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Innovative and environment-friendly strategies for processing tomato (*Solanum lycopersicum* L.) production

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General Index

Abstract

Processing tomato is a worldwide economic important crop and his yield and quality are strictly affected by fertilizer applications. In fact, the processing tomato yield is lower in organic systems in comparison with conventional ones. In addition, most cultivated genotypes are sensitive to chilling in all growth stages and extremely dependent on irrigation water. In this view, the present PhD project aimed to increase the tolerance to abiotic stresses and the sustainable production of processing tomato exploiting the biodiversity of the species and the beneficial effect of the soil microbiota.

Focusing on the abiotic stresses that limit processing tomato growth at seedling stage, we evaluated the effects and the interactions between beneficial microorganisms (*Funneliformis mosseae*, *Rhizophagus intraradices* and *Paraburkholderia graminis*) and processing tomato genotypes ('Pearson', 'H3402' and 'Everton') under chilling or drought stresses (chapters three and four). Our results showed that *F. mosseae* was the most effective in reducing the chilling damage and in mitigating the effects of drought on morphological and physiological traits. In addition, specific genotype x microbiota x stress interactions were also revealed.

The use of rootstocks ('RS01658654' and 'Tomito'), in combination with or without beneficial microorganisms (alone and in consortia) (*Funneliformis mosseae, Paraburkholderia graminis* and *Azospirillum brasiliensis*), was studied in order to improve marketable yield and quality under organic cropping system (chapters five and six). Interestingly, 'H3402' grafted onto 'Tomito' and inoculated with *A. brasiliensis* was observed to display early flowering in greenhouse. While in the field, grafting plus beneficial microorganisms (*P. graminis*, *A. brasiliensis* and their consortium) increased marketable yield, fruit quality and reduced the number of fruits affected by blossom-end rot.

Finally, we hypothesized that differences in processing tomato performances associated to different forms of nitrogen could be determined, at least in part, by a differential recruitment of bacteria at the root-soil interface. To test this hypothesis, a single genotype was grown in open field and subjected to seven fertilizer treatments (same amount of N) (chapter seven). Using a cultivation-independent protocol we assessed crop yield, quality and microbiota

composition demonstrating that each treatment produced “distinct signatures”, represented by specific selective enrichment on both the rhizosphere and root community.

In our results, we provide evidence for the use of beneficial microorganisms and grafting to improve adaptation, yield and quality of processing tomato. However, specific beneficial microorganisms x genotype/cropping system interactions should be considered to produce *ad hoc* biostimulants. All the presented approaches could be a key strategy towards improved fertilization and irrigation water managements to increase fruit yield and quality, and we foresee an effective exploitation of the plant microbiota for agricultural purposes.

Sintesi

Il pomodoro da industria è una coltura economicamente importante a livello mondiale e la sua resa e la qualità sono strettamente legati all’uso dei fertilizzanti. Infatti, la resa del pomodoro da industria è inferiore nei sistemi biologici rispetto a quelli convenzionali. Inoltre, la maggior parte dei genotipi coltivati sono sensibili, in tutti gli stadi di sviluppo, alle basse temperature ed estremamente dipendenti all’irrigazione. In quest’ottica, lo scopo del presente progetto di dottorato è stato quello di migliorare la tolleranza agli stress abiotici e aumentare la produzione sostenibile del pomodoro da industria sfruttando la biodiversità delle specie e l’effetto positivo dei microorganismi del suolo.

Focalizzando l’attenzione sugli stress abiotici che limitano la crescita delle piantine del pomodoro da industria, abbiamo valutato l’effetto e le interazioni tra microrganismi benefici (*Funneliformis mosseae*, *Rhizophagus intraradices* and *Paraburkholderia graminis*) e diversi genotipi di pomodoro da industria (‘Pearson’, ‘H3402’ e ‘Everton’) sottoposti a stress idrico o termico (capitoli tre e quattro). I nostri risultati mostrano che *F. mosseae* è stato il più efficace a ridurre i danni dovuti all’esposizione delle piantine alle basse temperature e a mitigare gli effetti della siccità a livello fisiologico e morfologico. Inoltre, sono state osservate diverse interazioni significative tra genotipi, microrganismi e stress.

L’uso di portainnesti (‘RS01658654’ e ‘Tomito’), inoculati o meno con microrganismi benefici (da soli o in consorzio) (*Funneliformis mosseae*, *Paraburkholderia graminis* e *Azospirillum brasiliensis*), è stato studiato al fine di incrementare la resa commerciale e la

qualità dei frutti in sistemi biologici (capitoli cinque e sei). È interessante notare che 'H3402' innestato su 'Tomito' ed inoculato con *A. brasiliensis* ha avuto, in serra, una fioritura precoce, mentre in campo, lo stesso innesto inoculato con alcuni microrganismi benefici (*P. graminis*, *A. brasiliensis* ed il loro consorzio) ha mostrato un aumento della resa commerciale e della qualità dei frutti, ed una riduzione del numero di frutti affetti da marciume apicale.

Infine, abbiamo ipotizzato che le differenti performance del pomodoro da industria, associate all'utilizzo di forme diverse di azoto, fossero determinate, almeno in parte, da un differente reclutamento di batteri all'interfaccia radice-suolo. Per valutare quest'ipotesi, un singolo genotipo è stato coltivato in campo e soggetto a sette diversi tipi di trattamenti fertilizzanti (stessa quantità di azoto) (capitolo sette). Usando un protocollo indipendente dalla coltura, è stata valutata la resa, la qualità e la composizione del microbiota dimostrando che ogni trattamento è in grado di produrre delle caratteristiche distintive, rappresentate dall'arricchimento di specifici microorganismi delle comunità microbica della radice e della rizosfera.

In questo lavoro, sono state fornite prove sull'efficacia dell'uso di microrganismi benefici e dell'innesto per migliorare la resa e la qualità del pomodoro da industria. Tuttavia le interazioni tra specifici microrganismi benefici, genotipi e sistemi di coltivazione dovrebbero essere considerate per produrre biostimolanti *ad hoc*. Inoltre, tutti gli approcci presentati potrebbero essere la chiave per migliorare la gestione della fertilizzazione e dell'acqua di irrigazione, per aumentare la resa e la qualità dei frutti e anticipare l'efficace sfruttamento del microbiota delle piante per fini agricoli.

Chapter 1

General Introduction

1.1 Challenges of modern agriculture and possible solutions

1.1.1 Challenges of modern agriculture

Agriculture is mainly considered the science that cultivates the soil, growing crops and raising livestock for human nutrition. Furthermore, agriculture also provides no food products for fabrics and paper and energy production. In 2018, the European agri-food trade reached a value of €254 billion - €138 billion of exports and €116 billion of imports (European Union, 2019).

Nowadays, modern agriculture faces many challenges. Certainly, the first one is to increase the production of food to satisfy the increasing demands of a growing population. In fact, the worldwide population is expected to reach ca. 10 billion by mid-century (FAO, 2019; Schröder et al., 2019).

Over the past 50 years, agricultural intensification by use of high-yielding crop genotype, fertilizers, irrigation and plant protection products has contributed to increase crop production (Matson et al., 1997). Agricultural intensification, however, cause a high exploitation of natural resources and an increase of greenhouse gas emissions and environmental pollution. Furthermore, intensive agricultural management has contributed to biodiversity loss, soil erosion and changes to nitrogen and carbon cycles (Lorenz and Lal, 2016).

More than many other sectors, agriculture is depended on the weather and climate, and the global climate changes (such as rising temperatures, changing precipitation regimes, and increased atmospheric carbon dioxide levels) are influencing crop performances. In particular, climate changes are modifying the needs and issues linked to the production of different crops and increasing the incidence of extreme weather events (e.g., drought, extreme temperatures, etc.) affecting crop productivity and quality (Parry et al., 2004). Predictions have estimated that the cropping area affected by drought will be increased by two folds, and water resources declined by 30% by 2050 (Falkenmark, 2013).

Nowadays, consumers' concerns are increasing for the environment conservation and for their family's health and well-being causing an increase of the demands of sustainable foods (Compant et al., 2019; Valiante et al. 2019). In this view, it is necessary to develop and apply innovative strategies in order to increase productivity in an environment-friendly manner. To reach this objective, the agrosystems should rapidly adapt to environment and climate changes

moving towards a resource-efficient agriculture and food and feed supply chains which reduce the use of natural resources.

1.1.2 Possible solutions towards a more sustainable agriculture

One promising and sustainable approach to increase the crop production might be the exploitation of the wide variety of microorganisms present in the soil (Raklami et al., 2019) and the relationships that these microorganisms create with plants. In fact, soil-borne microorganisms create neutral, mutualistic, commensalistic or parasitic relationships with host plants (Wang et al., 2019). Numerous studies conducted with soil-borne microorganisms have revealed a series of beneficial activities for the host plants (reviewed e.g. in Dimkpa et al., 2009). The best performances are attributed to bacteria belonging to the genera *Arthrobacter, Azotobacter, Azospirillum, Bacillus, Enterobacter, Paraburkholderia, Pseudomonas, Serratia, Stropmtomyces* (reviewed e.g. in Dimkpa et al., 2009), and to fungi belonging to arbuscular mycorrhizal fungi (Bonfante and Genre, 2010). The main advantage concerns the improvement of the uptake of macro and micronutrients. The nutrients uptake may be improved indirectly by stimulating the root growth or directly by fixing nitrogen or by converting insoluble minerals (e.g., phosphorus) to bioavailable forms (Vessey, 2003). Urquiage et al. (2012) reported that Brazilian commercial and spontaneous cane varieties can obtain over 40 kg N ha^{-1} yr^{-1} from nitrogen fixation by microorganism-plant associated. A recent study showed that *Paraburkholderia graminis* can bind iron, an essential element for chlorophyll production, producing the siderophore gramibactin (Hermenau et al., 2018). Other compounds stimulating host plants, such as phytormones, can be produced by beneficial microorganisms. In addition, microorganisms can also induce plants to produce phytormones. For example, a study on wheat reported that *Azospirillum brasilense* can induce the gene of indole-acetic acid when inoculated on root surface (Rothballer et al., 2005). Beside the growth plant stimulation, beneficial microorganisms can help plants to cope with abiotic and biotic stresses. Recently, a study on chickpea has reported that *Azospirillum lipoferum* FK1 alleviated salinity stress damage by modulating osmolytes, antioxidants machinery and stress-related genes expression (El-Esawi et al., 2019). As outlined above, many positive effects can result by the use of plant growth promoting (beneficial) microorganisms. However, the interactions between crop and microorganisms are specific for each crop species (Berg and Smalla, 2009). Therefore, it is very

important to carry out experiments both in controlled condition and open field to verify the effectiveness towards specific crop.

Another way to increase crop production, in a sustainable manner, could be the exploitation of the available biodiversity of crop species. Over the years, the classical plant breeding has been extremely successful to develop elite crop genotypes with high yields and desirable traits. Although, the marker-assisted and genomic selections can improve the efficiency and reduce the time of the breeding process, multiple rounds of backcrossing and selection to obtain the elite genotypes are necessary (Wolter et al., 2019). Recently, as a result of scientific progress, a new generation of targeted genome editing technology, called CRISPR–Cas9, is catching on. This technique is extremely simple and versatile (Song et al., 2016) and can combine agronomically desirable traits with useful traits, like that present in wild lines, in a quick and economic way (Zsögön et al., 2018). Nonetheless, gene-edited crops are not allowed in Italy and in organic farming since they are still considered as conventional genetically modified (GM) organisms (Callaway, 2018). Among other techniques, grafting could be an alternative way to classical breeding process, allowing to achieve the benefits of two plants in shorter time and at lower cost. In fact, it is a technique that allows the union of living tissue of two or more plants by vascular connection. The use of selected rootstocks can improve many traits of scion like growth, development and fruit yield and quality (Djidonou et al. 2013; Flores et al. 2010; Venema et al. 2008). Selected rootstocks can also improve the plant tolerance against soil-borne pathogens such as *Ralstonia solanacearum* (Grimault and Prior 1994), *Fusarium* spp. (Polizzi et al. 2015), *Meloidogyne* spp. (Owusu et al. 2016; Yin et al. 2015), *Verticillium* spp. (Paplomatas et al. 2002). In addition, specific rootstocks could be exploited to induce resistance to abiotic stresses. Wang et al. (2017) reported that, under saline water irrigation, grafting reduces the stress damage by maintaining low Na^+ concentration and high K^+/Na^+ ratio in shoots, and improving chlorophyll a and b and carotenoid contents, stomatal conductance and transpiration rate. In tomato, the use of a low-temperature tolerant rootstocks increased shoot growth at suboptimal cultivation temperatures by stimulation of the leaf expansion rate (Venema et al., 2008). Zang et al. (2019) found that tomato grafted with drought-tolerant seedlings alleviated the phytotoxicity and oxidative damages caused by drought by regulating antioxidant enzymes. Also some physiological disorders can be minimized by using selected rootstocks (Lee et al., 2010). In fact, rootstocks can influence the production of phytohormones such as abscisic acid and cytokinins (Dong et al. 2008). A study reported that

tomato water use efficiency can be improved exploiting rootstock-derived hormonal signals which control leaf growth (Cantero-Navarro et al. 2016). Finally, different crop species can be combined (e.g. tomato grafted on potato) in order to harvest more than one final product like tomato and potato by the same plant (Lee et al., 2010).

1.2 Tomato (*Solanum lycopersicum* L.)

1.2.1 History, domestication and agronomic importance

Tomato is the second most cultivated crop in the world (Casal et al., 2019; Foolad 2007; Rothan et al., 2019). The worldwide production of tomatoes has increased ~ 300% during the last 40 years (Costa and Heuvelink 2007). In 2018, the production was ~ 182 million tonnes and the major producing countries are China, India, Turkey, USA, Egypt, Iran and Italy (FAO, 2019).

Tomato is a tropical plant that come from Andean region of South America (Chile, Bolivia, Ecuador, Colombia and Peru). However, nowadays it is grown in different areas of the world (Foolad 2007). Tomato belongs to the large family of Solanaceae which includes more than three thousand species (Peralta et al., 2008). The original name of cultivated tomato was *Solanum lycopersicum* L. (Linnaeus, 1753). However, in 1754, tomato was inserted in the new genus *Lycopersicum* and in the specie *esculentum* by Miller (Miller, 1754). Since then, a discussion on distinction of genus *Lycopersicum* from genus *Solanum* has started, as many researchers were not completely in agreement with the new classification (Foolad, 2007). Subsequently, studies on chloroplast DNA restriction site have modified again the phylogenetic classification of the Solanaceae replacing tomato in the *Solanum* genus (Knapp et al., 2004; Olmstead et al. 1999; Spooner et al.,1993).

The modern cultivated tomato genotypes come from domestication of their supposed wild progenitors, *Lycopersicon pimpinellifolium* and/or *L. esculentum* var. *cerasiforme* (Luckwill 1943; Jenkins 1948; Rick 1976). Classical breeders, exploiting tomato germplasm in attempts, aimed for creating larger-fruited genotypes with higher yields suitable for greenhouse and fresh market purposes (Lippman and Tanksley, 2001).

For its features, tomato is used as a model plant in many research areas like genetics, agronomic, physiology, etc. (Foolad, 2007; Rothan et al., 2019). In fact, this species is easy to cultivate under different environmental conditions, has an annual life cycle, is easy to cross (self-pollinated) and is a diploid species (2n = 2x = 24) with a genome of ~ 900 Mb that is fully

sequenced (Foolad 2007, Rothan et al., 2019). In addition, recently, Gao et al. (2019) have also constructed the tomato pan-genome using 725 representative accessions, revealing 4,873 genes absent from the reference genome.

Tomato is an important part of human diet as its consumption contributes to the uptake of vitamins (A and C), minerals and antioxidant compounds (Foolad, 2007). In addition, the significant amount of lycopene contained in the fruits, leads to the health promoting benefits of tomatoes. In fact, lycopene seems to play a role in the prevention of different health issues such as cardiovascular disorders, digestive tract tumors and in inhibiting prostate carcinoma cell proliferation in humans (Levy and Sharoni, 2004).

Tomato is cultivated for both fresh market and processing industry purposes. Genotypes suitable for canning purposes derived by specific breeding programs to obtain genotype suitable for mechanical harvest (Casals et al., 2019).

In canning industries, tomato fruits were used to produce many products like passata, tomato paste, whole peeled or diced tomatoes and vary type of juice and sauces (Foolad 2007). Processing tomato production was ~ 20.7% of the all tomato production. In 2018, the estimated total value of the worldwide processing tomato harvest was ~ 2.8 USD (WPTC, 2019). California (~ 11 million tons) and Italy (~ 4.6 million tons) are the worldwide producer leader (WPTC, 2019) (Figure 1).

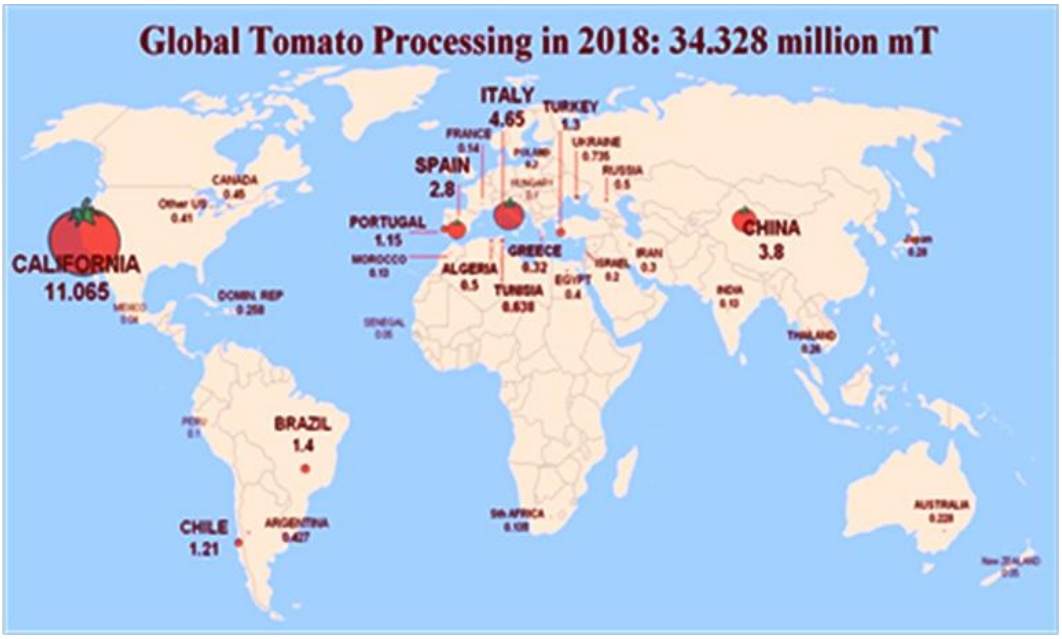

Figure 1: Tomato processing in 2018 in the world (WPTC, 2019)

1.2.2 Major issues related to processing tomato production

One of the main issues related to processing tomato production is the reduction of damage due to some abiotic stresses. In fact, environmental stresses limit plant growth and yield, leading to high losses. Among abiotic stresses, chilling and drought are the main stresses that affect processing tomato and to cope with these stresses would have important economic impacts on processing tomato yield (Shinozaki and Yamaguki-Shinozaki, 2000). In particular, chilling affects the development and productivity of crops originating from tropical and subtropical regions, such as the processing tomato, as they miss the genetic information to be or become chill tolerant (Allen et al., 2001). The optimal mean daily temperature of processing tomato range between 20 and 25 °C, the tomato growth is reduced or inhibited when the temperature drops below 12 °C and temperature below 0-2 °C can cause the death of tomato plants (Cao et al., 2015; Sadashiva et al., 2013). In addition, it is cultivated especially in temperate regions where chilling events are common during the initial phase of the growing season (Ronga et al., 2018). Unlike the fresh tomato that may also be cultivated in heated greenhouses (preserving plant to chilling occurrences), processing tomato plants are cultivated only in open fields where chilling occurrences cannot be avoided. When processing tomato plants are exposed to chilling, lamina withering, leaf chlorosis and local necrosis can be observed (Tatsumi and Murata 1981). Chilling stress induces severe damage affecting cell membrane integrity (Ronga et al., 2018). The chloroplasts are the primary visible sites of chilling injury (Kimball and Salisbury, 1973). All major components of photosynthesis, such as thylakoid electron transport, the carbon reduction cycle and control of stomatal conductance, can be disrupted (Allen et al., 2001) causing a reduction of chlorophyll fluorescence and photosynthetic activity (Yun et al., 1996). Finally, after chilling event, reactive oxygen species were generated causing damage by lipid peroxidation, protein degradation, breaking of DNA, and cell death (Tian and Li 2006).

Besides the damage due to chilling stress, processing tomato is extremely dependent on irrigation water, the average water requirement ranging from ~ 400 to 600 mm based on climatic conditions of the area (Rana et al., 2010). Therefore, a scarce water availability, during the growth cycle, could lead to a decrease in fruit yield and quality. When the potential transpiration rate exceeds the water absorption by the roots from the soil, the crops experience water stress.

Initially, crop water potential and turgor are reduced interfacing with normal functions (Shao et al., 2008). At the same time, plant stomata are closed reducing the gas exchange and leading to a decrease of carbon dioxide uptake with a reduction in photosynthetic activity (Chitarra et al., 2016; Osakabe et al., 2014). On the other hand, the lack of water affects the metabolism and cell structure and leads to the cessation of enzyme catalysing reactions (Shao et al., 2008). As for chilling stress, ROS substances, such as superoxide, hydrogen peroxide and hydroxyl radicals are produced (Lei et al. 1998). Additionally, drought stress reduces nutrient uptake, leading to a decrease in macro and micro element availability (Sardanrs et al., 2004). Finally, the water deficit affects the plant growth through the repression of gene expression related to cell division and proliferation (Claeys et al., 2013; Todaka et al., 2017).

For a sustainable production that respects the environment, a wide range of benefits can be expected from the production of processing tomato production in low input systems such as organic farming. Reviews and meta-analyses revealed that organic farming systems have higher soil carbon levels, better soil quality and less soil erosion compared with conventional systems (Tuomisto et al., 2012; Mondelaers et al., 2009; Gomiero et al 2011). Also the biodiversity is improved in organic systems (Crowder et al., 2010; Lynch et al., 2010; Mondelaers et al., 2009). In addition, synthetic pesticides are not allowed and there is a reduction of nitrate and phosphorus leaching and greenhouse gas emissions in comparison with conventional farming systems (Lee et al., 2015; Lynch et al., 2012; Reganold and Wachter, 2016). However, different studies showed that processing tomato yield was ~ 35% lower in low input systems compared to integrated/conventional systems (de Ponti et al., 2012; Ronga et al., 2015; Zaccardelli et al., 2012). Furthermore, Ronga et al. (2019) reported that the primary energy demand and the global warming potential were higher in organic farming system in comparison with conventional one when 1 ton of marketable fresh processing tomato fruits was considered.

Nitrogen is considered the main key limiting factor responsible for lower productivity in low input systems (Möller et al., 2008). Indeed, the low nitrogen mineralization availability, showed by organic fertilizers, rarely is able to satisfy the tomato nutrient requests (Ronga et al., 2015). In addition, Scholberg et al., (2000) reported that nitrogen deficiency can reduce tomato LAI, biomass, and fruit yield by 60 to 70%.

In light of the observations reported above, researchers, industries and farmers are called to develop strategies able to reduce the damage due to abiotic stresses and increase the

sustainability of the processing tomato production especially in low input cropping systems which themselves tend to be rather eco-friendly.

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Chapter 2

Research aims

The present PhD project aimed to increase the tolerance to abiotic stresses and the sustainable production of processing tomato (*Solanum lycopersicum* L.) exploiting the biodiversity of the crop species and the beneficial effect of the soil microorganisms.

Multi-disciplinary approaches - integrating agronomy, physiology, microbial genomics - were undertaken and, on these premises, 5 experiments were carried out:

- ✔ In the first experiment (reported in Chapter 3) the attention was focused on an abiotic stress (chilling) that limits processing tomato growth and yield during early transplant, and on the effect of some arbuscular mycorrhizal fungi and plant growth promoting rhizobacteria in improving chilling tolerance of processing tomato seedlings. In particular, the objectives of this research were to investigate: (i) *Funneliformis mosseae* and *Paraburkholderia graminis* C4D1M in avoiding processing tomato damage during severe chilling stress; (ii) the synergic effect of the two microorganisms inoculated as a consortium on chilling tolerance; (iii) the interactions between microorganisms and genotypes bred in different years.
- ✔ In the second experiment (reported in Chapter 4), we assessed, in a growth chamber, the physiological and morphological responses, at the seedling stage, of three reference processing tomato genotypes inoculated with the two arbuscular mycorrhizal fungi (AMF) *Funneliformis mosseae* and *Rhizophagus intraradices*, under two water regimes (fully-watered and reduced).
- ✔ In the third experiment (reported in Chapter 5), in order to improve marketable yield of processing tomato plants when produced in the organic cropping system, a non-commercial processing tomato genotype "TC266" was grafted onto an interspecific rootstock "RS01658654" (RT1) and transplanting in an open field in an organic farm. During two growing seasons, morphological, physiological and agronomic performances of grafted processing tomato were compared to no-grafted and self-grafted ones.
- ✔ In the fourth experiment (reported in Chapter 6), the effects of a cherry rootstock genotype 'Tomito' inoculated with different microbial plant biostimulants (alone and in consortium) were evaluated on agronomic performances of a commercial processing tomato genotype 'H3402' under controlled (greenhouse) conditions and in an organic open field trial.

- ✔ In the fifth experiment (reported in Chapter 7), we hypothesized that differences in processing tomato performances associated to different forms of nitrogen could be determined, at least in part, by a differential recruitment of bacteria at the root-soil interface. To test this hypothesis, we grew a single genotype of processing tomato, in the same soil type and subjected to seven fertilizer treatments using the same amount of nitrogen (150 kg ha^{-1}). Crop yield and quality were recorded, and microbiota composition using a cultivation-independent protocol was assessed.

Chapter 3

Arbuscular mycorrhizal fungi and plant growth promoting rhizobacteria avoid processing tomato leaf damage during chilling stress

Federica Caradonia, Enrico Francia, Caterina Morcia, Roberta Ghizzoni, Lionel Moulin, Valeria Terzi and Domenico Ronga, 2019. Arbuscular Mycorrhizal Fungi and Plant Growth Promoting Rhizobacteria Avoid Processing Tomato Leaf Damage during Chilling Stress. Agronomy, 9(6), 299. https://doi.org/10.3390/agronomy9060299

3.1 Introduction

Environmental stresses limit plant growth and yield, leading to high losses. In particular, chilling affects the development and productivity of crops originating from subtropical regions (Ma et al., 2018; Zhang et al., 2004), such as tomato (Rui et al., 2018). Tomato (*Solanum lycopersicum* L.) is an economically important horticultural crop (Ronga et al., 2017, 2019a), cultivated worldwide over ~4.7 million ha, and with a total production of ~177 million tons (FAO, 2019).

