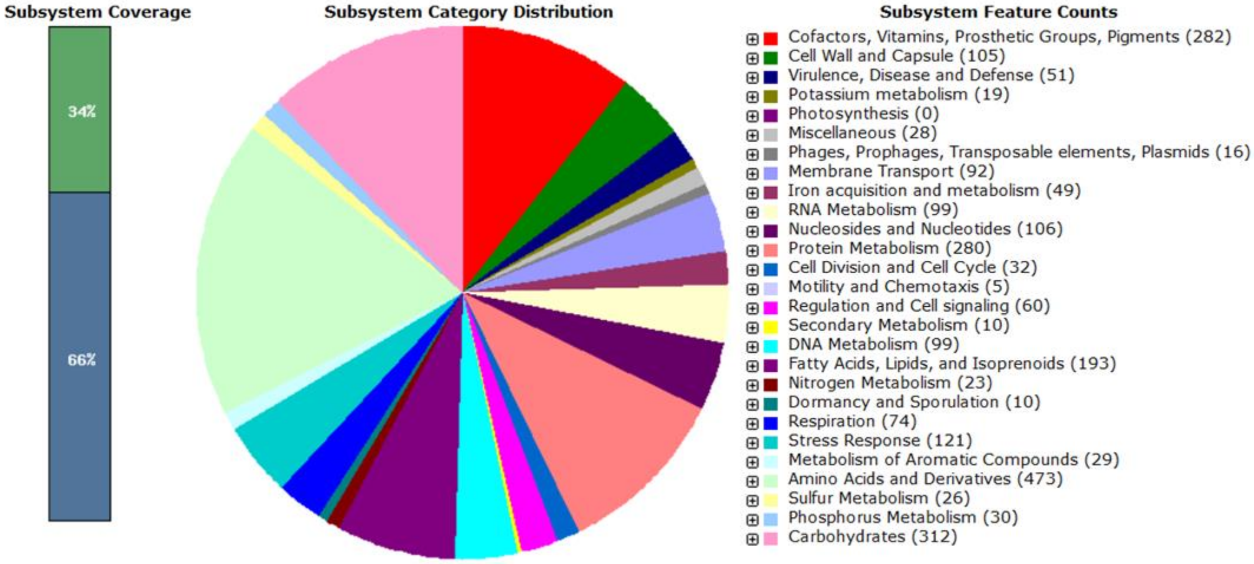

Figure 42 *Subsystem distribution of Streptomyces sp. DLS1568 (based on RAST annotation server)*

Table 23 Genes identified by RAST in the genome of Streptomyces sp. DLS 1568 and their role.

Category	***Subcategory***	***Subsystem***	***Putative role***
Iron acquisition and metabolism	Siderophores	Salmochelin-mediated Iron Acquisition	Glycosyltransferase IroB
Iron acquisition and metabolism	Siderophores	Siderophore assembly kit	Siderophore biosynthesis diaminobutyrate--2-oxoglutarate aminotransferase (EC 2.6.1.76)
Iron acquisition and metabolism	Siderophores	Siderophore assembly kit	Siderophore biosynthesis L-2,4-diaminobutyrate decarboxylase
Iron acquisition and metabolism	Siderophores	Siderophore assembly kit	ABC-type Fe3+-siderophore transport system, ATPase component
Iron acquisition and metabolism	Siderophores	Siderophore assembly kit	ABC-type Fe3+-siderophore transport system, permease 2 component
Iron acquisition and metabolism	Siderophores	Siderophore assembly kit	Thioesterase in siderophore biosynthesis gene cluster
Iron acquisition and metabolism	Siderophores	Siderophore assembly kit	ABC-type Fe3+-siderophore transport system, permease component
Iron acquisition and metabolism	Siderophores	Siderophore assembly kit	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase (EC