Tomato fruits are consumed fresh in salads and sandwiches or processed into products like whole peeled, diced products, juices, sauces and soups (Foolad, 2007). The genotypes suitable for processing tomato production are bred for adaptation to mechanical harvesting and canning purposes (Casals et al., 2019). Most of the cultivated processing tomato genotypes are sensitive to low temperatures (0–12 °C) in all growth stages (Foolad and Lin, 2000). The negative effects of chilling are more remarkable during germination and at the seedling stage (Ghanbari and Sayyari, 2018). Chilling damages cellular membranes, generates Reactive Oxygen Species (ROS) and accumulates toxic compounds (Allen and Ort, 2001; Kuk et al., 2003; Nayyar et al., 2005). In addition, the chloroplasts are the primary visible sites of chilling injury (Kimball and Salisbury, 1973) that causes a reduction of chlorophyll fluorescence and of photosynthetic activity (Yun et al., 1996).

Unlike the fresh tomato that may also be cultivated in heated greenhouses (preserving plant to chilling occurrences), processing tomato plants are cultivated only in open fields where chilling occurrences cannot be avoided. In Mediterranean environments, such as Italy and Spain, the growing season starts in March-April, when the probability of chilling events is still high (Ronga et al., 2018). A typical management practice applied by farmers is to transplant in the field processing tomato seedlings produced in nursery. Besides the possibility of chill spells in early spring, an increase of temperature and more frequent drought events have been reported in climatic models for the 21st century, especially in Southern European countries (Lovelli et al., 2017). Warmer temperatures can accelerate tomato phenology, resulting in lower total biomass accumulation with a negative impact on yield (Ventrella et al., 2012). It is therefore expected that the practice of early transplant will become increasingly widespread.

The use of beneficial microorganisms could be a sustainable way that allows a reduction of external inputs and improve tolerance to biotic and abiotic stresses (Daranas et al., 2018). In fact, microorganisms, such as arbuscular mycorrhizal fungi (AMF) or/and plant growth promoting rhizobacteria (PGPR), can increase plant tolerance to abiotic stresses like drought, salinity, metal toxicity and high temperature on many crops like wheat (*Triticum aestivum* L.), sunflower (*Helianthus annuus* L.), pea (*Pisum sativum* L.), barley (*Hordeum vulgare* L), rice (*Oryza sativa* L.), tomato (*Solanum lycopersicum* L.) grapevine (*Vitis vinifera* L.), lettuce (*Lactuca sativa* L.) and corn (*Zea mays* L.) (Calvo-Polanco et al., 2016; Grover et al., 2011; Ilangumaran and Smith, 2017; Lanfranco et al., 2017). AMF belong to phylum Glomeromycota and are the most widespread fungal symbionts of plants (Bonfante and Genre, 2010; Gutjahr and Parniske, 2013), including many agricultural crops (Smith and Read, 2008). AMF provide several benefits to host plants, like enhanced water and nutrient uptake, increased tolerance to soil-borne pathogens and environmental stresses, reduced sensitivity to heavy metals and positive contribution to soil structure (Baum et al., 2015; Cavagnato et al., 2012; Gosling et al., 2006; Hart et al., 2014; Subramanian et al., 2006; Vangelisti et al., 2018; Zouari et al., 2014). These benefits could be ascribed to the influence of AMF on plant physiology and, in particular, on plastid biosynthetic pathways, Krebs cycle and secondary metabolism (Vangelisti et al., 2018).

PGPR include a wide range of microorganisms which positively influence growth, yield and stress tolerance of plants through several direct and indirect mechanisms of actions colonizing both the rhizosphere and the endo-rhizosphere (Ahmad et al., 2008; Bhattacharyya and Jha, 2012; Ruzzi and Arocas, 2015; Shameer and Prasad, 2018). PGPR can induce physical and chemical changes in the plants by producing enzymes, osmolytes, siderophores and organic acids or/and by triggering the plants to produce hormones (Ilangumaran and Smith, 2017; Numanad et al., 2018; Ruzzi and Arocas, 2015; Shameer and Prasad, 2018). Ait Barka et al. (2006) reported an increase of phenolic compounds and starch in leaves and shoots of grapevine cv. Chardonnay after treatment with the endophytic bacterium *Paraburkholderia phytofirmans*. In addition, when explants of grapevine were exposed to 4 °C for 2 weeks, an increase of the content of proline was recorded compared to the un-inoculated control. Interestingly, some PGPR, such as *Agrobacterium* spp., *Azospirillum* spp., *Azotobacter* spp. and *(Para)Burkholderia* spp., can promote mycorrhiza colonization (in this case, the PGPR are named mycorrhiza helper bacteria–MHB) (Duponnois, 2006).

Although some evidence of the positive influence of AMF and PGPR under sub-optimal temperatures has been reported (Abdel Latef and Chaoxing, 2011; Ait Bakra, 2006; Liu et al., 2016) on tomato and cucumber (*Cucumis sativus* L.), a precise characterization of the physiological responses in terms of photochemical efficiency of photosystem II (PSII), integrity of cell membranes, recovery and regrowth capacity of inoculated tomato plants exposed to severe chilling (1 °C for 24 h) is missing. In fact, these parameters are very important because, as previously reported, the earliest visible damage caused by chilling stress is the impairment of the integrity of cell membranes and of photosystem activity. In addition, a study on four tomato recombinant inbred lines inoculated with AMF and PGPR showed that, during doughty stress, the microbial inoculant effects were depending on the recombinant inbred line considered (Calvo-Polanco et al., 2016).

Hence, the objectives of this research were to investigate: (i) the efficacy of *F. mosseae* and *P. graminis* C4D1M in avoiding injuries to cell membranes and reduction of PSII efficiency after severe stress (1 °C for 24 h); (ii) the synergic effect of the two microorganisms inoculated as a consortium; (iii) if the putative microorganism effects depended on the processing tomato genotype.

3.2 Materials and Methods

3.2.1 Plant Materials, Growing and Stress Conditions

In the present study, two experiments were carried out; in the first preliminary experiment the genotype 'Everton' was used, while the second experiment was performed by comparing three genotypes released in different years: an old and well-known genotype 'Pearson'; the most commonly transplanted in the Northern Italy 'H3402′; and the more recent cultivar 'Everton'. The main features of the three cultivars are summarized here: 'Pearson' was released in the mid−1930s by the University of California-DAVIS. This cultivar is bushy, self-topping, semi-determinate, has dense foliage, develops globular and large fruits, and is suitable for canning (Ronga et al., 2018). 'H3402′ was released in 2002 by HEINZ; it is determinate, rustic with good vigor, bushy, has a good yield, provides a medium oval fruit, and is suitable for canning (Ronga et al., 2018). 'Everton' was released in 2008 by ISI-Sementi, it is an all-flesh genotype, rustic with medium vigor, high yielding, and suitable for dicing production (also frozen). The seeds were kindly provided by Dr. M. Beretta, ISI Sementi Company, Fidenza,

Italy. Growth chamber experiments were conducted following a fully randomized experimental design. Each treatment consisted of nine plants with three replications. The seeds were germinated on moistened filter paper in a Petri dish at 25 °C for 3 days. Then the germinated seeds were transferred in the alveolar fixed seed trays (20 mm diameter holes, height of 60 mm in the first experiment; 30 mm diameter holes, height of 60 mm in the second experiment) filled with neutral peat composed of 23% organic carbon, 0.5% nitrogen (N) and dry apparent density 214 kg m^{-3} (Dueemme S.r.l., Reggio Emilia). Before transferring the germinated seeds in alveolar fixed seed trays, *F. mosseae* was mixed with peat 10% (*v/v*) (1 g of inoculum contained 10 propagules) as suggested by Rivero et al. (2015). The arbuscular mycorrhizal fungal inoculum was obtained from MycAgro, LabTechnopôle Agro Environnement, Bretenière, France.

Nine days after sowing, when cotyledons were completely unfolded (Meier, 2001), 1 mL of bacterial inoculum (10^7 CFU mL^{-1} of *P. graminis* C4D1M; determined according to a preliminary test) was added close to the plant's root collar. A single colony of bacterium was cultivated in a 250 mL Erlenmeyer flask containing 60 mL of Tryptone Soya Yeast extract broth. The flask was incubated at 28 °C at 150 rpm for 24 h. Then the suspension was centrifuged for 4 min at 8000× g, the pelleted was washed and suspended in sterilized distilled water. The bacterial concentration was estimated by Jasco V-550 UV-VIS spectrophotometer (600 nm) and adjusted by sterilized distilled water until reaching 10^7 CFU mL^{-1}. All treatments are summarized in Table 1.

Table 1. Beneficial microorganisms and genotypes used in the experiments. CTRL = seedlings without microorganism treatment, G = seedlings inoculated with *Funneliformis mosseae*, B = seedlings inoculated with *Paraburkholderia graminis*, G + B = seedlings inoculated with *Funneliformis mosseae* + *Paraburkholderia graminis*.

Experiment	Treatment	Genotype	CTRL	G	B	G + B
1 and 2	T1	Everton	x			
1 and 2	T2	Everton		x		
1 and 2	T3	Everton			x	
1 and 2	T4	Everton				x
2	T5	Pearson	x			
2	T6	Pearson		x		
2	T7	Pearson			x	
2	T8	Pearson				x
2	T9	H3402	x			
2	T10	H3402		x		
2	T11	H3402			x	
2	T12	H3402				x

The seedlings were kept in a growth chamber (Binder KBW 720, Tuttlingen, Germany) with a photoperiod of 16 h light and 8 h dark for 40 days under an irradiance of 180 µmol m^{-2} s^{-1} (white fluorescent tubes Fluora 18W/77, Osram, Munich, Germany), day/night temperatures of 25/19 °C (Ronga et al., 2018). After 40 days, when the seedlings reached the four-leaf stage, chilling treatment was performed at 1 °C for 24 h, as reported by Caffagni et al. (2014) and Ronga et al. (2018). The temperature was gradually decreased by 2 °C h^{-1} until it reached 1 °C. In addition, during the day the irradiance was decreased from 180 m^{-2} s^{-1} to 60 µmol m^{-2} s^{-1}. At the end of the stress period, the temperature was gradually raised by 2 °C h^{-1} until it reached 19 °C. After chilling treatment, seedlings were grown for two weeks in control conditions (25/19 °C day/night, 16 h photoperiod). The investigated parameters were recorded at the following four timings: (0) before the chilling stress, (24 h) at the end of chilling stress, (48 h) 24 h after stress (recovery), and (15 days) 15 days after stress (regrowth).

3.2.2 Morpho-Physiological and Agronomic Parameters

Before chilling stress, some morphological and agronomic parameters (height of plant, number of leaves, leaf area, leaf mass *per* area, stem diameter, leaves, stems, roots, and total dry weights) were recorded. The leaves of five seedlings were weighed and leaf area measured (using area meter LI-3000A, LI-COR, Lincoln, Nebraska, USA). In addition, specific leaf mass (SLM), a key parameter in plant growth and an important indicator of ecological strategies, was calculated as the ratio between leaf dry weight and leaf area. The different organs of the plant (leaves, stems and roots) were weighted and oven-dried at 65 °C until constant weight was reached to obtain the dry weight of single organs and the total dry weight.

The physiological parameters were recorded: before the chilling stress, immediately and one day after the end of the chilling stress, and at regrowth (15 days after the end of the stress). The leaf content of chlorophyll (CHL), flavonoids (FLAV, sum of adaxial and abaxial side of the leaf), and anthocyanins (ANTH) were estimated on the youngest fully expanded leaf using Dualex 4 Scientific (FORCE-A, Orsay, France) as suggested by Cerovic et al. (2012). Dualex 4 is a leaf-clip-type sensor that assesses, in a non-destructive way, physiological status of plants by transmittance and fluorescence measurements (Cerovic et al., 2012). In addition, nitrogen balance index (NBI) was calculated as the ratio between CHL and FLAV (Cerovic et al., 2005).

The electrolyte leakage method was used to assess the size of cell membrane damage at the end of chilling stress following the protocol reported by Caffagni et al. (2014) and Ronga et al. (2018). Briefly, two leaf disks of 0.5 cm diameter were put in a tube containing 25 mL of deionized water and stirred at 25 °C for 180 min. Electrolyte leakage (EL (%)) was expressed as (C.a − C.w)/(C.b − C.w) × 100 (Rizza et al., 1994), where Ca and Cb were the electrical conductivities of the samples (a) at the end of chilling stress and (b) after autoclaving, while Cw was the conductivity of the deionized water. The electrical conductivities were measured by conductivity meter GLP 31 (Crison instruments, Barcelona, Spain). In addition, the degree of injury of cell membranes was evaluated by the F_v/F_m ratio (maximal efficiency of PSII) at four timings: before the chilling stress (F_v/F_m0), at the end of chilling stress (F_v/F_m 24 h), after 24 h (F_v/F_m 48 h), and after 15 days (F_v/F_m 15 days). The photochemical efficiency of photosystem II was indirectly assessed by chlorophyll a fluorescence using a pulse amplitude-modulated fluorometer (PAM 2000, Walz, Effeltrich, Germany) connected to a Leaf Clip Holder (2030-B, Walz, Effeltrich, Germany).

Visual score evaluation (VS 15 days) was used to evaluate the plant regrowth 15 days after chilling stress. A five-point scale (5 = fully regrown, 4 = slightly yellowed leaf tips, 3 = half yellowed leaves 2 = half yellowed and half wrinkled leaves and no regrowth, 1 = fully wrinkled leaves and no regrowth, 0 = dead plant) was used (Ronga et a., 2018).

3.*2.3 Molecular Analysis*

The AMF root presence was evaluated with a qualitative real-time PCR approach. Subsamples of the tomato roots (three replicates/treatment) were finely ground in liquid nitrogen with a mortar and pestle. The grinded material (300 mg) was mixed with 500 µL of extraction buffer (40 mM Tris-acetate, 20 mM sodium acetate, 1 mM EDTA, 1% *w/v* SDS pH 7.8) and 5 µL RNase (500 µg μL^{-1}). After incubation at 37 °C for 600 s to digest contaminating RNA, 150 µL of NaCl was added. The suspension was centrifuged at 12,000× g for 1200 s at 4 °C and the supernatant mixed with 400 µL of chloroform and 400 µL of phenol, then centrifuged at 12,000× g for 1200 s. at 4 °C. The upper phase containing DNA was precipitated with 2 volumes of ethanol 95% (*v/v*). DNA was eluted with 50 µL elution buffer (10 mM Tris-HCl, 1mM EDTA, pH 7.8). The DNA concentration and quality were determined with a spectrophotometer at 260 and 280 nm (NanoDrop 1000, Thermo Fisher Scientific, Wilmington, DE, USA).

For qualitative real-time PCR the following primer pairs were used, according to Alkan et al. (2006): MOSF 5′-GAAGTCAGTCATACCAACGGGAA-3′, MOSR 5′-CTCGCGAATCCGAAGGC-3′. The amplification was carried out in 25 µL volume containing 12.5 µL of KAPA Sybr Fast qPCR kit (KAPA Biosystems, Wilmington, DE, USA), 0.3 µL of MOSF/MOSR primers (10 µm), 5 µL of template DNA (10 ng μL^{-1}) and 6.9 µL of water. Reactions were repeated twice with a 7300 real-time PCR Systems (Applied Biosystems, Foster City, CA, USA) and with the following cycling protocol: 95 °C for 180 s and 40 cycles of 95 °C for 3 s and 60 °C for 30 s. A melting curve analysis (95 °C for 15 s, 60 °C for 30 s, 95 °C for 15 s) was always included in each run to control for false-positive results caused by primer-dimer hybridization and non-specific amplifications. The presence of *F. mosseae* DNA in the root samples was estimated based on the comparison of Cycle threshold (Ct) that was automatically calculated by 7300 system software.

3.2.4 Statistical Analysis

In order to evaluate the effects of treatment or genotype, one-way analysis of variance was performed, while to evaluate treatment × genotype interaction, data were subjected to two-way ANOVA. Means were compared using Duncan's test at the 5% level. In addition, all recorded data during the experiment were analyzed by the Principal Component Analysis (PCA) model (Jackson, 1991; Wold, 1987) to evaluate the relationships between the analyzed objects and the original variables, and a biplot graph was used. All analyses were performed by using GenStat 17th software.

3.3. Results

3.3.1 Physiological, Morphological and Agronomic Parameters Evaluated before Chilling Stress

Measurements of morpho-physiological and agronomic parameters, such as the ratio between height and diameter, dry weights, and leaf content of chlorophyll, flavonoids and anthocyanins, represent a relevant indicator of the plant status already before stress exposure (Herrera et al., 2008; Muñoz-Huerta et al., 2013; Ronga et al., 2016). Accordingly, in order to evaluate plant status, some physiological and morphological parameters were assessed before chilling stress exposure (Table 2A and 2B)

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Table 2. Parameters measured before chilling stress in the first and second experiment (**A** and **B**, respectively). (1) Physiological parameters: F_v/F_m = photochemical efficiency of photosystem II (PSII), CHL = index of the chlorophyll content in leaf measured using a DUALX instrument, FLAV = index of the flavonoid content in leaf measured using a DUALX instrument, ANTH = index of the anthocyanin content in leaf measured using a DUALX instrument, NBI = nitrogen balance index. (2) Morphological non-destructive parameters: H = height of seedlings, D = stem diameter of seedlings, H/D = height to diameter ratio, LN (no.) = number of leaves *per* seedling, LA = leaf area, SLM = specific leaf mass. (3) Morphological destructive parameters: LDW = leaf dry weight, SDW = stem dry weight, RDW = root dry weight, TDW = total dry weight, FTL = fraction of total dry weight to leaves, FTR = fraction of total dray weight to roots. 0 = measured or recorded before stress, CTRL = seedlings without microorganism treatment, G = seedlings inoculated with *Funneliformis mosseae*, B = seedlings inoculated with *Paraburkholderia graminis*, G + B = seedlings inoculated with *Funneliformis mosseae* + *Paraburkholderia graminis*, TREAT = treatment, GENO = genotype. Data are presented as mean ± standard deviation (SD) (in the first experiment n = 5; in the second experiments n treatment = 15 and n genotype = 20). Different letters indicate statistically significant differences among treatments or genotypes by ANOVA followed by Duncan's test at $p < 0.05$, n.s. = not significant, ** = $p < 0.01$, * = $p < 0.05$.

A (1)

TREATMENT	**F_v/F_m0**		**CHL0**		**FLAV0**		**ANTH0**		**NBI0**	
CTRL	0.787 ± 0.04	n.s.	21.60 ± 2.5	c	0.507 ± 0.03	b	0.367 ± 0.10	n.s.	42.64 ± 4.5	n.s.
G	0.737 ± 0.06	n.s.	23.77 ± 1.9	bc	0.690 ± 0.29	ab	0.397 ± 0.02	n.s.	36.22 ± 9.4	n.s.
B	0.780 ± 0.05	n.s.	**28.83 ± 1.1**	**a**	0.763 ± 0.26	ab	0.353 ± 0.04	n.s.	31.64 ± 5.4	n.s.
G + B	0.780 ± 0.05	n.s.	23.73 ± 0.5	bc	**0.910 ± 0.12**	**a**	0.393 ± 0.06	n.s.	32.47 ± 6.5	n.s.
F values	0.939		**0.016**		**0.021**		0.841		0.091	

A (2)

TREATMENT	H0 (mm)		D0 mm)		H/D0		LN0 (no.)		LA0 (m^2 $plant^{-1}$)		SLM0 (g cm^{-2})	
CTRL	152.00 ± 16	n.s.	**2.83 ± 0.11**	**a**	53.60 ± 6.4	ab	3.67 ± 0.16	ab	0.0035 ± 0.0008	n.s.	$0.00319 \pm 8.2 \times 10^{-4}$	n.s.
G	143.00 ± 10	n.s.	2.73 ± 0.30	ab	52.49 ± 3.5	ab	3.71 ± 0.10	**a**	**0.0024 ± 0.0001**	n.s.	$0.00319 \pm 1.4 \times 10^{-4}$	n.s.
B	113.00 ± 30	n.s.	2.43 ± 0.05	b	46.38 ± 5.5	b	3.52 ±0.11	b	0.0025 ± 0.0009	n.s.	$0.00369 \pm 4.8 \times 10^{-4}$	n.s.
G + B	150.00 ± 20	n.s.	2.33 ± 0.13	b	**64.50 ± 12.6**	**a**	3.57 ± 0.15	ab	0.0030 ± 0.0008	n.s.	$0.00272 \pm 2.1 \times 10^{-4}$	n.s.
F values	0.055		0.010		**0.043**		**0.49**		0.331		0.268	

A (3)

TREATMENT	LDW0 (g $plant^{-1}$)		SDW (g $plant^{-1}$)		RDW0 (g $plant^{-1}$)		TDW0 (g $plant^{-1}$)		FTL0		FTR0	
CTRL	0.11 ± 0.03	n.s.	0.045 ± 0.016	ab	0.04 ± 0.06	n.s.	0.193 ± 0.044	n.s	58.00 ± 2.7	n.s	18.24 ± 1.6	c
G	0.08 ± 0.03	n.s.	0.039 ± 0.011	ab	0.04 ± 0.09	n.s.	0.163 ± 0.047	n.s	48.00 ± 8.5	n.s	26.96 ± 2.8	ab
B	0.09 ± 0.09	n.s.	**0.050 ± 0.004**	**a**	0.04 ± 0.05	n.s.	0.187 ± 0.014	n.s	49.00 ± 6.0	n.s	23.01 ± 2.8	b
G + B	0.08 ± 0.02	n.s.	0.029 ± 0.006	b	0.05 ± 0.10	n.s.	0.157 ± 0.046	n.s	51.00 ±1.9	n.s	**30.68 ± 2.1**	**a**
F values	0.569		**0.049**		0.458		0.734		0.275		**0.005**	

B (1)

TREATMENT	F_v/F_m0		CHL0		FLAV0		ANTH0		NBI0	
CTRL	0.787 ± 0.02	b	32.62 ± 3.2	ab	0.364 ± 0.23	b	**0.118 ± 0.04**	**a**	**112.80 ± 19.6**	**a**
G	**0.815 ± 0.01**	**a**	**35.13 ± 5.4**	**a**	**0.526 ± 0.21**	**a**	0.052 ± 0.02	ab	74.50 ± 28.4	b
B	**0.823 ± 0.01**	**a**	**37.13 ± 2.7**	**a**	**0.558 ± 0.29**	**a**	0.071 ± 0.02	ab	83.30 ± 23.4	b
G + B	**0.809 ± 0.02**	**a**	28.28 ± 4.3	b	0.255 ± 0.16	b	0.043 ± 0.01	b	**132.00 ± 21.2**	**a**
F values	**<0.001**		**0.012**		**<0.001**		**0.049**		**<0.001**	
GENOTYPE										
EVERTON	0.810 ± 0.03	n.s	32.08 ± 5.2	ab	0.284 ± 0.13	b	0.020 ± 0.01	b	**128.10 ± 25.5**	**a**
H3402	0.809 ± 0.02	n.s.	31.23 ± 3.4	b	**0.537 ± 0.30**	**a**	**0.097 ±0.02**	**a**	73.20 ± 15.2	c
PEARSON	0.806 ± 0.03	n.s.	**36.54 ± 3.1**	**a**	**0.456 ±0.24**	**a**	**0.097 ±0.02**	**a**	100.70 ± 19.8	b
F values	0.855		**0.049**		**<0.001**		**0.006**		**<0.001**	
TREAT*GENO	n.s.		n.s.		**		n.s.		n.s.	

B (2)

TREATMENT	H0 (mm)		D0 (mm)		H/D0		LN0 (no.)		LA0 (m^2 $plant^{-1}$)		SLM0 (g cm^{-2})	
CTRL	126.50 ± 13	b	**3.29 ± 0.46**	**a**	39.00 ± 5.8	b	4.60 ± 0.33	b	0.0054 ± 0.001	b	0.0025 ± 0.0006	n.s.
G	123.60 ± 21	b	3.04 ± 0.37	b	41.00 ±4.2	b	4.90 ± 0.62	ab	**0.0069 ± 0.001**	**a**	0.0025 ± 0.0007	n.s.
B	116.00 ± 20	b	2.80 ±0.27	c	41.00 ± 5.2	b	**5.30 ± 0.60**	**a**	0.0058 ± 0.002	ab	0.0027 ± 0.0005	n.s.
G + B	**146.90** ± 16	**a**	2.86 ±0.43	bc	**52.00 ± 6.9**	**a**	**5.00 ± 0.35**	**a**	0.0056 ± 0.002	ab	0.0026 ± 0.0004	n.s.
F value	**<0.001**		**<0.001**		**<0.001**		**0.011**		**0.048**		0.730	
GENOTYPE												
EVERTON	126.00 ± 18.1	b	2.90 ±0.20	b	43.00 ± 5.9	n.s.	4.80 ± 0.30	b	0.0049 ± 0.001	b	0.0024 ±0.0003	b
H3402	116.80 ± 20.2	c	2.70 ±0.35	c	44.00 ± 9.6	n.s.	5.00 ± 0.40	ab	0.0048 ± 0.001	b	**0.0028 ± 0.0003**	**a**
PEARSON	**142.00 ± 17.3**	**a**	**3.39 ±0.38**	**a**	42.00 ± 7.0	n.s.	**5.20 ± 0.30**	**a**	**0.0082 ± 0.002**	**a**	0.0025 ±0.0004	ab
F values	**<0.001**		**<0.001**		0.643		**0.049**		**<0.001**		0.043	
TREAT*GENO	**		n.s.		n.s.		n.s.		n.s.		n.s.	

B (3)

TREATMENT	LDW0 (g plant^{-1})		SDW0 (g plant^{-1})		RDW0 (g plant^{-1})		TDW0 (g plant^{-1})		FTL0		FTR0	
CTRL	0.12 ± 0.02	b	0.05 ± 0.01	n.s.	0.04 ± 0.02	n.s.	0.21 ± 0.05	b	61.00 ±5.5	n.s.	19.40 ± 5.7	n.s.
G	**0.15 ± 0.02**	**a**	0.06 ± 0.02	n.s.	0.06 ± 0.02	n.s.	**0.30 ± 0.06**	**a**	59.00 ± 9.6	n.s.	20.10 ± 5.3	n.s.
B	0.13 ± 0.03	ab	0.06 ±0.02	n.s.	0.05 ± 0.02	n.s.	0.25 ± 0.07	ab	61.00 ± 4.3	n.s.	21.40 ±3.0	n.s.
G + B	**0.15 ± 0.02**	**a**	0.06 ± 0.02	n.s.	0.05 ± 0.02	n.s.	0.27 ± 0.06	ab	58.00 ± 8.7	n.s.	19.90 ± 6.1	n.s.
F value	**0.049**		0.286		0.182		**0.035**		0.907		0.736	
GENOTYPE												
EVERTON	0.11 ± 0.03	b	0.05 ± 0.01	b	0.04 ± 0.02	b	0.20 ± 0.06	b	59.00 ± 7.7	n.s.	21.80 ± 5.9	n.s.
H3402	0.12 ± 0.04	b	0.05 ± 0.01	b	0.04 ± 0.02	b	0.23 ± 0.09	b	61.00 ± 9.1	n.s.	19.30 ± 5.5	n.s.
PEARSON	**0.18 ± 0.05**	**a**	**0.08 ± 0.02**	**a**	**0.10 ± 0.02**	**a**	**0.35 ± 0.09**	**a**	60.00 ± 3.6	n.s.	19.60 ± 3.2	n.s.
F value	**<0.001**		**0.049**		**0.002**		**<0.001**		0.456		0.276	
TREAT*GENO	n.s.		n.s.		n.s.		n.s.		n.s.		n.s.	