			1.3.1.28) of siderophore biosynthesis
Iron acquisition and metabolism	Siderophores	Siderophore assembly kit	Siderophore related permease
Iron acquisition and metabolism	Siderophores	Siderophore assembly kit	Isochorismatase (EC 3.3.2.1) of siderophore biosynthesis
Iron acquisition and metabolism	Siderophores	Siderophore assembly kit	Siderophore synthetase large component, acetyltransferase
Iron acquisition and metabolism	Siderophores	Siderophore assembly kit	Siderophore synthetase component, ligase
Iron acquisition and metabolism	Siderophores	Siderophore assembly kit	2,3-dihydroxybenzoate-AMP ligase (EC 2.7.7.58)
Iron acquisition and metabolism	Siderophores	Siderophore assembly kit	Siderophore biosynthesis non-ribosomal peptide synthetase modules
Iron acquisition and metabolism	Siderophores	Siderophore assembly kit	Isochorismate synthase (EC 5.4.4.2) of siderophore biosynthesis
Nitrogen Metabolism	Nitrogen Metabolism - no subcategory	Allantoin Utilization	Allantoinase (EC 3.5.2.5)
Nitrogen Metabolism	Nitrogen Metabolism - no subcategory	Allantoin Utilization	2-hydroxy-3-oxopropionate reductase (EC 1.1.1.60)
Nitrogen Metabolism	Nitrogen Metabolism - no subcategory	Allantoin Utilization	Glyoxylate carboligase (EC 4.1.1.47)

Nitrogen Metabolism	Nitrogen Metabolism - no subcategory	Allantoin Utilization	Allantoicase (EC 3.5.3.4)
Nitrogen Metabolism	Nitrogen Metabolism - no subcategory	Allantoin Utilization	Glycerate kinase (EC 2.7.1.31)
Nitrogen Metabolism	Nitrogen Metabolism - no subcategory	Nitrate and nitrite ammonification	Assimilatory nitrate reductase large subunit (EC:1.7.99.4)
Nitrogen Metabolism	Nitrogen Metabolism - no subcategory	Nitrate and nitrite ammonification	Nitrate/nitrite transporter
Nitrogen Metabolism	Nitrogen Metabolism - no subcategory	Nitrate and nitrite ammonification	Nitrite reductase [NAD(P)H] small subunit (EC 1.7.1.4)
Nitrogen Metabolism	Nitrogen Metabolism - no subcategory	Nitrate and nitrite ammonification	Nitrite reductase [NAD(P)H] large subunit (EC 1.7.1.4)
Nitrogen Metabolism	Nitrogen Metabolism - no subcategory	Nitrate and nitrite ammonification	Nitrate/nitrite response regulator protein
Nitrogen Metabolism	Nitrogen Metabolism - no subcategory	Ammonia assimilation	Nitrogen regulatory protein P-II
Nitrogen Metabolism	Nitrogen Metabolism - no subcategory	Ammonia assimilation	Glutamate-ammonia-ligase adenylyltransferase (EC 2.7.7.42)
Nitrogen Metabolism	Nitrogen Metabolism - no subcategory	Ammonia assimilation	Ammonium transporter
Nitrogen Metabolism	Nitrogen Metabolism - no subcategory	Ammonia assimilation	Glutamine synthetase type II, eukaryotic (EC 6.3.1.2)

Nitrogen Metabolism	Nitrogen Metabolism - no subcategory	Ammonia assimilation	Glutamate synthase [NADPH] large chain (EC 1.4.1.13)
Phosphorus Metabolism	Phosphorus Metabolism - no subcategory	High affinity phosphate transporter and Control of PHO regulon	Phosphate regulon transcriptional regulatory protein PhoB (SphR)
Phosphorus Metabolism	Phosphorus Metabolism - no subcategory	High affinity phosphate transporter and Control of PHO regulon	Phosphate transport system permease protein PstC (TC 3.A.1.7.1)
Phosphorus Metabolism	Phosphorus Metabolism - no subcategory	High affinity phosphate transporter and Control of PHO regulon	Phosphate transport ATP-binding protein PstB (TC 3.A.1.7.1)
Phosphorus Metabolism	Phosphorus Metabolism - no subcategory	High affinity phosphate transporter and Control of PHO regulon	Polyphosphate kinase (EC 2.7.4.1)
Phosphorus Metabolism	Phosphorus Metabolism - no subcategory	High affinity phosphate transporter and Control of PHO regulon	Phosphate transport system permease protein PstA (TC 3.A.1.7.1)
Phosphorus Metabolism	Phosphorus Metabolism - no subcategory	High affinity phosphate transporter and Control of PHO regulon	Phosphate ABC transporter, periplasmic phosphate-binding protein PstS (TC 3.A.1.7.1)
Phosphorus Metabolism	Phosphorus Metabolism - no subcategory	Phosphate metabolism	Phosphate regulon transcriptional regulatory protein PhoB (SphR)