Considering the physiological parameters, the seedlings treated with B showed always the highest values of chlorophyll leaf content (CHL0), +33% and +14%, in the first and second experiments, respectively, in comparison with the non-inoculated control (Table 2A and 2B). In the second experiment, the genotype 'Pearson' showed the highest leaf content of chlorophyll while 'Everton' showed the lowest leaf content of flavonoids and anthocyanins and the highest NBI value (Table 2B). Regarding the morphological and agronomic parameters, in the second experiment, the seedling, generally, showed a higher development (D0, LN0, LDW0, SDW0, RDW0 and TDW0) in comparison with the seedling of the first experiment. In both experiments, the non-inoculated control showed the highest diameter and G + B showed the highest H/D0 ratio (+20.3% and +33.8%, in the first and in the second experiments, respectively, in comparison with the non-inoculated control). In the first experiment the inoculated seedlings showed the highest fraction of total dry weight to roots (G + 47.8%; B + 26.1% and G + B +68.2% in comparison with the non-inoculated treatment) (Table 2A). On the other hand, the inoculated seedlings showed the highest total dry weight (G + 42.9%, B + 19.0% and G + B + 28.6% in comparison with the non-inoculated treatment) in the second experiment.

'Pearson' ranked first for many parameters (H0, D0, LN0, LA0, LDW0, SDW0, RDW0 and TDW0); while the modern genotype 'Everton' and 'H3402′ showed similar morphological characteristics for LA0, LDW0, SDW0, RDW0 and TDW0 (Table 2B).

No interaction was observed between treatments and genotypes apart for H0 and FLV0. Regarding H0, 'H3402′ inoculated with B showed the lowest values, while 'Pearson' inoculated with G + B the highest ones. For FLV0 'Everton' non-inoculated and 'H3402′ inoculated with G + B showed the lowest values, while 'H3402′ inoculated with B showed the highest ones.

In order to verify the photochemical efficiency of PSII before chilling exposure, F_v/F_m values were measured. All the seedling showed F_v/F_m values higher than 0.600 and in the second experiment G, B and G + B highlighted higher values than the non-inoculated treatment.

3.3.2 Effects of AMF and PGPR Inoculations on Leaf Damage and Performance after Chilling Stress

In order to verify whether AMF and PGPR inoculations help processing tomato seedlings to overcome chilling stress, the size of cell membrane damage and the photochemical efficiency of PSII were assessed (Table 3A and 3B) at the end of chilling stress and after 24 h.

Table 3. Parameters measured at the end of chilling stress and after 24 h in the first and second experiment (A and B, respectively). F_v/F_m = photochemical efficiency of photosystem II (PSII), EL = electrolyte leakage, 24h = measured or recorded immediately after the stress, 48h = measured or recorded 24 h after the end of the stress, CTRL = seedlings without microorganism treatment, G = seedlings inoculated with *Funneliformis mosseae*, B = seedlings inoculated with *Paraburkholderia graminis*, G + B = seedlings inoculated with *Funneliformis mosseae* + *Paraburkholderia graminis*, TREAT = treatment, GENO = genotype. Data are presented as mean ± standard deviation (SD) (in the first experiment n = 5; in the second experiments n treatment = 15 and n genotype = 20). Different letters indicate statistically significant differences among treatments or genotypes by ANOVA followed by Duncan's test at $p < 0.05$, n.s. = not significant, ** = $p < 0.01$, * = $p < 0.05$.

A

TREATMENT	**F_v/F_m 24 h**		**EL% 24 h**		**F_v/F_m 48 h**	
CTRL	0.510 ± 0.18	n.s.	**86.94 ± 0.3**	**a**	0.102 ± 0.050	b
G	0.490 ± 0.19	n.s.	**42.79 ± 1.6**	d	**0.460 ± 0.332**	**a**
B	0.380 ± 0.20	n.s.	67.95 ± 0.9	c	0.131 ± 0.057	ab
G + B	0.333 ± 0.23	n.s.	77.27 ± 9.5	bc	0.350 ± 0.249	ab
F value	0.099		<0.001		0.032	

B

TREATMENT	F_v/F_m 24 h		EL% 24 h		F_v/F_m 48 h	
CTRL	0.572 ± 0.06	b	**55.48 ± 11.8**	**a**	0.528 ± 0.10	c
G	**0.728 ± 0.05**	**a**	36.21 ± 10.87	b	**0.776 ± 0.03**	**a**
B	**0.718 ± 0.07**	**a**	38.89 ± 10.69	b	0.719 ± 0.11	ab
G + B	**0.662 ± 0.08**	**a**	42.26 ± 11.2	b	0.707 ± 0.06	b
F value	<0.001		<0.001		<0.001	
GENOTYPE						
EVERTON	0.640 ± 0.12	n.s.	45.32 ± 16.26	n.s.	**0.701 ± 0.10**	**a**
H3402	0.689 ± 0.10	n.s.	39.84 ± 12.32	n.s.	0.645 ± 0.14	b
PEARSON	0.681 ± 0.12	n.s.	44.47 ± 10.27	n.s.	**0.702 ± 0.12**	**a**
F value	0.271		0.092		0.041	
TREAT*GENO	n.s.		n.s.		n.s.	

At the end of chilling stress, all the treatments with microorganisms reduced the EL% 24 h values and the treatment G displayed lower values of EL% 24 h in comparison with the non-inoculated seedlings (−49.21% and −65.26% in experiment 1 and 2, respectively). In addition, in the second experiment, all the treatments with microorganisms reported higher F_v/F_m 24 h ratios.

In both experiments, all the treatments with microorganisms increased the F_v/F_m 48 h ratio and the seedlings inoculated with G showed the highest values (+351.0% and +47.0%, in the first and in the second experiment, respectively, in comparison with the non-inoculated seedlings) 24 h after chilling treatment. Considering the genotype effect, ‘Everton’ and ‘Person’ recorded values higher compared to ‘H3402′.

3.3.3 Effects of AMF and PGPR Inoculation after Regrowth

In order to evaluate the effects of microorganism inoculations on regrowth capacity, some parameters were also evaluated 15 days after the end of the stress (Table 4A and B).

Table 4. Parameters measured 15 days after chilling stress (regrowth) in the first and second experiment (A and B, respectively). Fv/Fm = photochemical efficiency of photosystem II (PSII), CHL = index of the chlorophyll content in leaf measured using a DUALX instrument, FLAV = index of the flavonoid content in leaf measured using a DUALX instrument, ANTH = index of the anthocyanin content in leaf measured using a DUALX instrument, NBI = nitrogen balance index, VS 15 days = visual score recorded, 15 days = measured or recorded at regrowth (15 days after stress), CTRL = seedlings without microorganism treatment, G = seedlings inoculated with *Funneliformis mosseae*, B = seedlings inoculated with *Paraburkholderia graminis*, G + B = seedlings inoculated with *Funneliformis mosseae* + *Paraburkholderia graminis*, TREAT = treatment, GENO = genotype. Data are presented as mean ± standard deviation (SD) (in the first experiment n = 5; in the second experiments n treatment = 15 and n genotype = 20). Different letters indicate statistically significant differences among treatments or genotypes by ANOVA followed by Duncan's test at $p < 0.05$, n.s. = not significant, ** = $p < 0.01$, * = $p < 0.05$.

A

TREATMENT	F_v/F_m 15 days		CHL 15 days		FLAV 15 days		ANTH 15 days		NBI 15 days		VS 15 days (val. 0–5)	
CTRL	0.544 ± 0.10	b	16.67 ± 2.7	n.s.	1.167 ± 0.41	n.s.	0.327 ±0.10	n.s.	15.42 ± 5.17	n.s.	3.33 ± 0.3	b
G	**0.787 ± 0.09**	**a**	20.70 ± 8.0	n.s.	1.123 ± 0.24	n.s.	0.340 ±0.08	n.s.	20.24 ± 12.9	n.s.	**4.00 ± 0.3**	**a**
B	**0.708 ±0.09**	**a**	16.20 ± 2.7	n.s.	1.127 ± 0.25	n.s.	0.303 ±0.03	n.s.	14.87 ± 3.87	n.s.	**4.00 ± 0.3**	**a**
G + B	**0.762 ±0.07**	**a**	15.03 ± 0.3	n.s.	1.357 ±0.07	n.s.	0.453 ±0.01	n.s.	11.11 ± 0.80	n.s.	3.67 ± 0.4	ab
***F* values**	**0.01**		0.452		0.578		0.142		0.397		**0.045**	

B

TREATMENT	F_v/F_m 15 days		CHL 15 days		FLAV 15 days		ANTH 15 days		NBI 15 days		VS 15 days (val. 0–5)	
CTRL	0.544 ± 0.08	C	26.48 ± 4.6	b	0.953 ± 0.16	c	**0.435 ± 0.10**	**a**	28.77 ± 7.8	b	2.49 ± 0.37	c
G	0.787 ± 0.02	Ab	**37.51 ± 4.9**	**a**	**1.250 ± 0.23**	**a**	**0.402 ± 0.08**	**a**	31.14 ± 4.3	b	**4.39 ± 0.27**	**a**
B	0.768 ± 0.03	B	**35.15 ± 3.8**	**a**	1.090 ± 0.13	b	0.381 ± 0.10	ab	32.96 ± 6.3	b	3.87 ± 0.29	b
G + B	0.802 ± 0.02	**a**	**34.34 ± 4.1**	**a**	0.872 ± 0.13	c	0.323 ± 0.05	b	**40.03 ± 6.3**	**a**	3.85 ± 0.28	b
F values	**<0.001**		**<0.001**		**<0.001**		**0.011**		**<0.001**		**<0.001**	
GENOTYPE												
EVERTON	**0.750 ± 0.08**	**a**	31.33 ± 6.5	b	1.040 ± 0.25	n.s.	0.373 ± 0.08	n.s.	31.83 ± 7.9	n.s.	3.71 ± 0.64	b
H3402	0.725 ± 0.01	ab	**34.67 ± 5.8**	**a**	1.000 ± 0.22	n.s.	0.369 ± 0.10	n.s.	35.43 ± 7.1	n.s.	**3.91 ± 0.76**	**a**
PEARSON	0.701 ± 0.01	b	**34.11 ± 5.2**	**a**	1.080 ± 0.17	n.s.	0.413 ±0.10	n.s.	32.41 ± 7.1	n.s.	3.33 ± 0.82	c
F values	**0.001**		**0.031**		0.377		0.241		0.169		**<0.001**	
TREAT*GENO	**		n.s.		n.s.		n.s.		n.s.		n.s.	

In both experiments, the treatment containing microorganisms increased the F_v/F_m 15 days ratio in comparison with the non-inoculated control. In the second experiment the highest F_v/F_m 15 days ratio was showed by G + B (+44.4%, in comparison with the non-inoculated seedlings). When the interaction between genotype and treatment was considered, the best F_v/F_m 15 days ratios were shown by 'Everton' inoculated with G and 'H3402′ inoculated with G + B. For chlorophyll assessment, in the second experiment, all the treatments with microorganisms increased the values of CHL 15 days. In general, after chilling stress, in both experiments the FLAV 15 days values increased and NBI 15 days values decreased in comparison with the values measured before chilling stress (FLAV0 and NBI0). In the second experiment, treatment G showed the highest values of FLAV 15 days, while G + B recorded the lowest value of ANTH 15 days. At the end of the regrowth period, the long-term effect of treatments was evaluated also by VS 15 days (Figure 1). In the first experiment, the seedlings inoculated with G and B showed the best regrowth capacity recording both a value of VS 15 days of 4.0. In addition, treatment G also confirmed the best regrowth capacity in the second experiment (VS 15 days = 4.4). Regarding the genotype effect, in the second experiment, 'H3402′ showed the highest VS 15 days while 'Pearson' was the most damaged.

Figure 1. Seedlings of 'Pearson' (**A**), 'H3402′ (**B**), and 'Everton' (**C**) at regrowth. In each square, from left to right: CTRL, G, B and G + B. CTRL = seedlings without microorganism treatment, G = seedlings inoculated with *Funneliformis mosseae*, B = seedlings with *Paraburkholderia graminis* inoculated, G + B = seedlings inoculated with *Funneliformis mosseae* + *Paraburkholderia graminis.*

3.3.4 AMF Root presence in Tomato Seedling after Chilling Stress

Based on real-time analysis the presence of *F. mosseae* DNA was confirmed in the processing tomato roots inoculated with *F. mosseae* and with *F. mosseae* + *P. graminis* (Figure 2). In contrast, all non-inoculated roots (controls) and the *P. graminis* inoculated roots were negative for *F. mosseae* DNA presence (flat lines). Significant differences were found for AMF presence among treatments, but not among genotypes ($p = 0.586$).

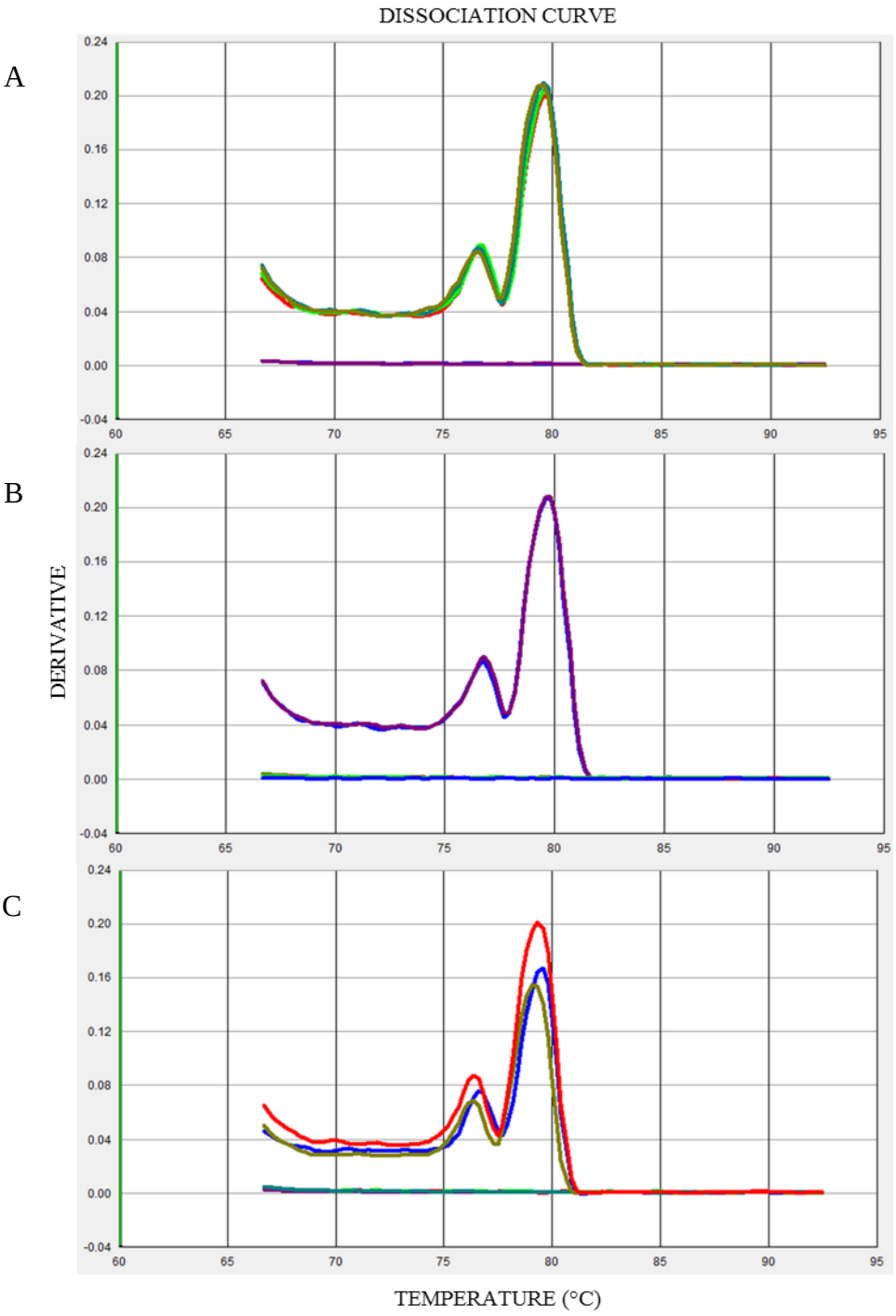

Figure 2. Real time dissociation curves to confirm mycorrhizal inoculation in 'Pearson' (**A**), 'H3402′ (**B**) and 'Everton' (**C**), respectively. Single peaks are obtained from three technical replicates (each peak of different color represents a replicate). Flat lines are no template control (NTC) technical replicates.

3.3.5 Relationships between Treatments and Evaluated Parameters

The correlations among treatments and evaluated parameters were studied using PCA. Figure 3 (first experiment) and Figure 4 (second experiment) show the biplots of the PCA

models calculated for each experiment. The contributions of the two first principal components are 46.81% (PC1) and 29.60% (PC2) for the first experiment and 54.80% (PC1) and 31.52% (PC2) for the second one. In both Figures, differences among treatments and recorded parameters are visible and the first principal component gives an indication on the effect of treatments. The non-inoculated seedlings were always found on the top left quadrant while genotype inoculated with G and G + B were always found on the positive side. In both experiments, the seedlings inoculated with G were associated with VS15 days, F_v/F_m 48 h and F_v/F_m 15 days, while seedlings inoculated with G + B were linked to H/D0 ratio. In both experiments D0, NBI0, EL24h, and FTL0 were associated with the control (non-inoculated seedlings).

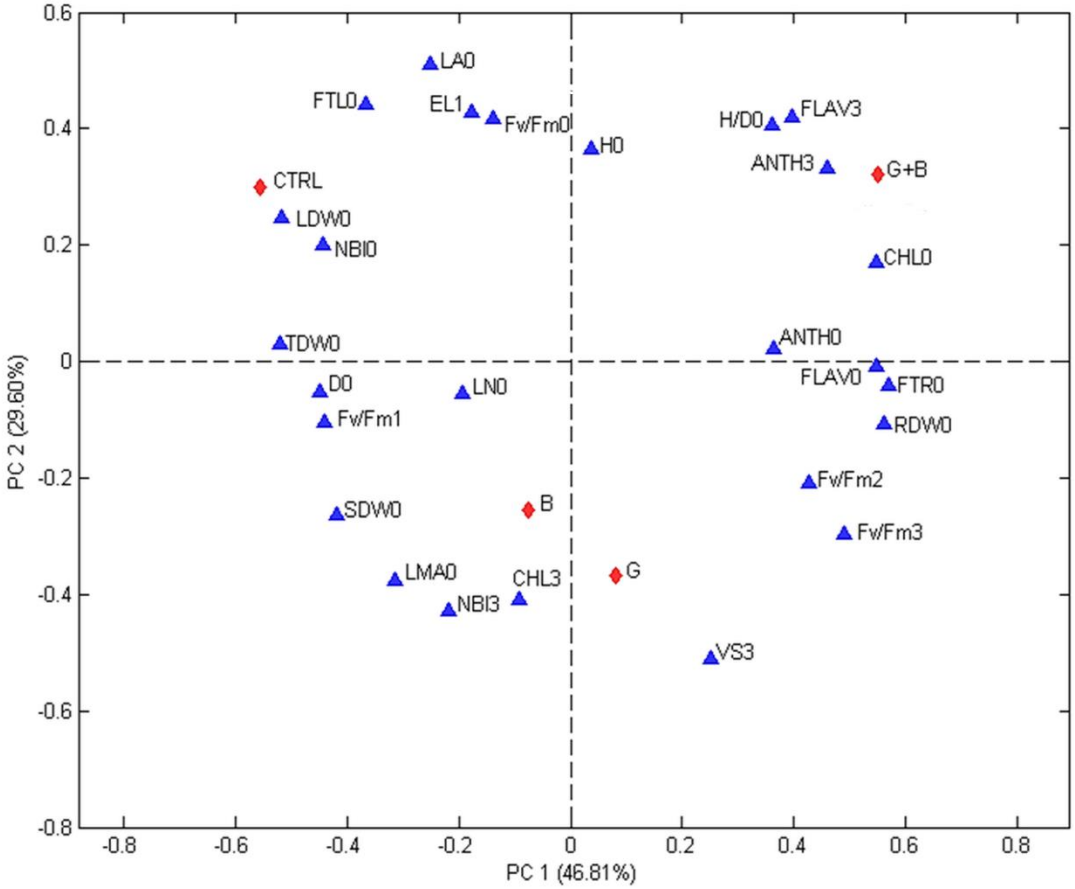

Figure 3. Biplot of Principal Component Analysis results of first experiment. The studied parameters (blue triangles) are: H/D = height to diameter ratio, CHL = index of the chlorophyll content in a leaf measured using a DUALX instrument, FLAV = index of the flavonoids content in leaf measured using a DUALX instrument, ANTH = index of the anthocyanins content in leaf measured using a DUALX instrument, NBI = nitrogen balance index, LN (no.) = number of leaves per seedling, LA = leaf area, LDW = leaf dry weight, SDW = stem dry weight, RDW = root dry weight, TDW = total dry weight, FTL = fraction of total dry weight to leaves, FTR

= fraction of total dray weight to roots, LMA = leaf mass *per* area, F_v/F_m = photochemical efficiency of photosystem II (PSII), EL = electrolyte leakage, VS 3 = VS 15 days = visual score recorded, 0 = measured or recorded before stress, 1 = measured or recorded immediately after the stress, 2 = measured or recorded 24 h after the end of the stress, 3 = measured or recorded at regrowth (15 days after stress). The studied treatments (red diamonds) are: CTRL = seedlings without microorganism treatment, G = seedlings inoculated with *Funneliformis mosseae*, B = seedlings inoculated with *Paraburkholderia graminis*, and G + B = seedlings inoculated with *Funneliformis mosseae* + *Paraburkholderia graminis*.

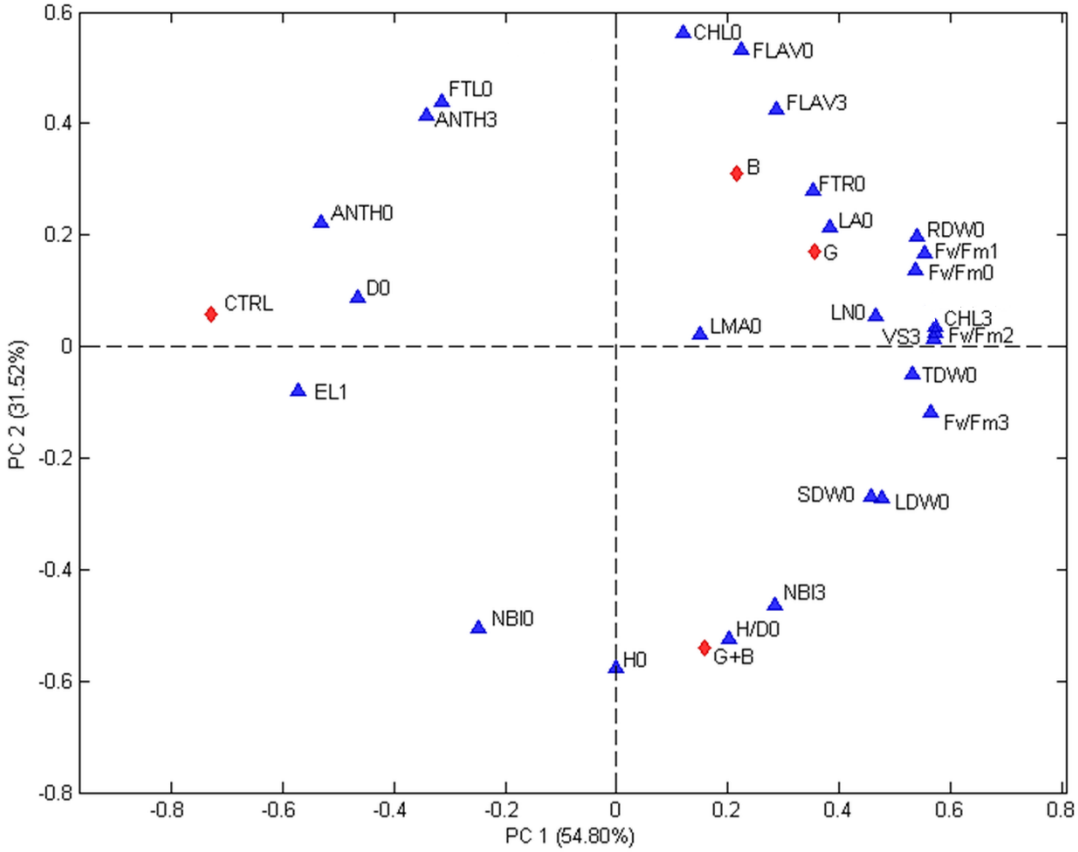

Figure 4. Biplot of Principal Component Analysis results on second experiment. The studied parameter (blue triangles) are: H/D = height to diameter ratio, CHL = index of the chlorophyll content in leaf measured using a DUALX instrument, FLAV = index of the flavonoid content in leaf measured using a DUALX instrument, ANTH = index of the anthocyanin content in leaf measured using a DUALX instrument, NBI = nitrogen balance index, LN (no.) = number of leaves per seedling, LA = leaf area, LDW = leaf dry weight, SDW = stem dry weight, RDW = root dry weight, TDW = total dry weight, FTL = fraction of total dry weight to leaves, FTR = fraction of total dray weight to roots, LMA = leaf mass *per* area, F_v/F_m = photochemical efficiency of photosystem II (PSII), EL = electrolyte leakage, VS 3 = VS 15 days = the visual score recorded, 0 = measured or recorded before stress, 1 = measured or recorded immediately after the stress, 2 = measured or recorded 24 h after the end of the stress, 3 = measured or recorded at regrowth (15 days after stress). The studied treatments (red diamonds) are: CTRL = seedlings without microorganism treatment, G = seedlings inoculated with *Funneliformis mosseae*, B = seedlings inoculated with *Paraburkholderia graminis*, and G + B = seedlings inoculated with *Funneliformis mosseae* + *Paraburkholderia graminis*.