Phosphorus Metabolism	Phosphorus Metabolism - no subcategory	Phosphate metabolism	Inorganic pyrophosphatase (EC 3.6.1.1)
Phosphorus Metabolism	Phosphorus Metabolism - no subcategory	Phosphate metabolism	Pyrophosphate-energized proton pump (EC 3.6.1.1)
Phosphorus Metabolism	Phosphorus Metabolism - no subcategory	Phosphate metabolism	Phosphate transport system permease protein PstC (TC 3.A.1.7.1)
Phosphorus Metabolism	Phosphorus Metabolism - no subcategory	Phosphate metabolism	Phosphate transport ATP-binding protein PstB (TC 3.A.1.7.1)
Phosphorus Metabolism	Phosphorus Metabolism - no subcategory	Phosphate metabolism	Phosphate transport system permease protein PstA (TC 3.A.1.7.1)
Phosphorus Metabolism	Phosphorus Metabolism - no subcategory	Phosphate metabolism	Exopolyphosphatase (EC 3.6.1.11)
Phosphorus Metabolism	Phosphorus Metabolism - no subcategory	Phosphate metabolism	Probable low-affinity inorganic phosphate transporter
Phosphorus Metabolism	Phosphorus Metabolism - no subcategory	Phosphate metabolism	Predicted ATPase related to phosphate starvation-inducible protein PhoH
Potassium metabolism	Potassium metabolism - no subcategory	Potassium homeostasis	putative Glutathione-regulated potassium-efflux system protein KefB
Potassium metabolism	Potassium metabolism - no subcategory	Potassium homeostasis	Trk system potassium uptake protein TrkA

Potassium metabolism	Potassium metabolism - no subcategory	Potassium homeostasis	Potassium uptake protein TrkH
Potassium metabolism	Potassium metabolism - no subcategory	Potassium homeostasis	Large-conductance mechanosensitive channel
Potassium metabolism	Potassium metabolism - no subcategory	Potassium homeostasis	Potassium channel protein
Potassium metabolism	Potassium metabolism - no subcategory	Potassium homeostasis	Osmosensitive K+ channel histidine kinase KdpD (EC 2.7.3.-)
Potassium metabolism	Potassium metabolism - no subcategory	Hyperosmotic potassium uptake	Trk system potassium uptake protein TrkA
Potassium metabolism	Potassium metabolism - no subcategory	Hyperosmotic potassium uptake	Potassium uptake protein TrkH
Potassium metabolism	Potassium metabolism - no subcategory	Glutathione-regulated potassium-efflux system and associated functions	Glutathione-regulated potassium-efflux system protein KefB
Stress Response	Detoxification	Uptake of selenate and selenite	Various polyols ABC transporter, ATP-binding component
Stress Response	Detoxification	Uptake of selenate and selenite	Sulfate and thiosulfate import ATP-binding protein CysA (EC 3.6.3.25)
Virulence, Disease and Defense	Resistance to antibiotics and toxic compounds	Copper homeostasis	Copper-translocating P-type ATPase (EC 3.6.3.4)
Virulence, Disease and Defense	Resistance to antibiotics and toxic compounds	Copper homeostasis	Copper chaperone