3.4 Discussion

Processing tomato is a globally important horticultural crop (Hagassou et al., 2019). It is generally grown in high-input conventional systems (Ronga et al., 2015) and typically transplanted during spring, a period in which chill spells could still occur in Mediterranean environments. Hence, to improve agricultural sustainability, innovative strategies, able to improve processing tomato tolerance to environmental stresses, avoiding high yield losses (Ronga et al., 2019b), are required. The wide variety of beneficial microorganisms present in the rhizosphere could be used to help crops to overcome abiotic stresses and to reduce external inputs, thereby facilitating sustainable agricultural production (Sarkar et al., 2018). In the present work, the physiological responses (efficacy of PSII, cell membrane injuries in terms of EL%), recovery and regrowth capacity of processing tomato genotypes inoculated with *F. mosseae*, *P. graminis* and their consortium were evaluated under severe chilling stress (1 °C for 24 h). Before chilling stress, physiological, morphological and agronomic parameters were measured to evaluate the effect of the treatments and the genotypes in the absences of stress. Leaf chlorophyll content value (CHL) is considered to be a good indicator of the status of plants, because it is the key pigment involved in photosynthesis (Muñoz-Huerta et al., 2013). In the present study, the seedlings inoculated with *P. graminis* showed an increase of leaf chlorophyll content (CHL0) (Table 2A and 2B). These results could be due to different reasons. Some studies reported capability of bacteria of the genus *Paraburkholderia* to fix nitrogen asymbiotically (Cantliffe, 1993; NeSmith and Duval, 1998). In addition, a recent study (Hermenau et al., 2018) has showed that *Paraburkholderia graminis* can produce gramibactin, a siderophore that can bind iron, an essential element for chlorophyll production. Among the three genotypes, the values of leaf chlorophyll content were higher in genotype 'Pearson' (Table 2B), confirming the results showed by Ronga et al. (2018).

The height to diameter ratio of seedlings is an important parameter to assess the seedling quality in nursery production (Herrera et al., 2008; Muñoz-Huerta et al., 2013; Ronga et al., 2016). In the present study, the treatment containing the consortium (G + B) increased the height/diameter ratio of seedlings (Table 2A and B) conferring more vigor to seedlings. Seedlings grown in nursery are often sown in alveolar fixed seed trays with very small holes, allowing the growth of higher number of plants per unit area. However, the container size can affect the development of seedlings (Raison and Chapman, 1976; Thomashow, 1999). In fact,

in the second experiment, where the alveolar fixed seed trays with bigger holes were used, the seedlings had higher development (D0, LN0, LDW0, SDW0, RDW0 and TDW0). These results are consistent with findings previously reported by NeSmith and Duval (1998). In addition, in the second experiment, the total dry weight values, recorded before chilling stress, were higher confirming the previous results showed by reference (Liu et al., 2016) under optimal growth temperature condition. In contrast, in the first experiment, there were not differences among treatments, therefore, it possible to presume an interaction between treatments and the higher amount of soil available to seedlings in the second experiments. However, this theory should be validated by further experiments.

Low temperature stress is known to reduce the development of the plant due to leaf tissue damage. In particular, injury of the membranes, with an increase in permeability, is the main effect caused by chilling stress (Ma et al., 2018; Raison and Chapman, 1976; Thomashow, 1999). Electrolyte leakage (EL) is a useful parameter to measure cell membrane damage (Sarkar et al., 2018; Bajji et al., 2002); however, this parameter is influenced by plant and leaf age as well as leaf position on the plant (Adam et al., 2000; Bajji et al., 2002; Bandurska et al., 1995; Premachandra and Shimada, 1987). Hence, in both experiments the measurements were conducted at the same seedling age (40 days after sowing) using the upper fully expanded leaves. In the present study, the obtained data proved the protective activity of studied microorganisms towards cell membranes. However, only in the first experiments, significant differences among the treatments containing microorganisms were found. This could be ascribed to the different agronomic performance of the seedlings. In fact, seedlings with higher development are more tolerant to environmental stresses (Herrera et al., 2008). Therefore, it is possible to hypothesize that the higher tolerance of seedlings could have attenuated or hidden the different effects of the treatments at recovery. In addition, this hypothesis was supported by lesser EL values shown by the non-inoculated control in the second experiment in comparison with the first one.

The chloroplast is the primary site of a chilling injury (Kratsch and Wise, 2000). Chilling stress affected the photosystem putatively, leading to a photoinhibition of photosynthesis due to photoinactivation of catalase and a decline of variable fluorescence (Feierabend et al., 1992). Photosynthetic efficiency is a good marker to assess the effects of treatments and genotypes, after chilling stress. F_v/F_m parameter gives an idea of the PSII efficiency and, consequently, the

damage of photosynthetic apparatus due to chilling stress (Maxwell and Johnson, 2000) and the ability for recovery and regrowth of the seedlings to occur. Before chilling stress, all seedlings showed F_v/F_m0 ratios ranging from 0.73 to 0.83 (Table 2A and B), which are the typical values (F_v/F_m ratio) of many higher plants (Feierabend et al., 1992). Instead, immediately after the chilling stress, the values of the F_v/F_m ratio were lower than the 0.73–0.83 range (Stirbet and Govindjee, 2011) (Table 2B and 3B). These data confirmed the results previously displayed by Caffagni et al. (2014) and Ronga et al. (2018). In addition, in the first experiment differences between control and treatments were not observed. By contrast, in the second experiment, the seedlings inoculated with microorganisms showed higher values of F_v/F_m 24 h in comparison with the control seedling. It is possible to suppose that there was an interaction between treatments and higher amount of soil available for seedlings in the second experiments. However, this theory should be validated by further experiments. On the other hand, when the PSII efficiency was evaluated 24 h after the end of chilling stress (F_v/F_m 48 h), independent of genotype, *F. mosseae* was the treatment that mainly preserved the PSII to chilling stress. The differences in protecting the PSII highlighted by the different treatments containing microorganisms could be due to different microhabitats of the two microorganisms (*F. mosseae* vs. *P. graminis*). In fact, *F. mosseae* is an endophyte (Bonfante and Genre, 2010) while *P. graminis* lives in the rhizosphere (Suárez-Moreno et al., 2012). Therefore, the seedling tissues could have protected *F. mosseae* from chilling stress (Calco-Polanco, 2016) and could have influenced his efficacy. In addition, a study performed by Liu et al. (2016) reported that *F. mosseae* increased content of redox compounds in the tomato roots under optimal temperature. Therefore, the presence of redox compounds before the chilling stress could also make seedlings more reactive to overcome the chilling stress.

A good regrowth capacity after stress is an important and desirable ability of crops, as it allows plants to develop, in short time, new leaves and shoots that are very important for recovering photosynthetic activity and carbon fixation. All the treatments containing microorganisms showed a high efficiency of PSII 15 days after the chilling treatment. However, when the seedling had higher development (in the second experiment), the seedlings inoculated with the consortium (G + B) showed the best efficiency of PSII and a less content of secondary metabolites in the leaf (FLAV3 and ANTH3). When the genotype was considered, the same results were achieved by using the most modern genotype “Everton”. VS 15 days is another method used to assess the ability of regrowth of the seedlings. Our results revealed that the best

performances were obtained when the seedlings were inoculated with *F. mosseae* or the genotype "H3402" was used. The opposed results between F_v/F_m 15 days and VS 15 days could be due to different aspects that they consider: while the F_v/Fm ratio only considers the efficacy of PSII, the VS 15 days considers the regrowth capacity of the whole plant in general (i.e., appearance of new leaves).

Knowledge of the interaction between genotype and treatment could help farmers in choosing the best microorganism to help plants to overcome environmental stress. Some studies on rice and tomato have revealed that different genotypes of rice and tomato responded differently to different microorganism inoculations (Garcia de Salomone et al., 2012; Santamaría and Bayman, 2005). Also in our study, the three processing tomato genotypes showed different responses to the different treatments containing microorganisms. Interestingly, the more recent genotypes "Everton" achieved a higher F_v/F_m 15 days result when inoculated with *F. mosseae*, while "H3402" achieved the higher F_v/F_m 15 days result when inoculated with the consortium. When we considered the interaction between *F. mosseae* and *P. graminis*, no differences were observed on the AMF presence. These results agree with some studies (Mohameda et al. 2019; Roesti et al., 2006; Zubek et al., 2009), in which bacteria treatments did not influence the AMF root presence.

Also, the analysis of biplots confirmed the ability of microorganisms to help processing tomato seedlings during chilling stress. Interestingly, *F. mosseae*, F_v/F_m 48 h and F_v/F_m 15 days and VS 15 days were closely associated, proving that processing tomato seedlings successfully overcome chilling stress when inoculated with *F. mosseae*. In addition, FTL0 was always opposed to *F. mosseae* and F_v/F_m 15 days, suggesting that *F. mosseae* induced a reduction of the biomass allocated to leaf (improving biomass allocated to roots. This lower biomass allocated to leaf may lead to lower damage during chilling stress. However, further studies are necessary to corroborate this hypothesis.

3.5 Conclusions

Chilling damage could limit processing tomato growth and production in open field. The present work provided evidence for the use of arbuscular mycorrhizal fungi (AMF) and plant growth promoting rhizobacteria (PGPR), alone and in consortium, in order to avoid the damage of processing tomato seedlings due to exposure to severe chilling stress (1 °C). The

present study has revealed that *F. mossae* was the most effective treatment in reducing electrolyte leakage, while increasing the efficacy of PSII and regrowth capacity of seedlings. Also, a different tolerance of genotypes was observed. In particular, the modern genotypes inoculated with microorganisms showed a better regrowth capacity. Interestingly, the alveolar fixed seed trays dimensions can influence the seedling growth; therefore, reduction seedlings density within the alveolar fixed seed trays could be a useful practice for the nursery sector in order to provide farmers with more vigorous seedlings. Since in the present work the physiological pathways and the derived metabolites were not investigated, further studies are necessary in order to fully understand the mechanisms triggered when processing tomato seedlings are inoculated with microorganisms. Further investigations are being undertaken to assess the activity of the microorganisms and their consortia in real nursery conditions, where fertilizers and plant protection products are used, and in the open field, where competition with other microorganisms occurs.

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Chapter 4

Interaction of tomato genotypes and arbuscular mycorrhizal fungi under reduced irrigation

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4.1 Introduction

Crop growth, yield and fruit quality are influenced by many abiotic factors such as water, temperature, solar radiation and salinity. When the potential transpiration rate exceeds the water absorption by the roots from the soil, crops experience water stress. Water limitation causes the closure of plant stomata leading to a decrease of carbon dioxide (CO2) uptake followed by a reduction in photosynthetic activity (Chitarra et al., 2016; Osakabe et al., 2014). Additionally, drought stress reduces nutrient uptake, leading to a decrease in macro and micro element availability (Sardans et al., 2004). Finally, water deficits affect the plant growth through the repression of gene expression related to cell division and proliferation (Claeys and Inze, 2013; Todaka et al., 2017).

Processing tomato is one of the most economically important and widespread horticultural crops in the world (Calvo-Polanco et al., 2016; Dell'Amico et al., 2002; Ronga et al., 2019b), and in 2018 its production was ~ 34 million tons (WPTC, 2019). In addition, processing tomato is extremely dependent on irrigation water, the average water requirement ranging from ~ 400 to 600 mm during the growing season based on climatic conditions of the area (Rana et al., 2000). Successful open field transplanting of nursery-grown seedlings is one of the key factors in producing high yielding horticultural crops such as processing tomato. Nonetheless, in many cases the lack of closed and cooling systems for the production of tomato seedlings leads to an excessive use of irrigation water. Hence, the excessive use of water for irrigation (Cammarano et al., 2019a), combined with the effects of climate change (e.g. the increased frequency of heat waves) (Cammarano et al., 2019b; Ruiz-Lozano et al., 2016), will limit the availability of fresh water for irrigation even at early crop growth stages. As processing tomato requires huge volumes of irrigation water for growth, a more scarce water availability during the growth cycle could lead to a decrease in fruit yield and quality (Bisbis et al., 2019).

Usually, when plants are subjected to water limitation, several strategies are used to overcome drought stress (Farooq et al., 2009), and both morphological and physiological changes are observed as tolerance/resistance mechanisms used to cope with stress conditions (Bakr et al., 2018). In particular, plants accelerate the phenological development, improve root growth and consequently water uptake, and control the transpiration by stomata regulation in order to avoid cellular damage caused by the stress (Bark et al., 2018). However, during severe

and extended drought conditions, these mechanisms are not effective enough to preserve crop growth, fruit yield and quality.

AM fungi are ubiquitous in soil and stablish symbiotic relationships with the roots of many cultivated plants (Bonfante and Genre, 2015; Dell'Amico et al., 2002; Krak et al., 2012). Plant-AM fungal interaction is mutualistic as the fungi can help plants to overcome abiotic and biotic stresses and to improve mineral nutrients and water uptake, while the host plant provides photosynthates (Bonfante and Genre, 2015) and lipids (Bravo et al., 2017). During drought stress, AM fungi may influence physiological and cellular processes of the host plants (Sanchez-Romera et al., 2018). Dell'Amico et al. (2002) reported that *Glomus clarum* was able to stimulate tomato growth (cultivar Amalia) under drought stress in greenhouse, and the effects were more evident on leaves and shoots than on roots. Likewise, Duc et al. (2018) reported that the use of AM fungus *Septoglomus constrictum* improved stomatal conductance, leaf water potential, leaf relative water content and the activity of photosystem II of genotype Moneymaker under combined heat and drought stress when grown in greenhouse. However, studies have also revealed that microorganism effects depend on plant genotypes (Caradonia et al., 2019; Garcia de Salamone et al., 2012).

Although there are many studies on the mitigation of crop drought stress by AM fungi conducted in open field or on adult plants grown in greenhouse (Chitarra et al., 2016), researches with processing tomato seedlings (at four true expanded leaves stage, corresponding to 35-40 days after sowing) are scarce. Studies on tomato seedlings are necessary as at this stage seedlings are transplanted in open field. Therefore, in the present study we assessed the interactions among three genotypes of processing tomato seedlings, and two AM fungi (*Funneliformis mosseae* and *Rhizophagus intraradices*), under two water regimes (fully-watered and reduced) in order to provide useful information to nursery growers.

4.2 Materials and Methods

4.2.1 Plant material and experimental design

The experiment was conducted in a growth chamber located at the University of Modena and Reggio Emilia (Reggio Emilia, Italy) following a fully randomized experimental design with five biological repetitions *per* treatment. Three genotypes of processing tomato (Pearson, H3402 and Everton) were used in the experiment. Pearson is an old genotype released in the

mid-1930s, with a semi-determinate growth habit, large fruits, suitable for canning; H3402 is a modern genotype released in 2002, with determinate growth habit, high vigour and medium oval fruit, suitable for medium and late transplanting and canning purposes; Everton is a modern genotype released in 2008, with determinate growth habit, medium vigour, with oval fruit suitable for medium transplanting and canning purposes (Caradonia et al., 2019; Ronga et al., 2018).

Processing tomato seeds were provided by ISI Sementi S.p.A. (Fidenza, Italy) and Furia Seed (Monticelli Terme, Italy). Seeds were germinated on moistened filter paper in Petri dishes at 25°C, and transferred after germination to pots (7 x 7 x 8 cm, 0.4 L) (one germinated seed *per* pot) containing the same quantity of neutralized peat (23% organic carbon, 0.5% nitrogen, pH 6, electrical conductivity 0.25 dS m^{-1} and dry apparent density 214 kg m^{-3}; Dueemme S.r.l., Reggio Emilia). *F. mosseae* and *R. intraradices* (10 propagules per 1 g) were separately mixed with peat 10:100 (w/w) as suggested by Rivero et al. (2015). Pure inocula of AM fungi were obtained from MycAgro, LabTechnopôle Agro Environnement, Bretenière, France. The same amount of propagules / peat mix was added in pots before transferring the germinated seeds to pots. After seedling emergence, each pot was covered with a transparent plastic sheet in order to reduce the water evaporation.

Plants were cultivated at 25°C day/19°C night with a 16h photoperiod under an irradiance of 180 µmol m^{-2} s^{-1} (white fluorescent tubes Fluora 18W/77, Osram, Munich, Germany). The seedlings fully-watered until 21 days after the sowing. Subsequently, the seedlings were subjected to one of two different irrigation regimes for 15 days: fully-watered and reduced irrigation regimes based on relative soil water content (RSWC) that was controlled gravimetrically weighing the pots every day (Bernardo et al., 2017). In particular, every day the amount of water lost by transpiration was added to each pot in order to keep the soil water content at the desired levels of volumetric soil moisture (100% and 55%, respectively). Table 1 summarizes all the treatments of the experiment.

Table 1. Genotypes, microorganisms and water irrigation regimes of experiment

Genotype	Microorganisms	Fully-watered irrigation regime	Reduced irrigation regime
Everton	Control without AM fungi	X	X
Everton	*Funneliformis mosseae*	X	X
Everton	*Rhizophagus intraradices*	X	X
Pearson	Control without AM fungi	X	X
Pearson	*Funneliformis mosseae*	X	X
Pearson	*Rhizophagus intraradices*	X	X
H3402	Control without AM fungi	X	X
H3402	*Funneliformis mosseae*	X	X
H3402	*Rhizophagus intraradices*	X	X

4.2.2 Morphological and physiological parameters assessed

Five seedlings *per* treatment were assessed at the end of drought stress. The impact of the different treatments on processing tomato seedling growth was assessed recording the number of leaves, leaf area, seedling height, stem diameter, height/diameter ratio, plant dry weight. Total and partitioned (leaves, stems and roots) dry weights were obtained oven-drying the fresh biomass at 65°C until constant weight. Leaf area was measured using area meter LI-3000A (LI-COR, Lincoln, Nebraska, USA). In addition, leaf mass *per* area (LMA), a key parameter in plant growth and an important indicator of plant functioning, was calculated as the ratio between leaf dry weight and leaf area.

Leaf chlorophyll, flavonoid and anthocyanin content and nitrogen balance index were considered as physiological parameters. The leaf content of chlorophyll (Chl), flavonoids (Flav) and anthocyanins (Anth) was estimated on the youngest fully expanded leaf using Dualex 4 Scientific (Dx4) (FORCE-A, Orsay, France). The nitrogen balance index (NBI) was calculated as the ratio between Chl and Flav (Cerovic et al., 2005).

In order to understand the responses of seedlings to the different water regime, the total water used by plants (TWU) was calculated as the sum of all the water applied during the experiment, while the water use efficiency (WUE) was calculated as the ratio between the total dry weight of the seedlings and the total water used.

4.2.3 Qualitative Real-time PCR analysis of AMF presence

Root subsamples were randomly chosen from three plants *per* treatment. Roots were washed with tap water, frozen in liquid nitrogen and stored at -80°C for the analysis. The AM fungal root presence was evaluated with a qualitative real-time PCR approach. Frozen root samples were finely pulverized in a sterile mortar using liquid nitrogen. As reported in Caradonia et al. (2019) the powder (300 mg) was mixed with 500 μL of extraction buffer (40 mM Tris-acetate, 20 mM sodium acetate, 1 mM EDTA, 1% w/v SDS pH 7.8) and 5 μL RNase (500 μg μL^{-1}). After incubation at 37°C for 600 s to digest contaminating RNA, 150 μL of NaCl was added. The suspension was centrifuged at 12,000× g for 1200 s at 4°C and the supernatant mixed with 400 μL of chloroform and 400 μL of phenol, then centrifuged at 12,000× g for 1200 s at 4°C. The upper phase containing DNA was precipitated with 2 volumes of ethanol 95% (v/v). DNA was eluted with 50 μL elution buffer (10 mM Tris-HCl, 1mM EDTA, pH 7.8). The DNA concentrations were determined using NanoDrop 1000 spectrophotometer at 260 and 280 nm (Thermo Fisher Scientific, Wilmington, DE, USA).

For qualitative real-time PCR the following primer pairs were used for *F. mosseae* (Alkan et al., 2006): MOSF 5′-GAAGTCAGTCATACCAACGGGAA-3′, MOSR 5′-CTCGCGAATCCGAAGGC-3′. While GI-mtLSU-499F 5′-GAGGGAGTGGCAGTTTCTT-3′ and GI-mtLSU-632R 5′-GCATTCTTAGCCCAGCTATG-3′ were used for *R. intraradices* (Krak et al., 2002).

In order to check the amplification of DNA, all the samples were also amplified using primer pairs coding for Elongation factor 1-alpha X144449, a tomato housekeeping gene:

EF1AFFxMF 5’ -CTCCGTCTTCCACTTCAGGAC-3’, EF1AFFxMR 5’ -GTCACAACCATACCAGGCTTG (Løvdal et al., 2009).

The amplification was carried out in 25 µL volume containing 12.5 µL of SYBR Green PCR, 2X GoTaq qPCR Master Mix (Promega), 0.25 µl of 100X Reference Dye (Promega), 0.3 µL of forward and reverse primers (10 µm), 5 µL of template DNA (10 ng μL^{-1}) and water to 25 µL. Reactions were repeated twice with a 7300 real-time PCR Systems (Applied Biosystems, Foster City, CA, USA) and with the following cycling protocol: 50°C for 120 s, 95°C for 600 s and 40 cycles of 95°C for 15 s and 60°C for 60 s. A melting curve analysis (95°C for 15 s, 60°C for 30 s, 95°C for 15 s) was always included in each run to control for false-positive results caused by primer-dimer hybridization and non-specific amplifications.

4.2.4 Statistical analysis

The data were subjected to a three-way analysis of variance (ANOVA) to examine the effects of genotype, AM fungal inoculation and irrigation regime by GenStat 17.0th edition. Means were compared using Duncan’s test at the 5% level. In order to evaluate the relationships between treatments and parameters analysed, all data were analysed by Principal Component Analysis (PCA) model (Jackson, 1991; Wold et al., 1987) using PLS Toolbox software (Eigenvector Research Inc, Wenatchee, WA, USA). Difference between groups were assessed by Multivariate analysis (MANOVA).

4.3 Results

3.1. Physiological and morphological results

The genotype and mycorrhizal treatment main effects on leaf chlorophyll content were not statistically significant (Table 2). However, the interaction between genotype and mycorrhizal inoculation was significant. In particular, the chlorophyll content was the highest in the genotype Everton inoculated with *R. intraradices* under reduced irrigation regime (Table S1).

Table 2. Physiological parameters measured at the end of the experiment. Chl = index of the leaf chlorophyll content measured using Dx4, Flav = index of the leaf epidermal flavonoid content measured using Dx4, NBI = nitrogen balance index, Anth = index of the leaf anthocyanin content measured using Dx4, M- = control without mycorrhizae, MF+ = seedlings inoculated with *Funneliformis mosseae*, MR+ = seedlings inoculated with *Rhizophagus intraradices*, G = genotype, M = mycorrhizal treatment, I = irrigation regime. Data are presented as mean ± standard deviation (SD) (n genotype and mycorrhizae = 30, n irrigation = 45). Identical letters indicate differences among treatments or genotypes that are not statistically different by three-way ANOVA followed by Duncan's test at $p < 0.05$, ns = not significant, * = interaction among variables

Treatment	Chl		Flav		NBI		Anth	
Genotype (n = 30)								
Everton	29.1±9.2	ns	0.732±0.09	b	43.12±9.5	a	0.210±0.02	ns
H3402	28.1±4.4	ns	0.823±0.14	a	34.89±6.8	b	0.213±0.04	ns
Pearson	28±5.2	ns	0.719±0.18	b	40.46±8.3	a	0.213±0.02	ns
Mycorrhizae (n = 30)								
M-	26.9±7.6	ns	0.750±0.16	ns	38.60±7.4	ns	0.218±0.02	a
MF+	28.9±7.1	ns	0.777±0.16	ns	39.51±8.4	ns	0.201±0.04	b
MR+	29.4±6.6	ns	0.746±0.12	ns	40.36±10.8	ns	0.217±0.02	a
Irrigation (n = 45)								
fully-watered	24.7±5.5	b	0.784±0.15	a	34.28±5.8	b	0.223±0.03	a
reduced	32.1±5.5	a	0.731±0.13	b	44.70±8.4	a	0.201±0.02	b
G	0.051		0.002		<0.001		0.889	
M	0.086		0.553		0.491		0.032	
I	<0.001		0.037		<0.001		<0.001	
G*M	0.049		<0.001		0.067		0.194	
G*I	0.019		0.027		0.107		0.127	
M*I	0.058		0.253		0.002		0.263	
G*M*I	0.101		0.263		0.043		0.359	

Table S1. Physiological parameters measured at the end of the experiment. Chl = index of the leaf chlorophyll content measured using Dx4 Flav = index of the leaf epidermal flavonoid content measured using Dx4, NBI = nitrogen balance index, Anth = index of the leaf anthocyanin content measured using Dx4, M- = control without mycorrhizae, MF+ = seedlings inoculated with *Funneliformis mosseae,* MR+ = seedlings inoculated with *Rhizophagus intraradices,* G = genotype, M = mycorrhizae treatment, I = irrigation regime. Data are presented as mean ± standard deviation (SD, n = 5). Identical letters indicate differences among treatments or genotypes that are not statistically different by one-way ANOVA followed by Duncan's test at $p < 0.05$, ns = not significant.

Genotype	Treatment	Irrigation	Chl		Flav		NBI		Anth	
Everton	M-	fully-watered	22.32±3.7	g	0.74±0.09	bc	38.98±3.3	defgh	0.22±0.003	abcd
		reduced	32.77±1.8	abcd	0.65±0.03	c	50.14±2.6	ab	0.19±0.003	bcd
	MF+	fully-watered	21.69±2.1	g	0.72±0.05	bc	36.52±3.4	efghi	0.22±0.008	abc
		reduced	31.69±2.6	bcde	0.69±0.05	bc	45.67±3.2	bcd	0.19±0.009	bcd
	MR+	fully-watered	27.75±2.4	cdefg	0.86±0.09	ab	32.83±5.0	fghi	0.24±0.033	a
		reduced	39.35±6.6	a	0.72±0.03	bc	54.58±5.6	a	0.20±0.015	abcd
H3402	M-	fully-watered	25.67±2.7	defg	0.87±0.09	ab	29.75±4.2	i	0.24±0.014	a
		reduced	31.18±1.3	bcdef	0.99±0.17	a	32.18±4.9	ghi	0.23±0.015	abc
	MF+	fully-watered	23.68±1.8	fg	0.75±0.09	bc	31.69±2.2	ghi	0.18±0.101	d
		reduced	33.88±1.8	abc	0.77±0.08	bc	44.16±3.6	bcde	0.20±0.085	abcd
	MR+	fully-watered	23.95±1.1	fg	0.78±0.08	bc	30.90±4.12	hi	0.23±0.010	ab
		reduced	30.40±2.4	bcdef	0.77±0.15	bc	40.63±6.0	cdef	0.20±0.005	abcd
Pearson	M-	fully-watered	25.80±0.6	defg	0.63±0.02	c	40.86±1.0	cdef	0.22±0.009	abc
		reduced	24.30±1.4	efg	0.61±0.04	c	39.71±4.0	cdefg	0.21±0.009	abcd
	MF+	fully-watered	27.69±2.2	cdefg	0.99±0.03	a	31.13±10.5	hi	0.23±0.036	abc
		reduced	35.13±6.4	ab	0.73±0.09	bc	47.88±4.7	abc	0.18±0.021	cd
	MR+	fully-watered	24.97±2.7	efg	0.72±0.17	bc	35.83±4.9	fghi	0.24±0.013	ab
		reduced	30.01±5.6	bcdef	0.63±0.03	c	47.37±7.9	abc	0.20±0.015	abcd

Leaf flavonoid content was affected by genotype, irrigation regime, and by interactions genotype x AM fungal inoculation and genotype x irrigation (Table 2). The highest values were showed by Everton inoculated with *R. intraradices* under fully-watered irrigation regime, H3402 without mycorrhizal inoculation under reduced irrigation regime and Pearson inoculated with *F. mosseae* under fully-watered irrigation regime.

NBI was affected by interaction among AM fungal inoculation, irrigation regime and genotype. NBI values were the highest in Everton inoculated with *R. intraradices* under reduced irrigation regime. On the other hand, H3402 inoculated with *F. mosseae* showed the highest NBI values under reduced irrigation regime (Table S1). Finally, Pearson showed the highest values inoculated with AM fungus (independent of AM fungal species) under reduced irrigation regime.

Interestingly, AMF inoculation and irrigation regimes influenced Anth values. The seedlings inoculated with *F. mosseae* showed the lowest Anth values in comparison with the seedlings without inoculation or inoculated with *R. intraradices* (Table 2).

Plant height is one of the most important parameters in processing tomato seedlings when grown in nursery. In this study, plant height was influenced by interaction among genotype x AM fungal inoculation x irrigation regime (Table 3). The highest value was displayed by Pearson inoculated with *R. intraradices* under fully-watered irrigation regime (Table S1). On the other hand, the lowest value was recorded by Pearson inoculated with *R. intraradices* under reduced irrigation regime.

Table 3. Morphological non-destructive parameters measured at the end of the experiment. H = height of seedlings, D = stem diameter of seedlings, H/D = height to diameter ratio, LN (no.) = number of leaves per seedling, LA = leaf area, LMA = leaf mass *per* area, M- = control without mycorrhizae, MF+ = seedlings inoculated with *Funneliformis mosseae*, MR+ = seedlings inoculated with *Rhizophagus intraradices*, G = genotype, M = mycorrhizae treatment, I = irrigation regime. * = interaction. Data are presented as mean ± standard deviation (SD) (n genotype and mycorrhizae = 30, n irrigation = 45). Identical letters indicate differences among treatments or genotypes that are not statistically different by three-way ANOVA followed by Duncan's test at $p < 0.05$, ns = not significant, * = interaction among variables.

Treatment	H (mm)	D (mm)	H D^{-1}	LN (no. $plant^{-1}$)	LA (m^2 $plant^{-1}$)	LMA (g m^{-2})
Genotype (n = 30)						
Everton	277.0±36 a	4.6±0.4 ns	59.86±5.9 ns	7.2±0.6 a	0.047±0.013 b	10.1±2.9 ab
H3402	264.4±51 b	4.6±0.4 ns	58.13±11.0 ns	6.4±0.4 c	0.059±0.016 a	11.2±4.5 a
Pearson	282.8±42 a	4.8±0.7 ns	59.49±6.3 ns	6.8±0.7 b	0.045±0.014 b	8.8±3.7 b
Mycorrhizae (n = 30)						
M-	276.0±37 ns	4.6±0.6 ns	59.92±5.6 ns	6.7±0.7 ns	0.047±0.014 b	10.3±3.0 a
MF+	275.5±49 ns	4.8±0.6 ns	57.63±6.3 ns	6.9±0.8 ns	0.050±0.018 ab	11.2±5.0 a
MR+	272.7±46 ns	4.6±0.5 ns	59.93±11.0 ns	6.8±0.6 ns	0.054±0.013 a	8.6±2.7 b
Irrigation (n = 45)						
fulyl-watered	313.1±18 a	5±0.5 a	63.28±7.5 a	7.0±0.6 a	0.057±0.015 a	8.7±2.5 b
reduced	236.3±25 b	4.3±0.4 b	55.04±6.2 b	6.6±0.6 b	0.043±0.013 b	11.3±4.5 a
G	<0.001	0.071	0.401	<0.001	<0.001	0.004
M	0.736	0.132	0.148	0.506	0.025	0.002
I	<0.001	<0.001	<0.001	0.001	<0.001	<0.001
G*M	0.056	0.170	0.214	0.004	0.654	0.086
G*I	0.016	0.004	<0.001	0.283	0.092	<0.001
M*I	0.253	<0.001	<0.001	0.560	0.018	<0.001
G*M*I	<0.001	0.217	<0.001	0.019	0.004	<0.001

Table S2 Morphological non-destructive parameters measured at the end of the experiment. H = height of seedlings, D = stem diameter of seedlings, H D^{-1} = height to diameter ratio, LN (no.) = number of leaves per seedling, LA = leaf area, LMA = leaf mass *per* area, M- = control without mycorrhizae, MF+ = seedlings inoculated with *Funneliformis mosseae*, MR+ = seedlings inoculated with *Rhizophagus intraradices*, F = genotype, M = mycorrhizae treatment, I = irrigation regime. * = interaction. Data are presented as mean ± standard deviation (SD, n = 5). Identical letters indicate differences among treatments or genotypes that are not statistically different by one-way ANOVA followed by Duncan's test at $p < 0.05$, ns = not significant.

Genotype	Treatment	Irrigation	H (cm)		D (mm)		H D^{-1}		LEAVES (no.)		LA (m^2 $plant^{-1}$)		LMA (g m^{-2})	
Everton	M-	fully-watered	32.12±1.4	ab	4.76±0.1	cdefg	67.59±3.8	ab	7.75±0.8	a	0.053±0.012	cde	9.15±0.1	def
		reduced	23.50±1.5	cd	4.20±0.2	hi	56.07±3.7	cde	6.83±0.4	cde	0.034±0.001	fg	13.52±2.2	bc
	MF+	fully-watered	30.90±0.9	ab	5.16±0.3	abc	60.12±4.7	cde	7.70±0.4	ab	0.055±0.016	bcde	11.46±2.4	bcd
		reduced	25.40±1.0	c	4.32±0.3	ghi	59.11±4.1	cde	6.80±0.4	cde	0.041±0.012	efg	8.98±2.4	def
	MR+	fully-watered	30.00±1.5	b	4.87±0.1	cdef	61.67±3.0	bcd	7.10±0.5	abcd	0.043±0.003	defg	10.72±2.9	cde
		reduced	24.25±1.0	c	4.49±0.4	defgh	54.57±6.5	defg	7.13±0.4	abcd	0.054±0.010	bcde	6.56±1.4	f
H3402	M-	fully-watered	30.50±1.5	ab	4.95±0.3	cde	61.74±4.3	bcd	6.70±0.4	cdef	0.056±0.01	bcde	10.05±3.3	cdef
		reduced	24.30±1.1	c	4.42±0.3	efghi	55.04±2.7	def	6.50±0.2	def	0.058±0.006	bcd	13.06±2.4	bc
	MF+	fully-watered	31.50±2.1	ab	5.01±0.2	bcd	62.91±2.9	bc	6.50±0.2	def	0.076±0.005	a	6.44±0.6	f
		reduced	19.60±3.1	e	4.11±0.4	hi	47.57±5.5	g	6.00±0.7	f	0.042±0.013	defg	17.31±4.1	a
	MR+	fully-watered	31.12±1.4	ab	4.35±0.6	fghi	73.45±8.9	a	6.38±0.2	def	0.070±0.018	ab	9.18±2.0	def
		reduced	21.62±1.2	de	4.50±0.2	defgh	48.09±1.6	fg	6.50±0.2	def	0.055±0.002	bcde	10.95±1.7	cd
Pearson	M-	fully-watered	30.38±1.5	ab	5.47±0.3	ab	55.72±3.0	cde	6.50±0.2	def	0.049±0.004	cdef	6.95±0.7	ef
		reduced	24.78±0.5	c	3.91±0.1	i	63.36±2.4	bc	6.10±0.5	ef	0.031±0.007	g	8.64±1.2	def
	MF+	fully-watered	32.50±1.6	ab	5.61±0.2	a	57.89±0.8	cde	7.00±0.5	bcd	0.054±0.012	bcde	7.96±2.2	def
		reduced	25.40±1.5	c	4.38±0.2	fghi	58.20±5.9	cde	7.30±0.8	abc	0.032±0.009	g	14.84±4.5	ab
	MR+	fully-watered	32.80±1.8	a	4.83±0.5	cdefg	68.44±5.9	ab	7.30±0.4	abc	0.062±0.007	abc	6.28±0.4	f
		reduced	23.80±0.6	cd	4.46±0.6	efgh	53.37±3.1	efg	6.480.8	def	0.043±0.008	defg	7.84±3.4	def

A significative interaction between AM fungal inoculation and irrigation regime was found for stem diameter. The highest values were achieved by Pearson, H3402 and Everton inoculated with *F. mosseae* under fully-watered regime. However, H3402 inoculated with *F. mosseae* under reduced irrigation regime showed the lowest value in comparison with Everton and Pearson.

For the ratio between plant height and stem diameter we noticed significant interactions among AM fungal inoculation, genotype and irrigation regime. The highest values of plant height and stem diameter ratio were achieved by H3402 and Person inoculated with *R. intraradices* and Everton without AM fungal inoculations under fully-watered irrigation regime.

Leaf traits (number of leaves, leaf area, leaf mass *per* area and leaf dry weight) were influenced by interactions among all the treatments (Tables 3 and 4). Everton and H3402 without mycorrhizal inoculation under fully-watered irrigation regime displayed the highest number of leaves (Table S2). On the other hand, Pearson showed the highest number of leaves when inoculated with *F. mosseae* under reduced irrigation regime and inoculated with *R. intraradices* under fully-watered irrigation regime (Table S2). H3402 and Pearson inoculated with *F. mosseae* under fully-watered irrigation regime achieved the highest leaf area values (Table S2). On the other hand, Everton displayed the highest leaf area values when inoculated with *F. mosseae* under fully-watered irrigation regime and inoculated with *R. intraradices* under reduced irrigation regime. In reduced irrigation regime, the decrease in leaf area of the seedlings was accompanied by an increase in leaf mass *per* area. These compensating changes led to little change in leaf dry mass *per* seedling (decreased in Everton inoculated with *F. mosseae* and increased in H3402 non-inoculated or inoculated with *F. mosseae*). H3402 showed the highest leaf dry weight values without inoculations under reduced irrigation regime, Pearson displayed the highest value inoculated with *F. mosseae* under reduced irrigation regime, while Everton inoculated with *F. mosseae* under fully-water irrigation regime (Table S3).

Stem dry weight, total dry weight and water use efficiency were influenced by interactions among genotype, AM fungal inoculation and irrigation regime (Tables 4 and 5). Stem dry weights and the total dry weights were highest in seedlings inoculated with *F. mosseae*. On the other hand, the highest water use efficiency values were reached by genotypes H3402 and Pearson. Finally, Pearson and H3402 inoculated with *F. mosseae* under reduced

irrigation regime achieved the highest value of water use efficiency and similar result was displayed by Pearson non inoculated (Table S4).

Root dry weight and root and shoot ratio were influenced by mycorrhizal treatments that interacted with genotype (Table 4). Root dry weight decreased in Everton inoculated with *F. mosseae* under reduced irrigation regime.

Table 4. Morphological parameters measured destructively at the end of the experiment. LDW = leaf dry weight, SDW = stem dry weight, RDW = root dry weight, TDW = total dry weight, Root Shoot^{-1} = root to shoot ratio, M- = control without mycorrhizae, MF+ = seedlings inoculated with *Funneliformis mosseae*, MR+ = seedlings inoculated with *Rhizophagus intraradices*, F = genotype, M = mycorrhizae treatment, I = irrigation regime. Data are presented as mean ± standard deviation (SD) (n genotype and mycorrhizae = 30, n irrigation = 45). Identical letters indicate differences among treatments or genotypes that are not statistically different by three-way ANOVA followed by Duncan's test at $p < 0.05$, ns = not significant, * = interaction among variables.

Treatment	LDW (g plant^{-1})		SDW (g plant^{-1})		RDW (g plant^{-1})		TDW (g plant^{-1})		ROOT SHOOT^{-1}	
Genotype (n = 30)										
Everton	0.452±0.13	b	0.233±0.08	ns	0.333±0.09	b	1.02±0.23	b	0.508±0.19	b
H3402	0.611±0.15	a	0.237±0.07	ns	0.495±0.17	a	1.34±0.35	a	0.578±0.12	a
Pearson	0.364±0.09	c	0.228±0.10	ns	0.243±0.10	c	0.83±0.27	c	0.406±0.10	c
Mycorrhizae (n = 30)										
M-	0.471±0.18	ns	0.206±0.09	b	0.372±0.23	ns	1.05±0.46	ab	0.518±0.18	ns
MF+	0.497±0.14	ns	0.284±0.08	a	0.376±0.10	ns	1.16±0.26	a	0.482±0.08	ns
MR+	0.459±0.17	ns	0.208±0.06	b	0.323±0.12	ns	0.99±0.29	b	0.493±0.19	ns
Irrigation (n = 45)										
fully-watered	0.482±0.14	ns	0.256±0.06	a	0.330±0.12	b	1.07±0.27	ns	0.440±0.12	b
reduced	0.469±0.18	ns	0.209±0.10	b	0.383±0.19	a	1.06±0.43	ns	0.555±0.17	a
G	<0.001		0.835		<0.001		<0.001		<0.001	
M	0.402		<0.001		0.085		0.018		0.546	
I	0.580		<0.001		0.015		0.885		<0.001	
G*M	0.055		<0.001		<0.001		<0.001		0.025	
G*I	<0.001		0.006		0.001		<0.001		0.909	
M*I	0.114		0.186		0.406		0.472		0.273	
G*M*I	0.043		<0.001		0.067		0.019		0.709	

Table S3 Morphological destructive parameters measured at the end of the experiment. LDW = leaf dry weight, SDW = stem dry weight, RDW = root dry weight, TDW = total dry weight, Root Shoot^{-1} = root to shoot ratio, M- = control without mycorrhizae, MF+ = seedlings inoculated with *Funneliformis mosseae*, MR+ = seedlings inoculated with *Rhizophagus intraradices*, F = genotype, M = mycorrhizae treatment, I = irrigation regime. Data are presented as mean ± standard deviation (SD, n = 5). Identical letters indicate differences among treatments or genotypes that are not statistically different by ANOVA followed by Duncan's test at $p < 0.05$, ns = not significant.

Genotype	Treatment	Irrigation	LDW (g plant^{-1})		SDW (g plant^{-1})		RDW (g plant^{-1})		TDW (g plant^{-1})		ROOT/SHOOT	
Everton	M-	fully-watered	0.49±0.11	cdefg	0.31±0.02	abc	0.34±0.04	cdef	1.13±0.13	cde	0.43±0.09	defg
		reduced	0.46±0.08	defg	0.11±0.01	h	0.37±0.14	cde	0.94±0.23	def	0.64±0.16	abc
	MF+	fully-watered	0.60±0.09	bcd	0.29±0.04	abc	0.40±0.06	bcd	1.30±0.17	bc	0.45±0.06	bcdefg
		reduced	0.36±0.09	gh	0.21±0.06	defg	0.25±0.05	efg	0.81±0.19	efg	0.44±0.05	cdefg
	MR+	fully-watered	0.46±0.13	defg	0.24±0.04	bcdef	0.31±0.11	def	1.02±0.25	cde	0.44±0.17	cdefg
		reduced	0.34±0.05	gh	0.24±0.08	bcdef	0.33±0.07	cdef	0.92±0.07	def	0.64±0.35	ab
H3402	M-	fully-watered	0.52±0.11	bcdef	0.23±0.05	cdef	0.48±0.18	bc	1.23±0.32	bcd	0.62±0.16	abcd
		reduced	0.75±0.15	a	0.28±0.08	abcd	0.73±0.20	a	1.77±0.40	a	0.71±0.11	a
	MF+	fully-watered	0.49±0.05	cdefg	0.27±0.01	bcde	0.37±0.04	cde	1.13±0.04	cde	0.49±0.07	bcdefg
		reduced	0.67±0.11	ab	0.26±0.12	bcdef	0.53±0.09	b	1.46±0.29	b	0.59±0.08	abcdef
	MR+	fully-watered	0.63±0.08	abc	0.20±0.02	defg	0.39±0.14	bcde	1.22±0.37	bcd	0.45±0.07	bcdefg
		reduced	0.60±0.09	bcde	0.18±0.04	fgh	0.47±0.06	bc	1.24±0.19	bcd	0.61±0.04	abcde
Pearson	M-	fully-watered	0.34±0.01	gh	0.19±0.04	efg	0.16±0.04	g	0.69±0.07	fg	0.31±0.07	g
		reduced	0.27±0.06	h	0.11±0.02	h	0.15±0.03	g	0.53±0.10	g	0.40±0.07	fg
	MF+	fully-watered	0.42±0.10	fgh	0.32±0.07	ab	0.31±0.03	def	1.05±0.03	cde	0.42±0.04	efg
		reduced	0.44±0.07	efg	0.36±0.05	a	0.40±0.04	bcd	1.20±0.15	bcd	0.50±0.04	bcdefg
	MR+	fully-watered	0.39±0.03	fgh	0.24±0.06	bcdef	0.22±0.07	fg	0.85±0.07	ef	0.34±0.07	g
		reduced	0.33±0.12	gh	0.14±0.04	gh	0.22±0.10	fg	0.69±0.25	fg	0.47±0.16	bcdefg

Table 5. Parameters measured at the end of the experiment in order to understand the responses of seedlings to the different water regime. TWU = Total water used, WUE = water use efficiency, M- = control without mycorrhizae, MF+ = seedlings inoculated with *Funneliformis mosseae*, MR+ = seedlings inoculated with *Rhizophagus intraradices*, F = genotype, M = mycorrhizae treatment, I = irrigation regime. Data are presented as mean ± standard deviation (SD) (n genotype and mycorrhizae = 30, n irrigation = 45). Identical letters indicate differences among treatments or genotypes that are not statistically different by three-way ANOVA followed by Duncan's test at $p < 0.05$, ns = not significant, * = interaction among variables.

Treatments	TWU ($g\ H_2O\ plant^{-1}$)		WUE ($g\ H_2O\ g\ dry\ weight^{-1}$)	
Genotype (n = 30)				
Everton	304.2±149	a	0.0039±0.001	b
H3402	287.1±108	a	0.0050±0.002	a
Pearson	227.1±126	b	0.0053±0.003	a
Mycorrhizae (n = 30)				
M-	266.6±139	ns	0.0051±0.003	a
MF+	283.4±133	ns	0.0050±0.003	a
MR+	268.4±119	ns	0.0042±0.002	b
Irrigation (n = 45)				
fully-watered	378.7±85	a	0.0030±0.001	b
reduced	166.9±61	b	0.0070±0.001	a
G	<0.001		<0.001	
M	0.530		<0.001	
I	<0.001		<0.001	
G*M	0.049		0.073	
G*I	0.036		<0.001	
M*I	0.103		<0.001	
G*M*I	0.655		0.005	

Table S4 Parameters measured at the end of the experiment in order to understand the responses of seedlings to the different water regime. TWU = Total water used, WUE = water use efficiency, M- = control without mycorrhizae, MF+ = seedlings inoculated with *Funneliformis mosseae*, MR+ = seedlings inoculated with *Rhizophagus intraradices*, F = genotype, M = mycorrhizae treatment, I = irrigation regime. Data are presented as mean ± standard deviation (SD, n = 5). Identical letters indicate differences among treatments or genotypes that are not statistically different by one-way ANOVA followed by Duncan's test at $p < 0.05$, ns = not significant.

Genotype	Treatment	Irrigation	TWU ($g\ H_2O\ plant^{-1}$)		WUE	
Everton	M-	fully-watered	421.60±61	ab	0.0027±0.0001	fg
		reduced	156.80±47	f	0.0061±0.0003	bc
	MF+	fully-watered	456.60±47	a	0.0028±0.0002	fg
		reduced	166.60±48	ef	0.0049±0.0003	d
	MR+	fully-watered	415.60±113	ab	0.0025±0.0002	fg
		reduced	208.10±13	ef	0.0044±0.0003	de
H3402	M-	fully-watered	389.00±99	abc	0.0032±0.0003	fg
		reduced	248.00±40	de	0.0071±0.0009	b
	MF+	fully-watered	378.50±59	abc	0.0031±0.0004	fg
		reduced	169.00±27	ef	0.0086±0.0009	a
	MR+	fully-watered	346.40±99	bc	0.0036±0.0007	ef
		reduced	191.70±36	ef	0.0065±0.0003	b
Pearson	M-	fully-watered	320.00±56	cd	0.0022±0.0003	g
		reduced	64.30±16	g	0.0084±0.0015	a
	MF+	fully-watered	377.80±52	abc	0.0028±0.0000	fg
		reduced	151.70±48	f	0.0084±0.0021	a
	MR+	fully-watered	303.00±84	cd	0.0029±0.0005	fg
		reduced	145.70±67	f	0.0053±0.00015	cd

4.3.2 Relationships among investigated treatments and parameters

The correlations among treatments and parameters were studied using PCA (Figure 1).

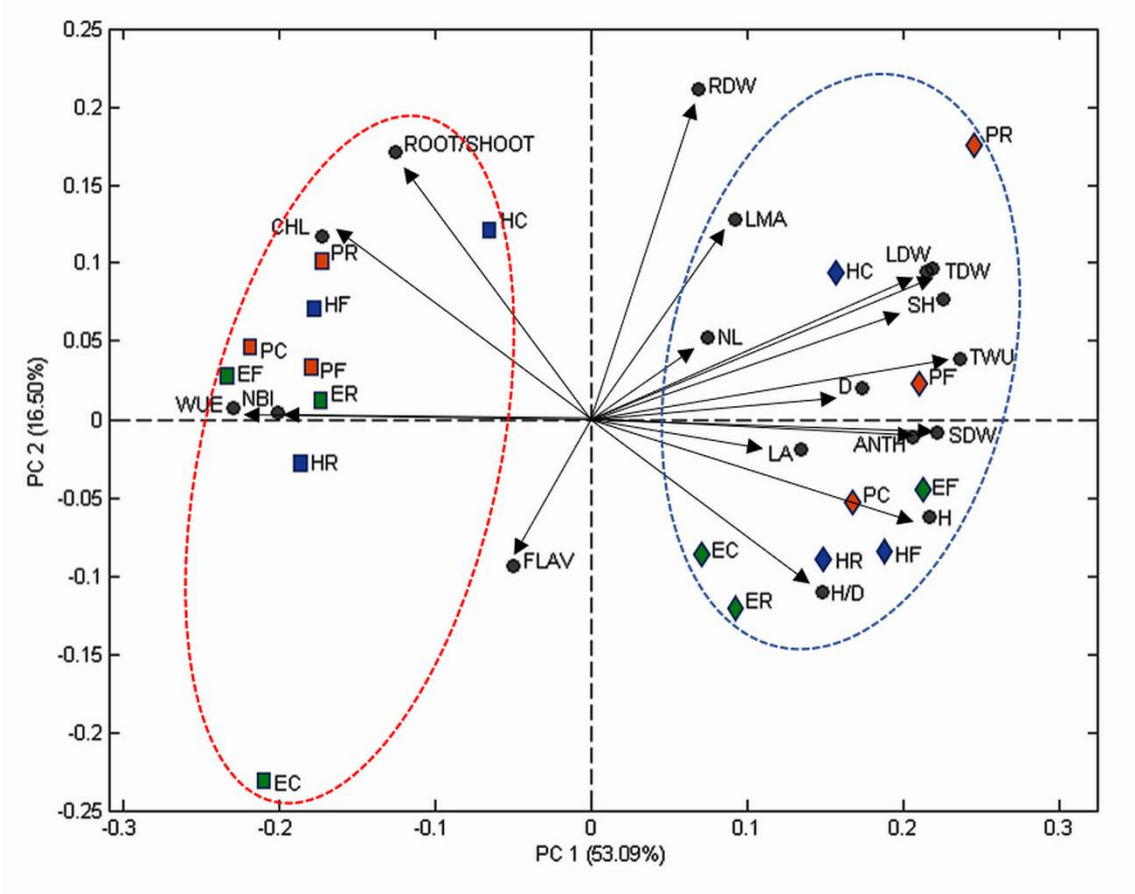

Figure 1: Biplot of Principal Component Analysis. CHL = index of the chlorophyll content in leaf measured using Dx4; FLAV = index of the flavonoids content in leaf measured using DUALX instrument; NBI = nitrogen balance index, ANTH = index of the anthocyanins content in leaf measured using Dx4, H = height of seedling; D = diameter of seedling; H/D = height and diameter ratio of seedling; NL = number of fully expanded leaves per seedling; LA = leaf area of the seedling; LMA = leaf mass *per* area of the seedling; LDW = leaf dry weight; SDW = stem dry weight; RDW = root dry weight; SH = shoot weight (LDW+SDW); TDW = total dry weight of the seedling; ROOT/SHOOT = root and shoot ratio; TWU = total water used by seedling; WUE = water use efficiency; PC = Pearson without mycorrhizal inoculation; PF = Pearson inoculated with *F. mosseae*; PR = Pearson inoculated with *R. intraradices*; HC = H3402 without mycorrhizal inoculation; HF = H3402 inoculated with *F. mosseae*; HR = H3402 inoculated with *R. intraradices*; EC = Everton without mycorrhizal inoculation; EF = Everton inoculated with *F. mosseae*; ER = Everton inoculated with *R. intraradices*; squares = reduced irrigation regime; diamonds = fully-watered irrigation regime; green = Everton; blue = H3402; red = Pearson: red ellipse groups all the genotypes under reduced irrigation regime; blue ellipse groups all the genotypes under fully-watered irrigation regime.

The contributions of the two first principal components were 53.1% (PC1) and 16.5% (PC2) and their sum explained 69.6% of the total variation. Irrigation regime clustered in two groups. In fact, all the combinations genotype x mycorrhizae treatment under reduced irrigation regime were on the negative side of the PC1, while all the combinations genotype x mycorrhizae treatment under fully-watered irrigation regime were on the positive side of PC1. Therefore, PC1 was clearly related with irrigation regime. In addition, MANOVA analysis confirmed that the two clusters were statistically different at $p < 0.05$. In addition, H3402 without mycorrhizal inoculation (HC) and Pearson inoculated with *R. intraradices* (PR) were in the positive square while H3402 inoculated with *R. intraradices* (HR) and Everton without mycorrhizal inoculation (EC) were in the negative one. In addition, WUE was positive correlated with CHL and root/shoot ratio (Figure S1). Finally, focusing the attention on the genotype, the old genotype Pearson that was not inoculated (PC) performed as well as PF under drought stress in terms of WUE, while the same trend was not observed in modern genotypes.