Virulence, Disease and Defense	Resistance to antibiotics and toxic compounds	Copper homeostasis	Copper resistance protein D
Virulence, Disease and Defense	Resistance to antibiotics and toxic compounds	Cobalt-zinc-cadmium resistance	Cobalt-zinc-cadmium resistance protein CzcD
Virulence, Disease and Defense	Resistance to antibiotics and toxic compounds	Cobalt-zinc-cadmium resistance	Probable Co/Zn/Cd efflux system membrane fusion protein
Virulence, Disease and Defense	Resistance to antibiotics and toxic compounds	Cobalt-zinc-cadmium resistance	Transcriptional regulator, MerR family
Virulence, Disease and Defense	Resistance to antibiotics and toxic compounds	Arsenic resistance	Arsenical-resistance protein ACR3
Virulence, Disease and Defense	Resistance to antibiotics and toxic compounds	Arsenic resistance	Arsenical resistance operon repressor
Virulence, Disease and Defense	Resistance to antibiotics and toxic compounds	Arsenic resistance	Arsenical pump-driving ATPase (EC 3.6.3.16)
Virulence, Disease and Defense	Resistance to antibiotics and toxic compounds	Arsenic resistance	Arsenate reductase (EC 1.20.4.1)
Virulence, Disease and Defense	Resistance to antibiotics and toxic compounds	Mercuric reductase	PF00070 family, FAD-dependent NAD(P)-disulphide oxidoreductase
Cofactors, Vitamins, Prosthetic Groups, Pigments	Cofactors, Vitamins, Prosthetic Groups, Pigments - no subcategory	Thiamin biosynthesis	Sulfur carrier protein adenylyltransferase ThiF

Cofactors, Vitamins, Prosthetic Groups, Pigments	Cofactors, Vitamins, Prosthetic Groups, Pigments - no subcategory	Thiamin biosynthesis	Glycine oxidase ThiO (EC 1.4.3.19)
Cofactors, Vitamins, Prosthetic Groups, Pigments	Cofactors, Vitamins, Prosthetic Groups, Pigments - no subcategory	Thiamin biosynthesis	Thiazole biosynthesis protein ThiG
Cofactors, Vitamins, Prosthetic Groups, Pigments	Cofactors, Vitamins, Prosthetic Groups, Pigments - no subcategory	Thiamin biosynthesis	Thiamin ABC transporter, ATPase component
Cofactors, Vitamins, Prosthetic Groups, Pigments	Cofactors, Vitamins, Prosthetic Groups, Pigments - no subcategory	Thiamin biosynthesis	Thiamine-monophosphate kinase (EC 2.7.4.16)
Cofactors, Vitamins, Prosthetic Groups, Pigments	Cofactors, Vitamins, Prosthetic Groups, Pigments - no subcategory	Thiamin biosynthesis	1-deoxy-D-xylulose 5-phosphate synthase (EC 2.2.1.7)
Cofactors, Vitamins, Prosthetic Groups, Pigments	Cofactors, Vitamins, Prosthetic Groups, Pigments - no subcategory	Thiamin biosynthesis	Thiamin ABC transporter, substrate-binding component
Cofactors, Vitamins, Prosthetic Groups, Pigments	Cofactors, Vitamins, Prosthetic Groups, Pigments - no subcategory	Thiamin biosynthesis	Thiamin ABC transporter, transmembrane component
Cofactors, Vitamins, Prosthetic Groups, Pigments	Cofactors, Vitamins, Prosthetic Groups, Pigments - no subcategory	Thiamin biosynthesis	Sulfur carrier protein ThiS

5. Discussions

The use of beneficial microorganisms in agriculture has increased in recent years. According to the Royal Society of London (2009), the need of sustainable agriculture is mandatory to preserve environmental safety. A possible strategy to achieve this goal could be using beneficial microorganisms in the cropping systems, such as BCAs and plant growth-promoting microorganisms (PGPMs), as a tool for sustainable food production without compromising ecosystems services (Abilash *et al.*, 2016). In fact, agricultural practices such as intensive fertilization, soil tillage and the abuse of pesticides are causing damage to the environment and humans (Tsiafouli *et al.*, 2015).

Seed is the basic input in agriculture (Gough et al., 2020); in fact, it is the most commercialized on a large scale globally among vegetable products. John *et al.* (2011) argue that using microbial inoculum to treat seeds is advantageous from both the economic and environmental point of view because with a small amount of inoculum, it is possible to treat a large amount of seeds and consequently, the inoculum can be disseminated on a large scale. For instance, the inoculation of seeds with nitrogen-fixing symbiont bacteria, such as *Rhizobium* and *Bradyrhizobium,* is a common practice that has been implemented in the agriculture of several countries (Ulzen *et al*., 2016). Moreover, according to Adesemoye and Egamberdieva (2013), co-inoculation of PGPR microorganisms significantly improved seed germination, considering that other PGPR can also exert beneficial effects on plant growth.