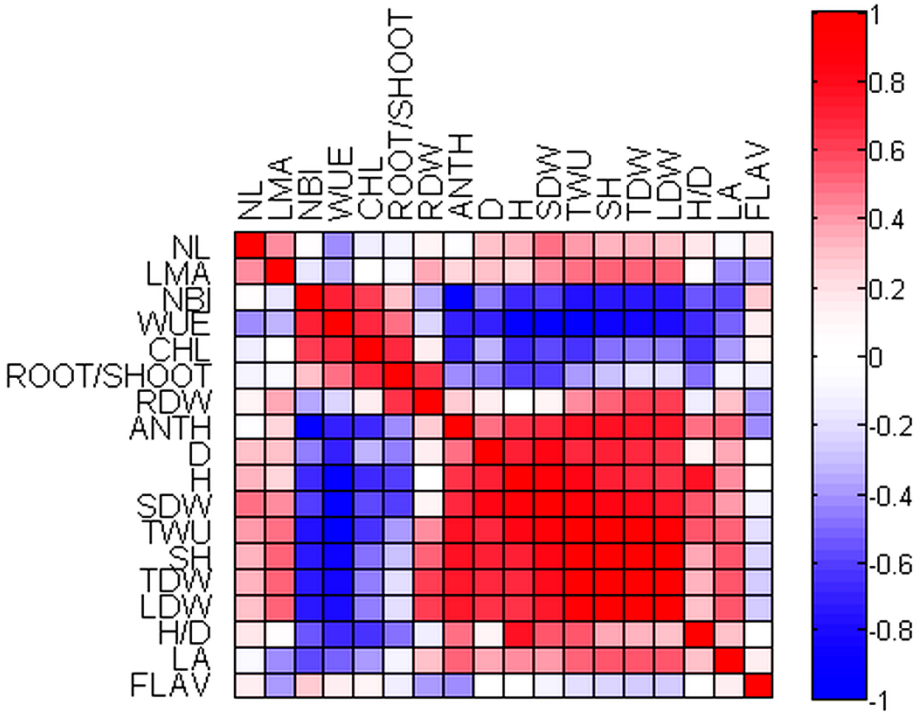

Figure S1: Correlation plot. CHL = index of the chlorophyll content in leaf measured using Dx4; FLAV = index of the flavonoids content in leaf measured using DUALX instrument; NBI = nitrogen balance index, ANTH = index of the anthocyanins content in leaf measured using Dx4, H = height of seedling; D = diameter of seedling; H/D = height and diameter ratio of

seedling; NL = number of fully expanded leaves per seedling; LA = leaf area of the seedling; LMA = leaf mass *per* area of the seedling; LDW = leaf dry weight; SDW = stem dry weight; RDW = root dry weight; SH = shoot weight (LDW+SDW); TDW = total dry weight of the seedling; ROOT/SHOOT = root and shoot ratio; TWU = total water used by seedling; WUE = water use efficiency.

4.3.3 AM fungal root presence

A melting curve profile was used to track the presence of inoculated mycorrhizal species. As reported in figure 2, all the samples were amplified by primer pairs of tomato housekeeping gene confirming the amplifiability of roots DNA. Based on SybrGreen PCR analysis, the presence of *F. mosseae* and *R. intraradices* DNA was confirmed in the processing tomato roots inoculated with *F. mosseae* and *R. intraradices*, respectively (Figures 3 and 4). In contrast, all non-inoculated roots (controls) were negative for *F. mosseae* and *R. intraradices* DNA presence (Figures 3 and 4).

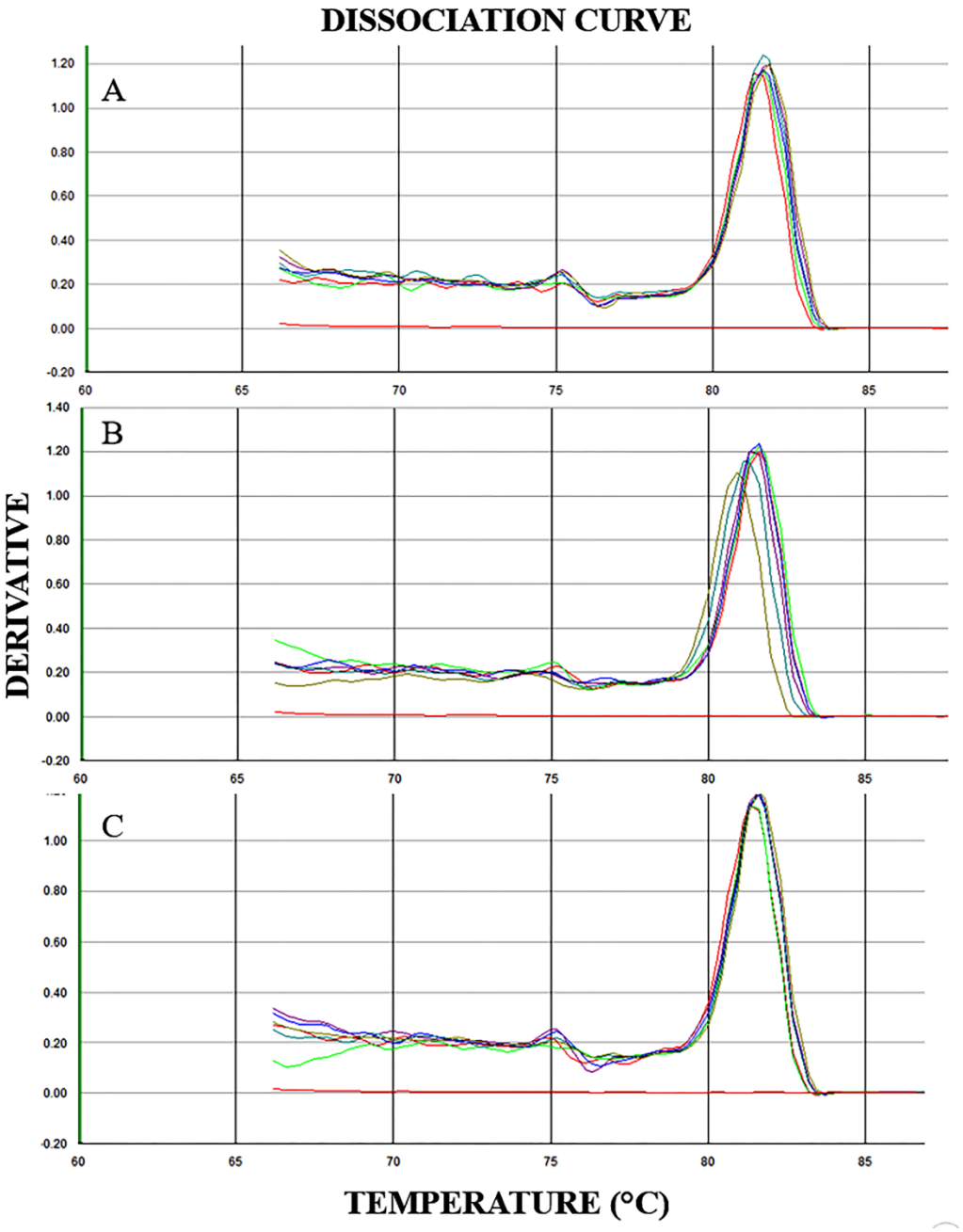

Figure 2. Dissociation curves to confirm the amplification of in root samples of Pearson (A), H3402 (B) and Everton (C). Single peaks are obtained from different samples (each peak of different color represents a samples). Flat lines are no template control (NTC) technical replicates

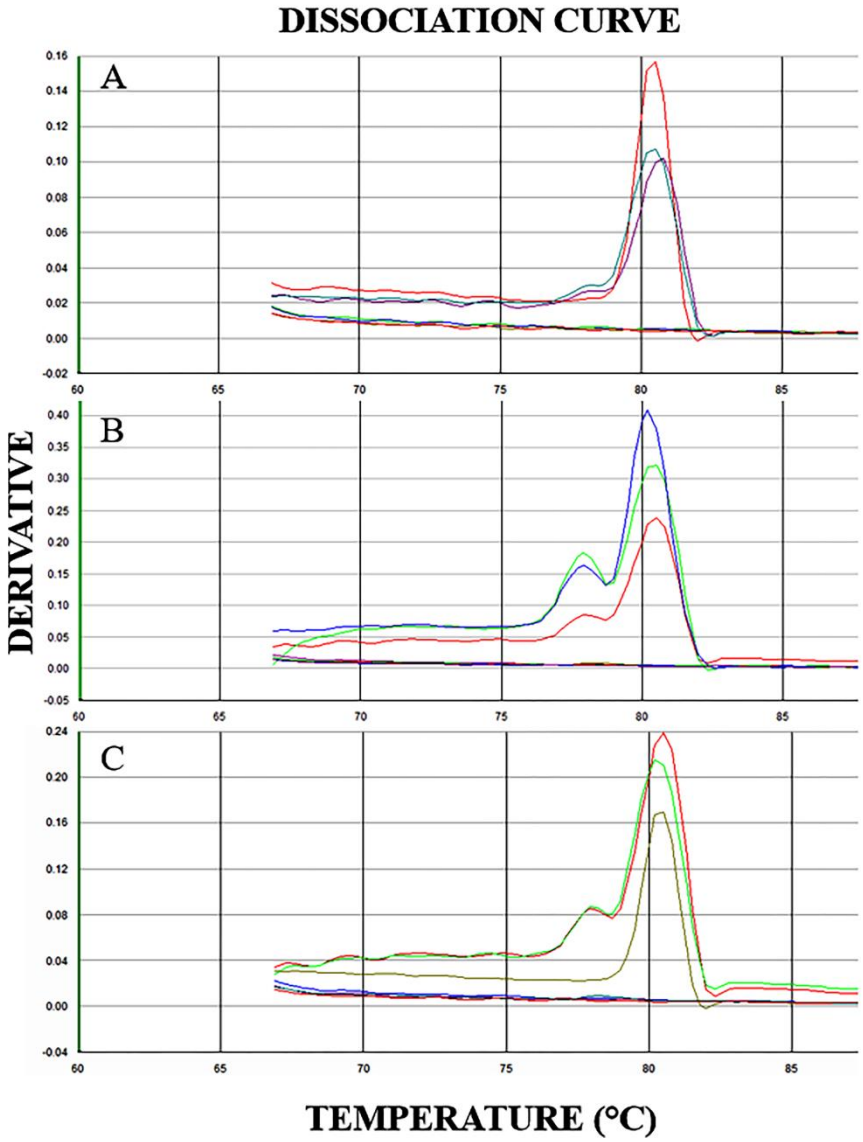

Figure 3. Dissociation curves to confirm *F. mosseae* presence in roots of 'Pearson' (**A**), 'H3402′ (**B**) and 'Everton' (**C**), respectively. Single peaks are obtained from different replicates (each peak of different color represents a replicate). Flat lines are no template control (NTC) technical replicates.

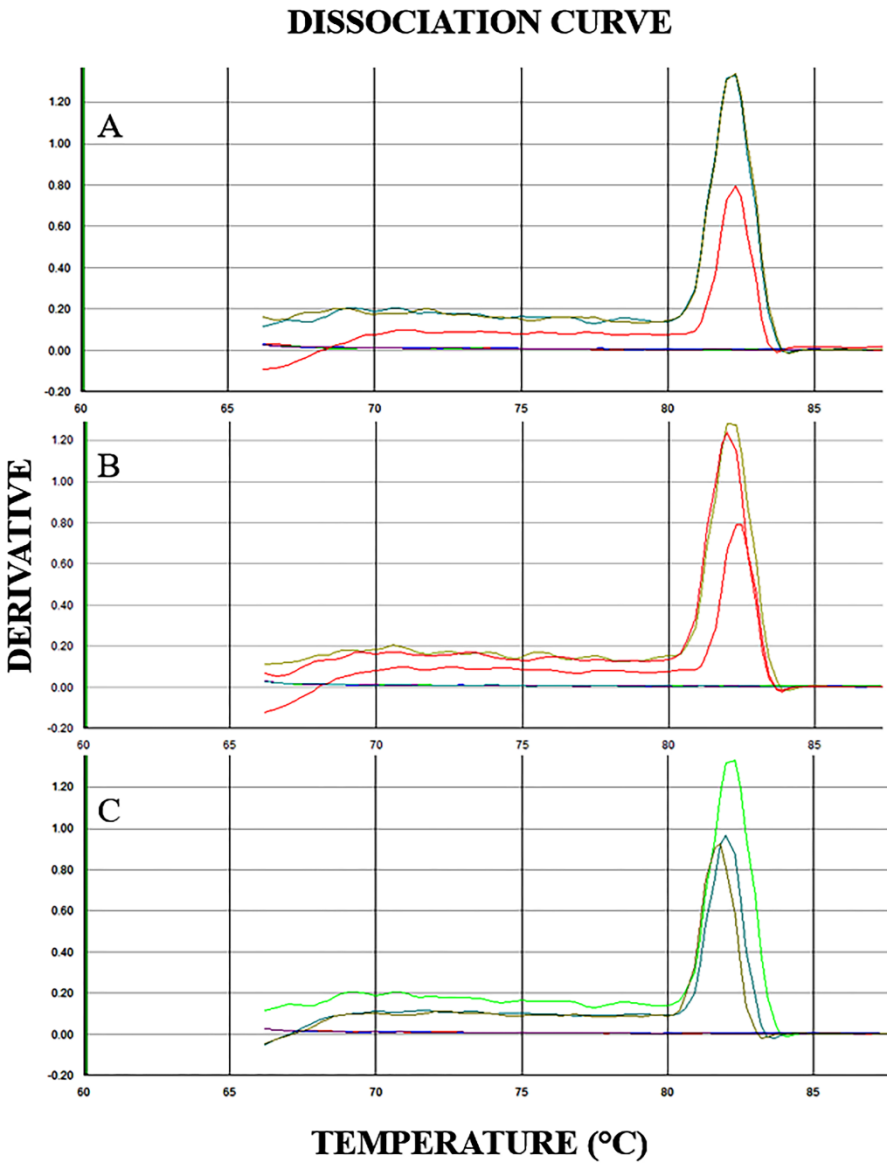

Figure 4. Dissociation curves to confirm *R. intraradices* presence in roots of 'Pearson' (**A**), 'H3402′ (**B**) and 'Everton' (**C**), respectively. Single peaks are obtained from different replicates (each peak of different color represents a replicate). Flat lines are no template control (NTC) technical replicates.

4.4 Discussion

The present study showed how different genotypes of processing tomato seedlings at stage of 35 to 40 days after germination responded to different mycorrhizal symbiosis under different irrigation regime.

Physiological parameters were assessed at the end of a drought stress period including leaf chlorophyll, flavonoid and anthocyanin contents. Chlorophyll is a key pigment involved in

photosynthesis while the accumulation of anthocyanins and flavonoids in plants is often linked to plant response to stress conditions (Caradonia et al., 2019; Krak et al., 2002). In our experiment, beneficial AM fungal effects were observed on leaf chlorophyll content for all genotypes. However, the beneficial effects varied with genotype, AM fungal specie and water availability (Table S1). These results agree with the findings by Londoño et al. (2019) who noticed that the inoculation with *Rhizophagus clarus* led to an increase in total chlorophyll concentration which varied between maize genotypes.

The content of leaf secondary metabolites, expressed as leaf anthocyanins and flavonoids, was influenced by genotype and AM fungal inoculation (Table 2 and S1). In fact, AM fungi are known to affect not only plant development and nutrient uptake, but also plant secondary metabolism especially in root and in shoot tissues (Lingua et al., 2013)

Observing morphological parameters (Table 3 and S2), an increase in chlorophyll content in seedlings under moderate drought stress was not reflected in an increase of the net assimilation rate corresponding to a higher plant growth. In fact, the seedlings under reduced irrigation regime were smaller in plant height, steam diameter and number of leaves. These data highlight the importance of irrigation water in processing tomato development also during the seedling production. Nonetheless, AM fungi positively influenced leaf area, and the seedlings inoculated with *R. intraradices* achieved the highest values. This observation, together with the increase of primary metabolism, might be an interesting aspect to take into account for seedling production in nursery conditions.

One of the most important parameters, both in agronomic and physiological studies, is the plant total dry weight. We observed a different partitioning of dry weight biomass to roots and stem; seedlings under drought stress had the highest allocation to the roots, while stem showed the highest value under normal irrigation. As previously reported, this pattern of biomass accumulation confirmed the ability of tomato plants to cope the drought stress inducing morphological changes. However, when genotype and the AM fungal inoculation effects were considered, there was a different behaviour between old and modern genotypes. Pearson recorded the highest values of root, stem, leaf and total dry weights when inoculated with *F. mosseae* under partial irrigation; while H3402 showed the highest dry weight values for the different tissues when not inoculated and grown under partial irrigation. The ability of tomato to produce high biomass under reduced irrigation was previously reported by Patanè et al.

(2011) in an experiment performed with the processing tomato (genotype Brigade) in open field. Finally, Everton performed better under fully-watered irrigation and when inoculated with *F. mosseae.* Our results confirmed that also the AM fungal effects on plant biomass depend on interactions with genotypes and cropping systems as previously showed by Londoño et al. (2019) and Steinkellner et al. (2011). In contrast, a negative effect of *R. intraradices* on plant biomass was observed. These results could be explained by a parasitic behaviour of AM fungi due to environmental conditions that limit plant growth and are not symbiotic (Jones et al., 2004; Smith et al., 1988; Veiga et al., 2011)

Water use efficiency is the most important parameter which assesses the ability of plant to maximize the use of the available water in the production of biomass. In our results, seedlings inoculated with *F. mosseae* showed higher values of WUE compared to those inoculated with *R. intraradices*. In fact, H3402 inoculated with *F. mosseae* displayed the highest values of WUE during drought stress. In contrast to these findings, Chitarra et al. (2016) and Volpe et al. (2018) showed that tomato plants inoculated with *R. intraradices* achieved the highest WUE. These differences could be due to the different growth conditions as Chitarra et al. (2016) worked under severe drought stress conditions and with adult plants) and a different genotype (San Marzano nano) confirming the importance to assess the interactions among the available AM fungi and genotype in different plant phenological stages and cropping systems.

The AM fungal root colonization was displayed for all the genotype x irrigation regimes, confirming the data reported by Steinkellner et al. (2011), who investigated old and modern genotypes of tomatoes bred for greenhouse and fresh market purposes.

Our findings highlighted that the breeding program for processing tomato (the introgression of favourable traits to harvesting machinery associated with reduced plant height, average fruit weight and increased marketable yields) (Ronga et al., 2019a) did not select against AM fungal symbiosis in processing tomato. These results corroborated those previously reported by De Vita et al. (2018) for durum wheat

4.5 Conclusions

The present study provides useful information to nursery growers on the application of AM fungi for the production of processing tomato seedlings during drought stress. Our results showed how AM fungi could improve drought tolerance and enhance tomato seedling growth.

The present study suggests that the two AMF studied activated different physiological strategies in processing tomato seedlings to cope with drought stress. However, *F. mosseae* seemed more effective than *R. intraradices* on influencing several morphological and physiological traits. In fact, Pearson and Everton inoculated with *F. mosseae* recorded the highest values of root, leaf and total dry weights under reduced and fully-watered irrigation regimes, respectively. In addition, seedlings of Pearson and H3402 inoculated with *F. mosseae* under reduced irrigation displayed the highest values of leaf chlorophyll content, nitrogen balance index and the water use efficiency. On the other hand, seedlings inoculated with *R. intraradices* recorded the highest values of leaf area. Our results confirmed the importance to develop *ad hoc* formulates based on AM fungal consortia that take into account the environmental conditions, the plant genotype and AM fungi interactions in order to achieve the best outcomes in terms of plant resilience/tolerance to adverse conditions. These aspects are particularly important in the current scenario of climate change characterized by a reduction of water availability for agricultural purposes. Hence, a multidisciplinary approach to investigate the interactions between the most cultivated genotypes and AM fungi is urgently needed and is fundamental to obtaining useful agronomic strategies that can promptly improve the sustainability of processing tomato production.

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Chapter 5

Interspecific rootstock can enhance yield of processing tomato (*Solanum lycopersicum* L.) in organic farming

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5.1 Introduction

Tomato (*Solanum lycopersicum* L.) is the second most commonly grown horticultural crop in the world. In 2017, the worldwide tomato production was ~ 182 million tonnes (FAOSTAT 2019) and 20.7% of this was used by the canning industries (WPTC 2019). California is the first world producer of processing tomatoes (~ 11 million tonnes) followed by Italy (~ 5 million tonnes) and China (~ 4 million tonnes) (WPTC 2019). However, only ~ 0.38 and ~ 0.16 million tonnes of processing tomatoes were produced organically in California and Italy, respectively, in 2017 (WPTC 2019). The productivity in organic system has been reported to be lower than that in conventional system (Ronga et al. 2017; Ronga, Gallingani, et al. 2019) and this gap in productivity has been thought to be linked with low nitrogen availability in the organic system, due to the type of fertilisers (organic) used, and also to the plant protection products approved for the use in the organic system (Clark et al. 1999; Ronga et al. 2015; Ronga, Gallingani, et al. 2019).

Although, some studies have investigated the factors influencing the productivity gap between the organic and the conventional farming systems, research to develop innovative solutions to reduce this gap are still limited. Recently, Ronga, Caradonia, et al. (2019) have assessed the use of biofertilisers (biochar and pelleted digestate) able to improve processing tomato productivity in the organic cropping systems. Furthermore, farmers only have access to a few genotypes of processing tomato that are suitable for low input systems such as organic cropping systems, and instead they often have to use genotypes developed for high input cropping systems (such as the conventional system). For this reason, studies to investigate the traits required to achieve high processing tomato yields under low input conditions are required (Lammerts van Bueren et al. 2011; Murphy et al. 2007).

Grafting is a widespread agronomic practice in horticulture, and it might provide an opportunity to improve the yield and quality of processing tomato in organic cropping systems. The grafting of commercial genotypes onto selected rootstocks, like an alternative to classic breeding, is an ancient practice for fruit trees (Goldschmidt 2014). However, only in the last century, the grafting has become more common also for vegetables belonging to Cucurbitaceae and Solanaceae (Goldschmidt 2014, Venema et al. 2008). The use of rootstocks could improve many traits of scion like growth, development and fruit yield and quality (Djidonou et al. 2013; Flores et al. 2010; Venema et al. 2008). Djidonou et al. (2013) found a higher number of fruits

and increased fruit weight in fresh market tomato grafted onto two interspecific rootstocks (*Solanum lycopersicum* L. x *S. habrochaites*) grown in a conventional farming system, which was thought to be due to the vigorous characteristics of the rootstocks (Beaufort' and 'Multifort'). Furthermore, plants of commercial tomato hybrid cv. 'Big Red' grafted onto 'Heman' (*L. hirsutum*) produced more fruits than non-grafted plants both in greenhouse and in open-field studies (Khah et al. 2006).

Rootstocks that are resistant to biotic stresses have also been reported to enhance the tolerance of commercial tomato genotypes to pathogens like *Ralstonia solanacearum* (Grimault and Prior 1994), *Fusarium* spp. (Hamdi et al. 2009; Polizzi et al. 2015), *Meloidogyne* spp. (Owusu et al. 2016; Yin et al. 2015), *Verticillium* spp. (Paplomatas et al. 2002) and tomato yellow leaf curl virus (Rivero et al. 2003). In addition, the use of rootstock could be exploited to induce resistance to abiotic stress such as drought, salinity and temperature (Santa-Cruz et al. 2002; Venema et al. 2008; Wang et al. 2017). Zhang et al. (2019) found that '606', a drought-tolerant tomato rootstock, increased the activity of antioxidant enzymes, reducing the phytotoxicity and oxidative damage caused by drought stress in a greenhouse experiment. In addition, rootstocks can influence the production of phytohormones such as ABA and cytokinins (Dong et al. 2008), can increase the water use efficiency (Cantero-Navarro et al. 2016) and the nutrient uptake (Albacete et al. 2015; Rivero et al. 2004). Leonardi and Giuffrida (2006), in a greenhouse experiments with *S. lycopersicum* (hybrid 'Pachino') and *Solanum melongena* L. (aubergine, hybrids 'Mission Bell'), found that the nutrient uptake was depended on the grafting combinations and that the combinations affected the content of phosphorous, calcium and sulphur in the plant tissues. In addition, the use of *Solanum torvum* Swartz as rootstock reduced the accumulation of Cd in shoots of cvs. 'Ziliren' (*S. melongena*) and 'Aoteyou' (*S. lycopersicum*) (Yuan et al. 2019). The results from a pot study conducted by Venema et al. (2008) showed that when tomato (cv. 'Moneymaker') was grafted on *S. habrochaites* LA 1777 Humb & Bonpl the growth rate of the shoots increased both under optimal (25°C) and suboptimal (15°C) temperature conditions in comparison with the non-grafted plants. Under sub-optimal temperature conditions, Bloom et al. (2004) found that the stomatal conductance of *L. hirsutum* declined during chilling stress. In addition, Cao et al. (2015) stated that seedlings of *S. habrochaites* had a lower chilling injury index after 8 days of chilling stress (4°C) in comparison with 47 other tomato lines. The authors noted that, to date,

only very few reported studies that have investigated the influence of rootstock on the marketable yield of processing tomato in organic farming have been reported so far.

Given that *S. habrochaites* can improve the tolerance of tomato plants to biotic and abiotic stresses and can increase the number of fruits, it is possible to hypothesise that the use of the interspecific rootstock 'RS01658654' (*S. lycopersicum* L. x *S. habrochaites*) could increase the processing tomato marketable yield when grown in the organic farming system. In order to assess the effect of the interspecific rootstock 'RS01658654' on yield and aiming to reduce the productivity gap between organic and conventional farming system, a non-commercial processing tomato genotype 'TC266' was grafted onto 'RS01658654'. The performance of the grafted plants were compared to non-grafted and self-grafted plants. Morphological, physiological and agronomic performances were assessed during two consecutive growing seasons, to give preliminary information to nurseries, farmers and the canning industry.

5.2 Material and methods

5.2.1 Plant material and grafting

The non-commercial processing tomato genotype 'TC226' (Tomato colors Soc. Coop, Sant'Agata Bolognese, Italy) was selected as scion and the 'RS01658654' (RT1) (De Ruiter™, Monsanto Agricoltura Italia S.p.A., Vegetable seeds division, Parma, Italy) was selected as rootstock, based on the results of preliminary tests conducted in the same area (M. Bonfiglioli from the Tomato colors Soc. Coop, Sant'Agata Bolognese, Italy, personal communication). The genotype 'TC226' has a determinate growth habit, a medium vigour and intermediate crop-cycle lenght, and blocky shape fruits; conversely, the genotype 'RT1' is an interspecific hybrid (*S. lycopersicum* L. x *S. habrochaites*) adaptable for every type of soil and with a medium vigour.