Seed-borne pathogens are one of the main problems affecting seed quality and agricultural production; they are responsible for the seed rot, reducing germination and in general, the quality of the seeds; as a consequence, there are losses in crops with severe economic losses (Agarwal and Sinclair, 1996). Chemical treatments with antimicrobial compounds contribute to the containment of seed-borne pathogen (Rothrock *et al.,* 2012). However, today we are witnessing a gradual withdrawal of chemicals and alternatives are being sought (Mancini and Romanazzi, 2014).

In the present study was isolated and characterized a microorganism belonging to streptomycetes. *Streptomyces* spp. shows different PGPR traits, and they have a key role in promoting plants via the increase of available nutrients in the soil or by producing secondary metabolites and protecting plants against pathogens. In addition, the dual-culture plate *in vitro* assay method evaluated the antagonist activity against several pathogenic fungi. This approach allowed to screen putative effective microorganisms capable of antagonizing the fungal pathogens of interest for horticultural crops. *Streptomyces sp*. DLS 1568 inhibited the fungal growth of *F. oxysporum* f. sp. *cepae*, *F. oxysporum* f. sp. *raphani*, *Sclerotinia* sp., *Stemphylium* sp., *Colletotrichum dematium*, *Pythium* sp.

with a percentage of inhibition between 60 and 65%. In contrast, the other microorganisms tested, such as *Pseudomonas* sp. PT65 and *Agrobacterium* sp. AR39 exhibited a lower fungal growth inhibition activity. Khamna et al. (2009) demonstrated that among the 396 strains of *Streptomyces* isolated from the soil, only 27 exhibited antifungal activity. The antifungal activity shown by *Streptomyces* is linked to the production of compounds such as secondary metabolites or extracellular hydrolytic enzymes (Tarkka, and Hampp, 2008), as well as compounds active agents important for the degradation of cellulose and chitin (Adegboy and Babalola, 2012). Although *Pseudomonas* sp. showed less antagonistic activity than *Streptomyces*; however, in the literature, it is reported as a bacterium capable of producing antifungal substances (Kim et al., 1999).

In the present study, we observed by *in vitro* test that *Streptomyces* sp. DLS1568 was capable of producing siderophores and solubilizing phosphate. The siderophores are small molecules secreted by many microorganisms that form stable complexes capable of chelating the iron present in the soil. These molecules produced by bacteria can promote plant growth through increased direct availability of iron to plants in iron-deficient conditions or by inhibiting the availability of iron to plant pathogens (Ahmad *et al*., 2008). Furthermore, Phosphorus (P) is one of the essential nutrients that are necessary for plant development and growth. In the natural environment several microorganisms in the soil are able to release phosphorus through solubilization and mineralization of soil phosphorus (Bhattacharyya and Jha, 2012). Viaene et al. (2016) reported that the *Streptomyces* genus is known as one of the major producers of bioactive compounds. These compounds include the production of antibiotics and extracellular enzymes.

The WGS of *Streptomyces* sp. DLS 1568 provided the presence of genes involved in plant growth promotion. In addition, the RAST annotations provided evidence for related genes and operons for iron acquisition, nitrogen, phosphorus and potassium metabolisms, suggesting that *Streptomyces* sp. DLS1568 could be involved in the biological control of plant pathogens and/or remodelling the soil microbiota.

Moreover, in this work, *Streptomyces* sp. DLS 1568 was employed to evaluate the impact on germination seed. From the germination tests on onion and fennel, it was observed that the number of plants germinated from seeds treated with *Streptomyces* sp. DLS1568 was significantly higher than in control. In contrast, the seedlings germinated from lettuce and rocket seeds that underwent the same treatment did not report significant differences from the control, although the germination rate was greater than 90%. In agreement with the advantages of this microorganism, a method has been developed for the application of *Streptomyces* sp. DLS1568 on the seeds with the help of a Bio Friendly 1 binder suitable for industrial processing. For instance, one kilogram of seeds was coated with 20 mL of bacteria inoculum supplemented with Bio Friendly 1, demonstrating the advantage of coating the seed to guarantee its quality. Furthermore, beneficial microorganisms can play a dual role, stimulating the growth and development of the plant and simultaneously they can protect against plant pathogens.

Field tests were carried out in a soil with *Fusarium* spp. historicity, in which were sown treated onion seeds (i.e. Fludioxonil, *Streptomyces* sp. DLS1568, *Streptomyces* sp. SA51 and Ozone). The assessment showed that *Streptomyces* sp. DLS1568 was greater than the control and the fungicide (Fludioxonil). Hence, *Streptomyces* sp. DLS1568 performed an antagonistic action against the pathogen during the first delicate stages of germination, comparable to the action of the fungicide.
The antagonistic action of the microorganism DLS1568 was also evident during the whole agronomic season of onion cultivation; this demonstrates the ability of the microorganism to colonize the plant as well as the seed, as was evident from the tests carried out in the laboratory with the use of *Streptomyces sp*. DLS1568 transformed with the GFP protein and was confirmed through the use of the confocal microscope.

Other field tests took place simultaneously in different locations, Conselice (RA) and Bagnile (CE). The results obtained showed the effectiveness of seed treatment with *Streptomyces* sp. DLS1568, on the growth and development of plants, was comparable to the IRF 360-1 (commercial product based on microorganisms). In addition, at the end of the agronomic crop season, the weight of onion bulbs and those germinated by plants whose seeds had been treated with *Streptomyces* sp. DLS1568 had a heavier weight compared to the control.

Moreover, this study highlighted the use of ozone to treat the seeds, reducing the microbial load and consequently helping to contain the seed-borne pathogens thanks to the activation of oxidizing molecules that play a role in the interaction with the microorganisms present in the seed (Graves, 2012). It has been reported that seed exposed to ozone undergoes physical changes on its surface, making it more likely to absorb water (Starič *et al.*, 2020). While inside the seed, the interaction with ozone molecules stimulates the production of reactive oxygen species involved in regulating biological processes such as growth and development (Graves, 2012).

It is also possible to assist in the use of ozone in the seed coating; Moroenyane *et al.* (2021) report that the microbial community of the seed has priority over the microbial community of the soil during the colonization process of the rhizosphere, but if the microbial community of the seed is destroyed the microbial community of the soil or the microorganisms used for the seed coating can colonize the rhizosphere more easily.

In this study, the action of ozone on seeds was evaluated to improve germination and reduce the microbial load on the seeds. During the tests, different treatment times were tested at the same ozone concentration in order to identify the best timing that reduces the microbial load and stimulates the germination of the seed, as if the exposure is too much prolonged on the one hand there is a reduction of the microbial load in the seed, but on the other hand it can compromise the vitality of the seed. Results showed that 45 min of exposure decreases the microbial load, does not compromise vitality and stimulates the germination of the seed. Furthermore, this timing obtained is ideal for industrial applications as times are important in the seed industry.

Numerous variables affect the ozone disinfection capabilities of the liquid during processing. The flow rate, ozone concentration, temperature, ozone state/phase, pH, and presence of organic matter are all intrinsic and extrinsic elements that impact ozone's efficacy. Numerous studies have been conducted on the effects of ozone on plant development and seed germination, for instance, Sudhakar et al. (2008) evaluated the influence of low-concentration ozone on tomato seedling development (0.1, 0.2, 0.3, and 0.4 ppm). Exposure to 0.2 ppm ozone for two minutes each day for ten days had a beneficial impact on the growth characteristics of tomato seedlings, including increased leaf area, root length, shoot size, and biomass output. According to Sudhakar et al. (2011), applying modest amounts of ozone to tomato seeds increased germination and resulted in seedlings with longer roots. On the other side, Wu, Doan, and Cuenca (2006) demonstrated that prolonged exposure to ozone gas had a detrimental impact on the germination ability of wheat seeds. Furthermore, ozone has been evaluated for its efficacy in eliminating infections from a variety of seed surfaces (Mohammada et al., 2019).