On 14 and on 18 April, in 2017 and 2018, respectively, the seeds were sown directly in alveolar fixed seed trays (510 x 310 x 42 mm) filled with neutral commercial peat composed of 23% organic carbon, 0.5% organic nitrogen and dry apparent density 214 kg m^{-3} (Dueemme S.r.l., Reggio Emilia) and germinated in the greenhouse at Coop Habitat (San Vito, Ferrara, Italy) under controlled conditions (Temperature: 25/19 °C; Relative humidity: ~ 60%) and a natural photoperiod. The rootstock was sown four days before the scion in order to avoid an

uneven development of seedlings. The grafting was performed by Coop Habitat when the seedlings had four trues leaves using the Japanese top grafting method, also known as tube-grafting or splice grafting (Lee et al., 2010). The rootstock plants were cut below the cotyledons to avoid the regrowth of rootstock. Grafting elastic tube-shaped clips with a stick were used to help the cohesion between scion and rootstock. The grafted seedlings were placed in a shaded (50%) healing chamber for 10 days until full recovery. The seedlings were produced following the European organic production Regulations (Council Regulation, 2007).

5.2.2 Growth condition and experimental design

The field trial was conducted for two consecutive growing seasons (2017 and 2018) at Coop. Agricola La Collina, an organic farm located in Reggio Emilia, Emilia Romagna Region, Northern Italy. For both years of cultivation wheat was the preceding crop in the experimental area. Principal weather conditions and soil characteristics are reported in Table 1 and Table 2, respectively.

Table 1. Weather conditions recorded during the two growing seasons (May – September, in 2017 and 2018, respectively)

Year	Month	Total Rainfall (mm)	Average Min Temperature (°C)	Average Max Temperature (°C)	Average Relative Humidity (%)
2017	May	84.0	13.5	25.0	56.0
2017	June	27.8	20.1	32.0	46.7
2017	July	4.4	20.6	32.7	40.7
2017	August	2.6	21.6	34	40.1
2017	September	72.6	14.6	24.5	61.8
2018	May	105.8	15.2	24.0	63.1
2018	June	110.6	18.3	28.7	52.7
2018	July	42.4	21.0	31.8	53.1
2018	August	12.8	21.6	32.2	48.7
2018	September	7.6	17.5	27.4	56.7

Table 2. Physical and chemical soil properties

Soil characteristics	2017	2018
Sand (%)	5.8	11.2
Silt (%)	54.1	67.5
Clay (%)	40.1	21.3
pH (-)	7.2	7.8
EC (dS m^{-1})	0.1	0.2
Limestone (%)	2.8	9.4
Exchangeable K (mg kg^{-1})	226.1	179.9
P total (mg kg^{-1})	34.4	55.0
N. tot. (‰)	1.5	1.3
Organic matter (%)	2.3	1.8
CEC (meq 100 g^{-1})	27.0	17.9

The grafted seedlings were transplanted on 25 May and 30 May in 2017 and 2018, respectively). The plant density was 2.5 plants m^{-2} with a spacing of 1.60 m between each row and 0.25 m between plants in the row. The experimental design was arranged in a completely randomized design with 3 repetitions each for each treatment and each replicate plot consisting of 12 plants. The non-grafted and the self-grafted genotypes TC226 were used as controls.

Seven months before transplanting, 40 t ha^{-1} of mature cow manure (N 0.5 – P 0.1 – K 0.3) were applied just before plowing. During the field studies, 351.0 and 224.4 L m^2 of irrigation water was distributed by drip irrigation, in 2017 and 2018, respectively. Weeds were

controlled by hand weeding and only plant protection products permitted for use in organic farming were used to control pests.

5.2.3 Parameters assessed during the experiments

After 20 days from transplanting (at full flowering) and at harvest (on 8 September 2017 and on 4 September 2018), agronomic and physiological parameters were recorded.

Morphological parameters

Some parameters regarding the plant growth were assessed by harvesting 6 plants *per* treatment: number of leaves and flowers, collar diameter of plants, height of plants, the ratio between plant height and collar diameter and the leaf area index (LAI). LAI was measured using fresh leaves that were run through the leaf area meter LI-3000A and linked to the number of plants in a square meter (destructive measurement).

Physiological parameters

Leaf chlorophyll, flavonoid and anthocyanin contents were estimated on the youngest fully expanded leaf using Dualex 4 Scientific (Dx4, FORCE-A, Orsay, France), an optical leaf-clip meter for assessment of the physiological status of plants without removing the leaves from plants (Cervic et al. 2012). NBI was calculated as the ratio between chlorophyll and flavonoid contents.

At harvest, the following physiological parameters were calculated: harvest index (HI), N-efficiency, crop water productivity and fruit water productivity. The HI was calculated as the ratio between fruit dry weight and the total plant dry weight. The N-efficiency was calculated as the ratio between fruit dry weight and the total amount of nitrogen supplied to the crop as reported by Craswell and Godwin (1984). The crop water productivity was calculated as the ratio between the plant dry weight (kg) and total water used by plants (mm) during the growing season as suggested by Cosentino et al. (2007) and Ronga, Villecco, et al. (2019), while fruit water productivity was calculated as the ratio between the marketable yield (kg) and the total water used by plants (mm) during the growing season (Padilla-Díaz et al. 2018).

Yield parameters

At harvest, when ~85% of the fruits were fully ripe, plants were sampled, weighted, and oven-dried at 65°C until constant weight to obtain the respective dry weight. Only fully ripe fruits were considered for the marketable yield, but for the number of fruits *per* plant all fruits (fully ripe, unripe, rotten and sunburnt) were considered.

Fruit quality parameters

Fruit quality was assessed by following parameters: average fruit weight (considering the marketable fully ripe fruits), dimension of fruit, number of fruit affected by blossom-end rot (BER), sunburnt and rotten fruits, pH and total soluble solid content (Brix°) (using 1 kg of marketable fruits *per* replicate plot). The Brix° parameter was determined using the digital refractometer HI 96814 (Hanna, Italy), while the pH was measured by pH meter pH 8+ DHS (XS INSTRUMENTS, Italy). In addition, Brix yield was calculated by multiplying the marketable yield by Brix° and dividing the result by 100.

5.2.4 Statistical analysis

One-way analysis of variance was performed by using GenStat 17th software after confirmation of the homogeneity of variance and normality. Means of grafted treatments (non-grafted, self-grafted and grafted) were compared by using Duncan's test at $p < 0.05$ of confident level. In addition, Principal Component Analysis (PCA) was performed by using PLS Toolbox software (Eigenvector Research Inc, Wenatchee, WA, USA), in order to evaluate the existing relationships with original variables.

5.3 Results

5.3.1 Seasonal weather conditions

Rainfall and relative humidity (RH) were higher in 2018 than in 2017. The wet and humid conditions in 2018 were very favorable for the spread of the oomycete *Phytophthora infestans* in the field and considering these abiotic and biotic stress recorded in the 2018, the results from the two years were analysed separately.

5.3.2 Growth parameters recorded at full flowering and harvest time

Results of the assessment of the morphological parameters are shown in Table 3. In both the years, significant effects of the rootstock RT1 were observed only for the number of flowers

and leaves. Plants grafted onto RT1 showed the highest number of flowers and leaves in comparison with the non-grafted plants.

5.3.3 Physiological parameters recorded at flowering and harvest time

Results of the assessment of the physiological parameters are shown in Tables 4 and 5. Considering the leaf physiological parameters (Table 4), significant effects of the grafting technique were observed at full flowering, in the second year. In fact, plants grafted on RT1 and self-grafted plants showed a higher value of leaf chlorophyll and NBI, and a lower value of leaf flavonoids compared with the non-grafted plants.

Considering the crop physiological parameters at harvest (Table 5), all traits were statistically affected by the rootstock. In particular, plants grafted onto 'RT1' showed increased N-efficiency, crop water productivity and fruit water productivity in comparison with non-grafted plants. Conversely, a reduction of HI was registered in self-grafted plants in comparison with the non-grafted and grafted onto 'RT1' plants.

5.3.4 Yield and quality traits

Plants grafted onto 'RT1' showed an increase of the marketable yield in comparison with the non-grafted plants, +26.6% and +20.1%, in 2017 and 2018, respectively, in comparison with the non-grafted plants (Table 6). Also, the fruit dry weight, number of fruits *per* plant and the plant dry weight were significantly influenced by rootstock 'RT1' in both years (Table 6). In fact, an increase in number of fruits, fruit dry weight and plant dry weight were recorded for the plants grafted onto 'RT1' in comparison with the non-grafted plants (Table 6).

For the fruit quality parameters (Table 7), not statistically significant effects of the grafting were displayed in both years, except for the Brix t ha^{-1} and the number of rotten fruits in the first year (Table 7), when the use of 'RT1' increased the Brix t ha^{-1} while the self-grafting increased the number of rotten fruits.

Table 3. Effects of grafting and rootstock on growth traits. TC226 non-grafted (TC), TC226 self-grafted (TC/TC), TC226 grafted on RT1 (TC/RT). Within year, means followed by the different letters are statistically significant at P<0.05; HCD-1 = ratio between height and collar diameter; n.s. = not significant.

Treatment	Year	Plant Height (cm)				Collar Diameter (cm)				HCD^{-1} (cm)		Flowers (number $plant^{-1}$)		Leaves (number $plant^{-1}$)		LAI (m^2 m^{-2})	
		Flowering		Harvest		Flowering		Harvest		Flowering		Flowering		Flowering		Flowering	
TC	**2017**	51.67	n.s.	84.33	b	1.58	n.s.	1.76	n.s.	32.94	n.s.	25.83	b	31.83	b	1.08	n.s.
TC/TC	**2017**	46.67	n.s.	76.33	b	1.68	n.s.	1.92	n.s.	27.62	n.s.	13.33	c	32.67	b	1.08	n.s.
TC/RT	**2017**	60.83	n.s.	93.83	a	1.50	n.s.	1.79	n.s.	40.56	n.s.	**33.00**	**a**	**43.83**	**a**	1.02	n.s.
F value				<0.01								<0.005		<0.05			
TC	**2018**	86.50	n.s.	86.17	n.s.	1.87	n.s.	1.93	n.s.	46.51	n.s.	11.07	b	69.17	b	2.34	n.s.
TC/TC	**2018**	80.00	n.s.	82.83	n.s.	1.67	n.s.	2.53	n.s.	48.11	n.s.	21.37	ab	68.50	b	2.26	n.s.
TC/RT	**2018**	86.00	n.s.	88.50	n.s.	1.87	n.s.	2.22	n.s.	46.05	n.s.	**30.80**	**a**	**84.50**	**a**	1.97	n.s.
F value												<0.05		<0.01			
Treatment		n.s.		<0.001		n.s.				n.s.		<0.001		<0.001		n.s.	

Table 4. Effects of grafting and rootstock on leaf physiological traits. TC226 non-grafted (TC), TC226 self-grafted (TC/TC), TC226 grafted on RT1 (TC/RT). Within year, means followed by the different letter are statistically significant at P<0.05; Chl = index of chlorophyll content in the leaves; Flv = index of flavonoid content in the leaves; Antho = index of anthocyanin content in the leaves; NBI = nitrogen balance index; n.s. = not significant.

Treatment	**Year**	**Chl**				**Flv**				**NBI**				**Antho**			
		Flowering		**Harvest**		**Flowering**		**Harvest**		**Flowering**		**Harvest**		**Flowering**		**Harvest**	
TC	**2017**	29.25	n.s.	26.37	n.s.	1.24	n.s.	1.26	n.s.	25.67	n.s.	23.03	n.s.	0.00	n.s.	0.06	n.s.
TC/TC	**2017**	30.10	n.s.	34.05	n.s.	1.42	n.s.	1.49	n.s.	22.89	n.s.	24.14	n.s.	0.00	n.s.	0.01	n.s.
TC/RT	**2017**	30.75	n.s.	34.55	n.s.	1.31	n.s.	1.35	n.s.	26.78	n.s.	26.71	n.s.	0.00	n.s.	0.00	n.s.
TC	**2018**	33.46	b	29.49	n.s.	**2.02**	**a**	**2.75**	**n.s.**	16.97	b	10.84	n.s.	**0.22**	**a**	**0.36**	**n.s.**
TC/TC	**2018**	**41.34**	**a**	**28.11**	**n.s.**	1.74	b	2.27	n.s.	**24.09**	**a**	**12.89**	**n.s.**	0.19	b	0.33	n.s.
TC/RT	**2018**	**41.40**	**a**	**35.47**	**n.s.**	1.76	b	2.19	n.s.	**24.40**	**a**	**17.04**	**n.s.**	**0.21**	**a**	**0.30**	**n.s.**
F value		<0.01				<0.05				<0.005				<0.001			
Treatment		<0.05		<0.05		n.s.		n.s.		n.s.		n.s.		<0.001		n.s.	

Table 5. Effects of grafting and rootstock on crop physiology traits at harvest time. TC226 non-grafted (TC), TC226 self-grafted (TC/TC), TC226 grafted on RT1 (TC/RT). Within year, means followed by the different letter are statistically significant at P<0.05; n.s. = not significant.

Treatment	Year	HI (%)		N-efficiency (kg kg^{-1})		Crop water productivity (kg mm^{-1})		Fruit water productivity (kg mm^{-1})	
TC	**2017**	**54.53**	**a**	20.94	b	1.92E-03	b	1.28E-02	b
TC/TC	**2017**	41.01	b	13.49	c	1.62E-03	c	9.37E-03	c
TC/RT	**2017**	**57.50**	**a**	**30.51**	**a**	**2.65E-03**	**a**	**1.63E-02**	**a**
F value		<0.05		<0.001		<0.001		<0.001	
TC	**2018**	**42.18**	**a**	11.11	b	1.32E-03	b	9.71E-03	ab
TC/TC	**2018**	32.21	b	10.38	b	**1.61E-03**	**a**	7.90E-03	b
TC/RT	**2018**	**44.53**	**a**	**14.08**	**a**	**1.58E-03**	**a**	**1.14E-02**	**a**
F value		<0.05		<0.05		<0.05		<0.05	
Treatment		<0.001		<0.001		<0.001		<0.001	

Table 6. Effect of rootstock on yield traits. TC226 non-grafted (TC), TC226 self-grafted (TC/TC), TC226 grafted on RT1 (TC/RT). Within year, means followed by the different letter are statistically significant at P<0.05; n.s. = not significant

Treatment	Year	Marketable yield (t ha^{-1})		Fruit dry weight (g plant^{-1})		Fruits (number plant^{-1})		Plant dry weight (g plant^{-1})	
TC	**2017**	51.5	b	170	b	35.55	b	310	b
TC/TC	**2017**	37.5	c	110	c	29.45	b	260	c
TC/RT	**2017**	**65.2**	**a**	**240**	**a**	**43.11**	**a**	**420**	**a**
F value			<0.001		<0.001		<0.05		<0.001
TC	**2018**	38.7	ab	90	b	28.03	b	210	b
TC/TC	**2018**	31.5	b	80	b	27.51	b	**260**	**a**
TC/RT	**2018**	**46.5**	**a**	**110**	**a**	**34.79**	**a**	**250**	**a**
F value			<0.05		<0.05		<0.05		<0.005
Treatment			<0.001		<0.001		<0.001		<0.001

Table 7. Effect of grafting and rootstock on quality. TC226 non-grafted (TC), TC226 self-grafted (TC/TC), TC226 grafted on RT1 (TC/RT). Within year, means followed by the different letter are statistically significant at P<0.05; n.s. = not significant

Treatment	Year	°Brix		Brix yield (t ha^{-1})		pH		BER fruit (number plant^{-1})		Sunburnt fruit (number plant^{-1})		Rotten fruit (number plant^{-1})		Fruit polar diameter (mm)		Fruit equatorial diameter (mm)		Average fruit weight (g)	
TC	**2017**	4.83	n.s.	2.43	b	4.28	n.s.	1.83	n.s.	4.17	n.s.	0.50	b	59.23	n.s.	47.98	n.s.	84.00	n.s.
TC/TC	**2017**	5.77	n.s.	2.11	b	4.31	n.s.	1.50	n.s.	0.83	n.s.	**2.50**	**a**	58.61	n.s.	48.40	n.s.	76.00	n.s.
TC/RT	**2017**	5.07	n.s.	**3.24**	**a**	4.44	n.s.	3.00	n.s.	3.67	n.s.	1.67	ab	56.69	n.s.	50.81	n.s.	78.00	n.s.
F value				<0.05								<0.05							
TC	**2018**	5.13	n.s.	1.95	n.s.	4.43	n.s.	1.67	n.s.	1.00	n.s.	6.00	n.s.	56.45	n.s.	45.84	n.s.	70.00	n.s.
TC/TC	**2018**	5.53	n.s.	1.74	n.s.	4.27	n.s.	5.67	n.s.	2.17	n.s.	10.67	n.s.	54.06	n.s.	44.29	n.s.	62.00	n.s.
TC/RT	**2018**	4.97	n.s.	2.22	n.s.	4.29	n.s.	0.50	n.s.	5.50	n.s.	9.50	n.s.	55.49	n.s.	45.37	n.s.	66.00	n.s.
Treatment			n.s.		<0.005		n.s.		n.s.		n.s.		n.s.		n.s.		n.s.		n.s.

5.3.5 Relationships among parameters, years and treatments

To identify the parameters associated with rootstock effects, Principal Component Analysis (PCA) was performed with all the variables that were statistically significant by ANOVA analysis. The contributions of the two principal components were 78.47% (PC1) and 21.53% (PC2) for the first years and 54.20% (PC1) and 45.80% (PC2) for the second year (Figure 1 and 2). In both biplots, the effect of rootstock 'RT1' (T3) was clearly associated with the positive side of PC1. Conversely, PC2 separated the grafted plants from non-grafted plants, giving an idea of the effect of grafting. In both years, T3 was associated with the majority of the investigated traits such as number of leaves, number of fruits, crop water productivity, fruit dry weight, Brix t ha^{-1}, marketable yield, height and N-efficiency. In addition, in both the investigated years, nutrient efficiency, Brix t ha^{-1}, fruit water productivity, plant height at harvest time and the number of fruits were strongly associated with the two main recorded parameters, marketable yield and fruit dry weight.

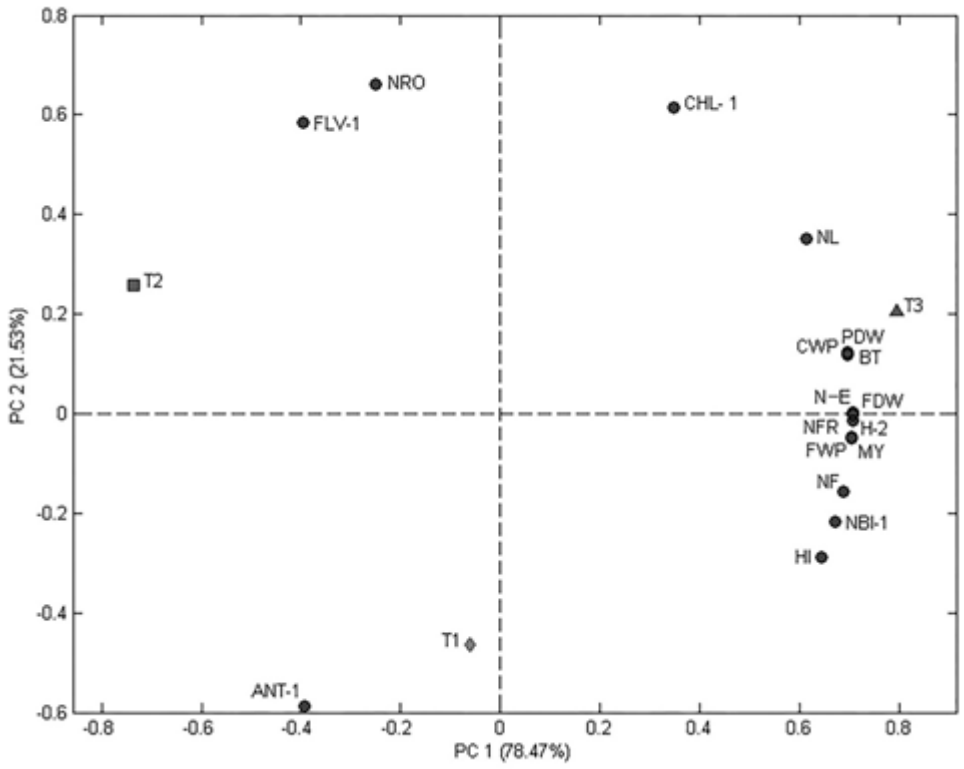

Figure 1. Biplot of PCA for the first year of field trail. ANT 1 = leaf anthocyanin content registered at full flowering; BT = Brix t ha-1; CHL 1 = leaf chlorophyll content registered at full flowering; CWP = Crop water productivity; FLV 1 = leaf flavonoid content registered at

full flowering; FDW = fruit dry weight; FWP = fruit water productivity; H 2 = height registered at harvest time; HI = Harvest Index; MY= marketable yield; NBI 1= NBI registered at full flowering; NL = number of leaves; NFR = number of fruits; NF = number of flowers; NRO = Number of Rotten fruits plant-1 ; N-E = nitrogen -efficiency; PDW = plant dry weight; T1 = TC226 non-grafted; T2 = TC226 self-grafted; T3 = TC226 grafted on RT1

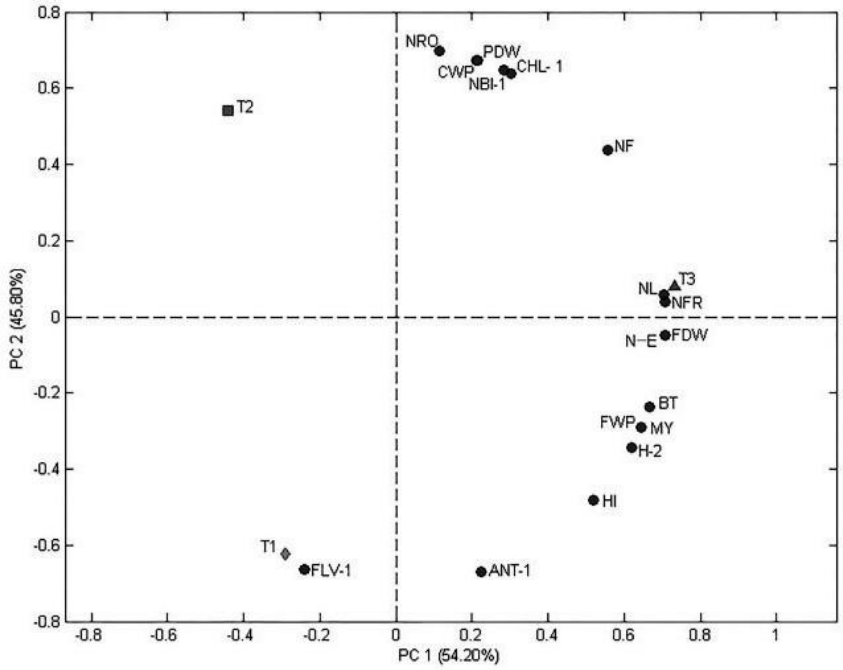

Figure 2. Biplot of PCA for the second year of field trail. ANT 1 = leaf anthocyanin content registered at full flowering; BT = Brix t ha^{-1}; CHL 1 = leaf chlorophyll content registered at full flowering; CWP = Crop water productivity; FLV 1 = leaf flavonoid content registered at full flowering; FDW = fruit dry weight; FWP = fruit water productivity; H 2 = height registered at harvest time; HI = Harvest Index; MY= marketable yield; NBI 1= NBI registered at full flowering; NL = number of leaves; NFR = number of fruits; NF = number of flowers; NRO = Number of Rotten fruits $plant^{-1}$; N-E = nitrogen -efficiency; PDW = plant dry weight; T1 = TC226 non-grafted; T2 = TC226 self-grafted; T3 = TC226 grafted on RT1

5.4 Discussion

Crop yield is the most important parameter that farmers consider in the choice of the cropping system (Clark et al. 1999). The use of grafting techniques with rootstocks in processing tomato production might be an option for improving marketable yield in organic cropping system.

Considering the plants at full flowering (Tables 3 and 4), in 2017, they were smaller (in terms of height, collar diameter, number of leaves) and the leaf chlorophyll content was lower in comparison with the plants grown in 2018. These seasonal differences could be due to less abundant and frequent rainfalls in the first year (Table 2). In fact, the lower rainfall (Table 2) may have induced the plants to nutrient stress. This data is in agreement with a study by Suchoff et al. (2019), in which plants of fresh tomato grafted on two different interspecific rootstocks ('Maxifort' and 'RST 106') were shorter when there was a low rainfall.

The results for this study revealed a positive influence of rootstock RT1 on the number of flowers *per* plant (Table 3). Plant age and gibberellins content - associated pathways may regulate the tomato floral induction (Silva et al., 2019). Although, currently, the molecular mechanisms of the root-to-shoot communication are not well known (Gaion et al. 2019), Satoh et al. (1996) noted a transport of signal factors from rootstock to scion in cucumber plants.

Considering the data recorded at harvest (Table 6), in the second year the plants reported a higher leaf content of flavonoids and anthocyanins, a lower dimension and number of fruits and a higher number of rotten fruits compared to the first year (Table 9). These results could be correlated to spread of late blight. In fact, the oomycetes *P. infestans* may devastate tomato plants in 7-10 days from infection (Fry, 2008) leading a decrease of yield and quality of fruits (Fontem et al. 1999).

In the present study, fruit water productivity was calculated as the ratio between marketable yield and total water used by plants during the growing season, while the N-efficiency was calculated as the ratio between fruit dry weight and the total amount of nitrogen provided. Therefore, these indexes are dependent on marketable yield and fruit dry weight, respectively. Plants grafted on RT1 showed higher N-efficiency and fruit water productivity compared with the controls in both the years. These results were in agreement with a study by Djidonou et al. (2013) in which the use of rootstock influenced both NUE (Nitrogen Use Efficiency) and WUE (water use efficiency). However, Djidonou et al. (2013) found that the positive effects of rootstocks decreased with increased of irrigation and fertilizer input.

The plant grafted onto interspecific rootstock RT1 showed the highest marketable yields (Table 6). The increase of marketable yield was correlated with the use of RT1 rather than the grafting technique as the self-grafted plants showed the lowest marketable yields. This finding

agreed with the results of Khah et al. (2006) who found that in a greenhouse experiment the tomato cv. 'Big Red' grafted on tomato rootstock 'Heman' (*L. hirsutum*) showed a higher yield in comparison with plants non-grafted and self-grafted. Also the determinate tomato cv. 'Florida 47' showed a higher total and marketable fruit yields when it was grafted onto 'Beaufort' or onto 'Multifort' (*S. lycopersicum* x *S. habrochaites*) and grown in the conventional cropping system (Djidonou et al., 2016). The results from this study agreed partially with those reported by Suchoff et al. (2019), who found that the vigorous rootstock 'Maxifort' (*S. lycopersicum* L. x *S. habrochaites*) grafted on fresh market tomato 'Tribune' increase the marketable yield. Suchoff et al. (2019) correlated the increase of marketable yield with an increase of the number and weight of fruits, while, in this study, no difference were found in fruit dimensions. This difference could be related to the different vigor of the rootstock used, the different attitude of the scion (fresh tomato *vs* processing tomato) or the different cropping system adopted.