Additionally, ozone treatment of cotton seeds resulted in aflatoxins being destroyed (Dwarakanath et al. 1968). Kang et al. (2015) investigated the antifungal activity of arc discharge plasma and ozone on diseased rice seedlings. Finally, Rodrigues et al. (2019) examined the efficacy of gaseous ozone concentrations of 15 and 25 g/m3 for 0 to 120 min exposure duration as a disinfectant against six plant diseases infecting soybean seeds (Penicillium sp., Phomopsis sp., Fusarium sp., Aspergillus sp., Alternaria sp., and Cercospora kikuchii).

In conclusion, microbial seed coating has the potential to be a cost-competitive and time-saving approach for crop production and protection, reducing application efforts and providing different and desirable characteristics to the seeds. *Streptomyces* sp. DLS1568 has great potential to become an essential constituent of agricultural practice as a biocontrol agent. This microorganism has the potential to be used to coat seeds, to improve plant growth promotion in a way that could lead to the increased sustainable production of agricultural products.

In addition, the use of plant beneficial microbes incorrect formulations provides a potential solution for a more sustainable agricultural future. However, the importance of continuing research on these microorganisms is still needed to design and implement industrial processes that are able to produce effective formulations with one or more microbial agents.

The ozone treatment improves germination, promotes growth and increased yield. Furthermore, the treatment of the seeds with ozone is feasible and advantageous, as it inactivates the seed-borne pathogens and stimulates the germination of the seed. The application of ozone on seeds can be an alternative to chemical treatments; in fact, the ozone molecule degrades in a short time, leaving no residues in the environment. The research focused on identifying the right concentration of ozone to be applied to the seed and establishing the ideal timing throughout the production process.

Overall, this thesis work has allowed the identification of a microorganism that can be an alternative to commercially available fungicides. Moreover, the patenting phase of the *Streptomyces* sp. DLS1568 is underway for future commercial uses.

6. References

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PUBLICATIONS

Soukaina Ben Othmen, **Gianmarco Conti Nibali**, Stefano Cassanelli, Sara Pipponzi, Emilio Stefani. (**2022**). Development and implementation of a viability-qPCR protocol to confirm sanitation of carrot 2 seeds from *Ca. Liberibacter solanacearum*. Submitted to ***PHYTOPATHOLOGY.***

REPORT OF ACTIVITIES

1st Year

PARTICIPATION TO THE SCHOOL COURSES

Date: 14/01/19; 15/01/19; 16/01/19; 28/01/19; 29/01/19; 30/01/19.

Title/subject: Scientific English

Date: 11/03/19; 18/03/19.

Title/subject: Introduction to MATLAB environment

Date: 29/05/19

Title/subject: The model organism *Saccharomyces cerevisiae* mitochondrial inheritance as case study

Date: 12/06/19; 14/06/19; 17/06/19

Title/subject: Applications of multivariate analysis in the agri-food context

Date: 04/07/19

Title/subject: Management and exploitation of bioresources the fundament roles of the microbial culture collections

Date: 25/09/19

Title/subject: Pest Risk Analysis and Management of Alien Pests

Date: 02/10/19

Title/subject: Model plants I

Date: 04/10/19

Title/subject: Animal models

Date: 09/10/19

Title/subject: Model plants II

2nd Year

Date: 07-08/10/2020

Title/subject: Infrared Spectroscopy

PARTICIPATION TO SYMPOSIA

Date: 08/11/2019

Location: Imola (BO)

Title: 1° Onion Day : Disseccamento fogliare fisiologico e da *Stemphylium* nella coltivazione della cipolla da mercato 121

3rd Year

Date: 27/05/2021; 03/06/2021

Title/subject: Functional Genomics Approaches in Crop Plants

Date: 18/06/2021

Title/subject: Regulation and use of bio-stimulants in agriculture

PARTICIPATION TO SYMPOSIA

Date: 28-30/06/2021

Location: Belgrade (online)

Title: 4th Annual Conference on “Integrating science on Xanthomonadaceae for integrated plant disease management in Europe”