The increase in marketable yield was correlated with the increase in the number of fruits and with the increase in the number of leaves (Figures 1 and 2, Tables 1 and 2 SM). In addition, the increase of marketable yield was correlated with fruit dry weight, which is another important trait involved in yield component (Figures 1 and 2, Tables 1 and 2 SM). These results suggested that the RT1 rootstock positively influenced these parameters.

Considering the fruit dry weight, the fruits from the plants grafted on RT1 rootstock had the highest dry weights indicating that the higher marketable yield was due to higher dry matter accumulation rather than to higher water accumulation. Conversely, some studies in the conventional greenhouse cropping systems reported an increase in the fruit moisture content of tomato plants grafted onto interspecific rootstocks (*S. lycopersicum* x *S. habrochaites*) compared with the fruits of self-grafted and non-grafted plants (Djidonou et al. 2016; Turhan et al. 2011).

The pH and Brix° are two important parameters to assess the quality of fruit of processing tomato (Huang et al. 2018). The results reported in the present study showed that the quality of fruits was not affected by the rootstock. These results were partially in agreement with a study performed by Turhan et al. (2011) in which pH was not affected by rootstock, while Brix° decreased.

In the first year, the rootstock RT1 influenced positively the Brix t ha^{-1}. This parameter (a measure of soluble sugars in relation to fruit yield) is very important in processing tomato: as it is directly related to the weight of finished products, it is used by the canning industry to set the final price payed to farmers. In addition, the grafting increased the number of rotten fruits. It was hypothesized that a different plant allocation of resources caused the lower dry matter in fruits (lower HI) making these more prone to the infection by pathogens.

Finally, the contribution of grafting on processing tomato grown in the organic cropping system could be important not only to reduce the losses due to stress but also to increase the yield in normal conditions. Currently the price of grafted seedling, ranged from 0.6 to 1.0 € per plant (Lee et al. 2010), and the price of organic processing tomato fruits ranged from 130 to 150 € t-1. Hence, considering these economic aspects, grafting is expected to become a viable solution for processing tomato just in case marketable yield can be increased (see, e.g. Ronga, Caradonia et al. 2019) and/or the grafting costs are further reduced. In this last sector, many benefits have been obtained from the adoption of robot technologies (Lee et al., 2010), and in the near future, grafting is expected to become a popular and sustainable way to improve yield also in processing tomato.

5.5 Conclusion

This study showed that the use of rootstock RT1 enhanced the marketable yield of processing tomato in an investigated organic cropping system without having to make changes to the management regimes for nutrients or plant protections. The effects of rootstock (RT1) on marketable yield were positively correlated with the increase of the number of fruits and leaves, but the quality of processing tomatoes was no affected. In addition, although the weather condition were different in the two years, in terms of marketable yield, the hybrid rootstock RT1 could be recommended for high yielding year, such as 2017. However, further investigation of the rootstock and environment interactions is necessary to verify the mechanisms of interaction and the cost-effectiveness. In addition, from an agronomic and physiological point of view, fruit and plant dry weights displayed the highest values in both the investigated years.

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Chapter 6

Biostimulants and cherry rootstock increased tomato fruit yield and quality in sustainable farming systems

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6.1 Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most produced horticultural crops in the world (Leogrande et al., 2012). In 2017, the world annual tomato yield exceeded 182 million tonnes over a cultivated area of ~ 5 million hectares (FAO, 2019). Tomato is found in many diets for its content in lycopene and other valuable anti-oxidant compounds (Raiola et al., 2014), and it is also considered a model plant for Solanaceae (Kimura and Sinha, 2008).

Nowadays, one of the main agriculture challenges is to increase crop yield in an eco-friendly manner, and a reduction of the use of synthetic products as fertilizers and plant protection products could increase the sustainability in crop production (Mura et al., 2013; Pretty, 2008; Ronga et al., 2019a). However, tomato yield and quality are strictly affected by fertilizer applications (Bettiol et al., 2004; Dumas et al., 2003; Ronga et al., 2015). Farneselli et al. (2013) reported that the sustainability of farming system for processing tomato production depends greatly on the management of soil nitrogen (N) availability. Furthermore, in OFS, where synthetic products are not allowed, the processing tomato yield is lower in comparison with the yield reached in CFS (Ronga et al., 2017).

Recently, the European Union has adopted a new regulation for fertilizer products, which replaces the previous one dating back to the year 2003. This regulation introduces and rules the use of plant biostimulants, substances or microorganisms improving the plants' nutrient use efficiency, tolerance to abiotic stresses, quality traits or increasing the availability of confined nutrients in the soil or rhizosphere (European Parliament and Council of the European Union, 2019). In particular, the plant biostimulants based on microorganisms include different fungi as mycorrhizal fungi (e.g. *Funneliformis mosseae,* etc.) and bacteria (such as *Azotobacter* spp., *Rhizobium* spp., and *Azospirillum* spp.) (Drobek et al., 2019). In a previous study, was displayed that *F. mosseae* was able to reduce the cell membrane injuries in processing tomato seedlings under chilling stress (Caradonia et. al., 2019). The same authors reported that *P. graminis* increased the leaf chlorophyll content in three different processing tomato genotypes (Caradonia et al., 2019).

Another study reported that *Azospirillum brasilense* Ab-V5 increased the growth and nitrogen use efficiency of maize seedlings under nitrogen deficit (Zeffa et al., 2019).

The principal characteristic of plant biostimulants, especially those based on single microorganism or microbial consortia, is the ability to reduce fertilizer applications improving yield and quality of agricultural crops. In addition, the combination of more sustainable strategies could increase the general positive effects on crop plants. In this point of view, microorganisms may be used to improve the sustainability of the tomato yield *per* hectare when cultivated in OFS (Ronga et al., 2019b).

Among the agronomic techniques, grafting is an alternative to classic breeding process to exploit, in a short time, favourable traits. In addition, rootstock can affect the growth, yield and fruit quality (Djidonou et al., 2013; Flores et al., 2010).

Cherry tomato is a type of small round tomato that is studied and appreciated mainly for its fruit quality and taste (Sanchez et al., 2019). However, it can be considered as an intermediate genotype between wild currant-type and domesticated (Wang et al., 2016), rustic and with a great productivity (da Silva et al., 2019). In addition, tomato genotypes producing small fruits (such as cherry types) are more tolerant to abiotic stress such as salinity than the other ones (Anastasio et al., 1987; Hagassou et al., 2019). Hence, the objective of this study was to investigate the agronomic effects of a cherry genotype, 'Tomito', when used as rootstock for the commercial processing genotype 'H3402' in combination with different microbial biostimulants (applied alone or in consortium) in order to increase yield and quality of processing tomato in sustainable farming systems.

6.2 Materials and methods

6.2.1 Plant material and treatments

In the present work two experiments were carried out. In the first experiment the agronomic performances of processing tomato seedlings grafted and inoculated with different plant growth promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi (AMF) were evaluated under controlled condition (greenhouse), while in the second experiment the same treatments, assessed in greenhouse, were evaluated in the open field in OFS. All the investigated treatments are reported in Table 1, genotype 'H3402' non-grafted and self-grafted were used as control.

Table 1. Information on genotypes, microorganisms and dose used in the experiments

Treatments	Genotype	Microorganisms	Dose	Information
T1	H3402xTomito	*Funneliformis mosseae*	2 g seedling^{-1} (1 g of inoculum contained 10 propagules)	Provided by MycAgro, LabTechnopôle Agro Environnement, Bretenière, France
T3	H3402xTomito	MICOSAT F UNO 40% Funghi simbionti (*Glomus* spp. GB 67, *Funneliformis mosseae* GP 11, *G. viscosum* GC 41) 18,60% C.F.U. g^{-1}: 12,4 x 10^7 Batteri della rizosfera (*Agrobacterium radiobacter* AR 39, *Bacillus subtilis* BA 41 e Streptomyces spp. SB 14) Funghi saprofiti (*Pochonia chlamydosporia* PC 50 e *Trichoderma harzianum* TH 01) Lieviti (*Pichia pastoris* PP 59)	10 g L^{-1}	Produced by CCS (Aosta, Italy)
T5	H3402xTomito	*Paraburkholderia graminis* C4D1M	1 mL seedling^{-1} (10^7 CFU mL^{-1})	CREA GB 's Collection
T6	H3402xTomito	*Azospirillum brasiliensis* sp. 245	1 mL seedling^{-1} (10^7 CFU mL^{-1})	CREA GB 's Collection
T8	H3402xTomito	*Funneliformis mosseae* + all bacteria	2 g of *F.mosseae* + 1 mL of each bacterium inoculum *per* seedling	
T10	H3402xTomito	all bacteria	1 mL of each bacterium inoculum *per* seedling	
T11	H3402	non-inoculated		
T12	H3402xH3402	non-inoculated		
T13	H3402xTomito	non-inoculated		

Two commercial processing tomato genotypes, 'H3402' (HEINZ, Pittsburgh, Pennsylvania, USA) and 'Tomito' (ISI Sementi SpA, Fidenza, Italy), were used for these experiments. The genotype 'H3402', used as scion, has a determinate growth habit and it is rustic with a good vigor, bushy, with a good yield and medium oval fruit. In addition, this genotype is suitable for canning and it is one of the most cultivated varieties in Southern Europe. On the other hand, the genotype 'Tomito', used as rootstock, is a cherry type tomato used both for fresh market and canning; it has a determinate growth habit and is rustic and vigorous.

The seeds were sown directly in plateaus (510 mm x 310 mm x 42 mm) filled with neutral commercial peat (23% organic carbon, 0.5% nitrogen and dry apparent density 214 kg m^{-3}, Dueemme S.r.l., Reggio Emilia) and germinated in greenhouse at Coop Habitat (San Vito, Ferrara, Italy) under controlled conditions (temperature: 25/19 °C; humidity: ~ 60%). The rootstock seeds were sown 4 days before the scion seeds in order to avoid an uneven development of seedlings. The grafting was performed by Coop Habitat when the seedlings had 4 trues leaves using the Japanese top grafting method also known as tube-grafting or splice grafting (Lee et al., 2010). The rootstock seedlings were cut below the cotyledons to avoid the regrowth of rootstocks. Grafting elastic tube-shaped clips with a stick were used to help the cohesion between scion and rootstock. The grafted seedlings were placed in a shaded (50%) healing chamber for 10 days until full recovery.

After 2 weeks from grafting, the seedlings were transplanting in pots (6.5 cm x 8 cm x 5.5 cm) filled with neutral peat (23% organic carbon, 0.5% nitrogen and dry apparent density 214 kg m^{-3}, Dueemme S.r.l., Reggio Emilia). Before to transplant seedlings in the pots, arbuscular mycorrhizal fungi (AMF) (2 g pot^{-1}) and the commercial product (Micosat F UNO, 10 g L^{-1}) were added in pots and mixed with peat (Table 1).

Immediately after the transplanting in pots, the bacterial inoculum (10^7 colony forming unit (CFU) mL^{-1}) (Table 1) was added close to the plant's root collar as reported by Caradonia et al. (2019). Single colonies of every bacterium were cultivated in 250 mL Erlenmeyer flasks containing 60 mL of Tryptone Soya Yeast extract broth. The flasks were incubated at 28°C at 150 rpm for 24 hours. Then the suspensions were centrifuged for 4 minutes at 8,000 x g, the pelleted were washed and suspended in sterilized distilled water. The bacterial concentrations were estimated by Jasco V-550 UV-VIS spectrophotometer (600 nm) and adjusted by sterilized distilled water.

6.2.2 Greenhouse experiment

After the seedlings transplant, ten seedlings *per* treatment were grown in greenhouse at University of Modena and Reggio Emilia with a photoperiod of 16 h light and 8 h dark and the day/night temperatures of 25/19°C. The seedlings were watered every two days with 50 mL of water *per* pot. Compo BIO fluid stillage (organic nitrogen 3%, potassium oxide 6% e organic carbon 10%, COMPO ITALIA S.R.L, Cesano Maderno (MB), Italy) was added in the irrigation water (10 mL L^{-1}) on seventh and fifteenth days.

Several parameters (plant height, steam diameter, height-to-steam diameter ratio, number of leaves, number of flowers, dry weight of leaves, stems and roots, total dry weight and leaf chlorophyll, flavonoid and anthocyanin contents) were recorded on six seedlings *per* treatment on 35^{th} day from microbial inoculations (corresponding to flowering stage). The content of chlorophyll (Chl), flavonoids (Flav) and anthocyanins (Antho) in leaves was estimated on the youngest fully expanded leaf using Dualex 4 Scientific (Dx4) (FORCE-A, Orsay, France). In addition, the nitrogen balance index (NBI) was calculated as the ratio between Chl and Flav as suggested by Cerovic et al. (2005). In order to determine the dry weight of different organs, leaves, stems and roots were oven-drying at 65°C until constant weight.

6.2.3 Field experiment

The effects of rootstock and biostimulant treatments on physiological and morphological parameters of the processing tomato seedlings, were also assessed in OFS. The field experiment was conducted at Coop. Agricola La Collina, an organic farm located in Reggio Emilia, Northern Italy, during the growing season 2018. The weather conditions registered during the experiment are reported in the Table 2.

Table 2. Weather conditions recorded during the growing season

Month	Total Rainfall (mm)	Average Min Temp. (°C)	Average Max Temp. (°C)	Average Relative Humidity (%)
May	105.8	15.2	24.0	63.1
June	110.6	18.3	28.7	52.7
July	42.4	21.0	31.8	53.1
August	12.8	21.6	32.2	48.7
September	7.6	17.5	27.4	56.7

The seedlings were grown and inoculated in the same way of the first experiment, and on 30th of May 2018 they were transplanted in open field (two weeks after the plant biostimulants inoculations). Plant density was 2.5 plants m^{-2} with a spacing of 1.60 m between each row and 0.25 m between plants in the row. The experimental design was arranged in a completely randomized design with 3 repetitions each consisting of 12 plants.

The soil had a silty loam texture (21.3% clay, 67.5% silt, 11.2% sand), a pH 7.8 (in H_2O), 1.3‰ total N (Kjeldahl method), 55 mg kg^{-1} available P (Olsen method), 179.9 mg kg^{-1} exchangeable K (Ammonium acetate), and 1.8 % organic matter (Walkey-Black method). The field was previously fertilized with mature cow manure (40 t ha^{-1}, N 0.5 – P 0.1 – K 0.3). During the growing season, irrigation water (224.4 L m^{-2}) was distributed by drip irrigation. Weeds were controlled by hand weeding while pests were controlled by using plant protection products allowed in OFS.

At fruit development (on 16th July 2018) and at harvest time (on 4th September 2018), the same parameters assessed in the greenhouse experiment were recorded on four and six plants *per* treatment, respectively, in order to evaluate the effects of the investigated treatments. In addition, the number and the weight of fruits were recorded in both the timeline. At harvest time, when the 85% of fruits were fully ripe, the plants were sampling and only the ripe fruits were considered for the marketable yield. Furthermore, leaf area index (LAI) was measured

using fresh leaves that were run through the leaf area meter LI-3000A and linked to number of plants in a square meter.

For fruit quality, the following parameters were evaluated: average fruit weight, number of fruits, number of fruits affected by blossom-end rot (BER), pH and Brix degree (°Brix). The total soluble solid content (°Brix) was determined using the digital refractometer HI 96814 (Hanna, Italy), while the pH was measured by pH meter pH 8+ DHS (XS INSTRUMENTS, Italy). In addition, Brix t ha^{-1} was calculated by multiplying the hectare marketable yield by the solid soluble content (°Brix) and dividing the result by 100.

6.2.4 Statistical analysis

All the investigated parameters were analysed by analysis of variance (ONE-way - ANOVA) using GenStat 17th (VSN International, Hemel Hempstead, UK). Means were compared using Bonferroni's test at the 5% level.

6.3 Results

6.3.1 Greenhouse experiment

The data recorded in the first experiment are shown in Tables 3-5. The use of the rootstock 'Tomito' increased the leaf chlorophyll content (Chl, +5.3% and +7.9%, respectively, in comparison with the non-grafted plants ('H3402') and self-grafted plants ('H3402' x 'H3402')) (Table 3). Nevertheless, the highest Chl values were achieved by grafted plants ('H3402 x 'Tomito') inoculated with the microbial consortium (*F. mosseae* + all the bacteria) (Table 3). The grafting technique influenced leaf flavonoid (Flv) content, increasing the Flv values of self-grafted *vs* non-grafted plants (+18%). However, the highest Flv values were achieved by grafted plants inoculated with the commercial product Micosat F UNO (+31.4%, in comparison with 'H3402') or with the microbial consortium (*F. mosseae* + all the bacteria; +24.7%, in comparison with 'H3402') (Table 3). A negative effect of grafting technique was recorded on plant height (-11.2%, in comparison with non-inoculated and non-grafted plants) (Table 4). Nevertheless, the use of 'Tomito' as rootstock increased the plant height. In addition, the highest values of plant height were achieved by grafted plants inoculated with *P. graminis*

C4D1M (+17.14%, in comparison with non-inoculated and non-grafted plants). On the other hand, grafting technique increased the number of leaves and flowers in comparison with the non-grafted plants (Table 4). Among microbial treatments, *F. mosseae*, Micosat F UNO, *P. graminis* and the bacterial consortium achieved the highest number of leaves while *A. brasiliensis* sp. 245 induced early flowering. Although, an increase of dry weights was recorded in all the grafted plants (Table 5), the plants inoculated with bacterial consortium displayed the highest values for leaf dry weight, steam dry weight and plant dry weight (+40%, +35.3% and +40%, respectively, in comparison with non-grafted and non-inoculated plants), while Micosat F UNO recorded the highest values for root dry weight (+46%, in comparison with non-grafted and non-inoculated plants).

Table 3. Treatment effects on physiological parameters in greenhouse experiment. The data are reported as mean ± standard deviation, means followed by the different letters are statistically significant at P <0.05; ns = not significant, Chl = index of chlorophyll content in the leaves; Flv = index of flavonoid content in the leaves; Antho = index of anthocyanin content in the leaves; NBI = nitrogen balance index. FM = 'H3402' grafted onto 'Tomito' and inoculated with *Funneliformis mosseae*, PG = 'H3402' grafted onto 'Tomito' and inoculated with *Paraburkholderia graminis* C4D1M, AB = 'H3402' grafted onto 'Tomito' and inoculated with *Azospirillum brasiliensis* sp 245, CM FM = 'H3402' grafted onto 'Tomito' and inoculated with *F. mosseae*, *P. graminis* C4D1M and *A. brasiliensis* sp 245, CM = 'H3402' grafted onto 'Tomito' and inoculated with *P. graminis* C4D1M and *A. brasiliensis* sp 245, 'H3402' = 'H3402' non-grafted and non-inoculated, H3402 x H3402 = 'H3402' self-grafted and non-inoculated, H3402 x Tomito = 'H3402' grafted onto 'Tomito' and non-inoculated

Treatments	Chl		Flv		Antho		NBI	
FM	27.11 ± 1.9	ab	1.09 ± 0.1	ab	0.24 ± 0.02	ns	25.31 ± 3.7	ns
MICOSAT F UNO	25.59 ± 1.3	ab	**1.17 ± 0.1**	**a**	0.25 ± 0.02	ns	22.27 ± 3.0	ns
PG	27.11 ± 1.9	ab	1.07 ± 0.1	ab	0.26 ± 0.01	ns	25.33 ± 1.9	ns
AB	25.66 ± 3.2	ab	1.04 ± 0.1	ab	0.28 ± 0.01	ns	24.95 ± 4.0	ns
CM FM	**29.43 ± 2.8**	**a**	**1.11 ± 0.1**	**a**	0.28 ± 0.03	ns	26.69 ± 4.1	ns
CM	25.88 ± 1.4	ab	1.07 ± 0.1	ab	0.27 ± 0.03	ns	24.52 ± 3.5	ns
H3402	23.93 ± 1.9	b	0.89 ± 0.1	b	0.24 ± 0.04	ns	26.88 ± 1.8	ns
H3402 x H3402	23.35 ± 3.0	b	1.08 ± 0.1	ab	0.29 ± 0.04	ns	21.72 ± 2.7	ns
H3402 x Tomito	25.20 ± 2.1	ab	1.05 ± 0.1	ab	0.26 ± 0.04	ns	24.09 ± 2.8	ns
F values		**0.002**		**0.015**		**0.111**		**0.093**

Table 4. Treatment effects on morphological no- destructive parameters in greenhouse experiment. The data are reported as mean ± standard deviation, means followed by the different letters are statistically significant at P<0.05; ns = not significant, HD^{-1} = ratio between plant height and collar diameter. FM = 'H3402' grafted onto 'Tomito' and inoculated with *Funneliformis mosseae*, PG = 'H3402' grafted onto 'Tomito' and inoculated with *Paraburkholderia graminis* C4D1M, AB = 'H3402' grafted onto 'Tomito' and inoculated with *Azospirillum brasiliensis* sp 245, CM FM = 'H3402' grafted onto 'Tomito' and inoculated with *F. mosseae, P. graminis* C4D1M and *A. brasiliensis* sp 245, CM = 'H3402' grafted onto 'Tomito' and inoculated with *P. graminis* C4D1M and *A. brasiliensis* sp 245, 'H3402' = 'H3402' non-grafted and non-inoculated, H3402 x H3402 = 'H3402' self-grafted and non-inoculated, H3402 x Tomito = 'H3402' grafted onto 'Tomito' and non-inoculated

Treatments	Plant height (cm)		Stem diameter (mm)		HD^{-1} (mm)		Number of leaves		Number of flowers	
FM	35.25 ± 2.3	bcd	4.87 ± 0.3	ns	72.45 ± 5.7	abc	**9.50 ± 1.2**	**a**	1±0.63	cd
MICOSAT F UNO	39.17 ± 2.1	ab	4.82 ± 0.2	ns	81.40 ± 6.7	ab	**9.66 ± 0.2**	**a**	2±0.89	bc
PG	**39.83 ± 3.1**	**a**	4.66 ± 0.5	ns	**86.51 ± 12.7**	**a**	**9.33 ± 0.8**	**a**	1±0.63	cd
AB	35.33 ± 1.8	abcd	4.90 ± 0.3	ns	71.84 ± 6.2	abc	8.50 ± 1.4	ab	**4.5±0.54**	**a**
CM FM	32.33 ± 1.8	de	4.70 ± 0.3	ns	68.92 ± 4.5	bc	9.00 ± 0.6	ab	1±0.63	cd
CM	37.33 ± 2.1	abc	5.06 ± 0.3	ns	73.89 ± 4.5	abc	**9.83 ± 0.7**	**a**	3±0.63	b
H3402	34.00 ± 2.5	cde	4.88 ± 0.4	ns	70.06 ± 8.5	bc	7.50 ± 1.0	b	0±0	d
H3402 x H3402	30.17 ± 1.2	e	4.60 ± 0.2	ns	65.57 ± 3.0	c	8.66 ± 0.5	ab	1±0.63	cd
H3402 x Tomito	35.00 ± 3.3	bcd	4.45 ± 0.3	ns	79.07 ± 11.3	abc	9.17 ± 0.7	ab	1.5±0.54	c
F values		**<0.001**		**0.07**		**<0.001**		**0.002**		**<0.001**

Table 5. Treatment effects on morphological no- destructive parameters in greenhouse experiment. The data are reported as mean ± standard deviation, means followed by the different letters are statistically significant at P <0.05; ns = not significant. FM = 'H3402' grafted onto 'Tomito' and inoculated with *Funneliformis mosseae*, PG = 'H3402' grafted onto 'Tomito' and inoculated with *Paraburkholderia graminis* C4D1M, AB = 'H3402' grafted onto 'Tomito' and inoculated with *Azospirillum brasiliensis* sp 245, CM FM = 'H3402' grafted onto 'Tomito' and inoculated with *F. mosseae, P. graminis* C4D1M and *A. brasiliensis* sp 245, CM = 'H3402' grafted onto 'Tomito' and inoculated with *P. graminis* C4D1M and *A. brasiliensis* sp 245, 'H3402' = 'H3402' non-grafted and non-inoculated, H3402 x H3402 = 'H3402' self-grafted and non-inoculated, H3402 x Tomito = 'H3402' grafted onto 'Tomito' and non-inoculated

Treatments	Leaf dry weight (g)		Steam dry weight (g)		Root dry weight (g)		Plant total dry weight (g)	
FM	1.53 ± 0.11	ab	1.23 ± 0.08	ab	0.59 ± 0.16	ab	3.41 ± 0.32	ab
MICOSAT F UNO	1.50 ± 0.26	ab	1.19 ± 0.27	ab	**0.76 ± 0.17**	**a**	3.68 ± 0.38	ab
PG	1.55 ± 0.21	ab	1.20 ± 0.14	ab	0.57 ± 0.08	ab	3.50 ± 0.34	ab
AB	1.45 ± 0.24	ab	1.26 ± 0.13	ab	0.58 ± 0.14	ab	3.52 ± 0.44	ab
CM FM	1.58 ± 0.11	ab	**1.30 ± 0.17**	**a**	0.53 ± 0.09	ab	3.52 ± 0.32	ab
CM	**1.71 ± 0.14**	**a**	**1.38 ± 0.08**	**a**	0.59 ± 0.05	ab	**3.85 ± 0.18**	**a**
H3402	1.22 ± 0.20	b	1.02 ± 0.09	b	0.52 ± 0.05	b	2.75 ± 0.20	c
H3402 x H3402	1.40 ± 0.09	ab	1.18 ± 0.06	ab	0.54 ± 0.13	ab	3.12 ± 0.18	bc
H3402 x Tomito	1.35 ± 0.08	ab	1.13 ± 0.09	ab	0.55 ± 0.11	ab	3.27 ± 0.31	abc
F values		**0.004**		**0.006**		**0.049**		**<0.001**

6.3.2 Field experiment

The interesting morpho-physiological effects observed in the greenhouse experiment were also assessed and compared with the results obtained in open field conditions under OFS. Unfortunately, during the growing season heavy rains and high moisture conditions allowed the spread of the oomicete *Phytophthora infestans* that was only partially controlled by foliar spray application using copper treatments in the field.

Measurements at fruit development

The data recorded at fruit development are shown in Tables 6 and 7. The treatments (rootstock, grafting technique and plant biostimulants) did not affect significatively the physiological parameters (Chl, Flv and Antho content and NBI). On the other hand, the grafting technique reduced the number of leaves (-17.9%) while the use of rootstock 'Tomito' increased this morphological parameter (+27.6%) in comparison with non-grafted and non-inoculated plants (Table 6). When we considered the number of fruits, the use of rootstock 'Tomito' increased this parameter (+76.3%), and the highest values were achieved combining grafting with inoculation of Micosat F UNO or *P. graminis* C4D1M (+89.1 % and +93.2%, respectively, in comparison with the non-grafted and non-inoculated plants) (Table 7).

The use of rootstock 'Tomito' influenced leaf and stem dry weight parameters (+29.9% and +55.9%, respectively, in comparison with the non-grafted non-inoculated controls) (Table 7). The highest values were recorded by grafted plants inoculated with *F. mosseae* + all the bacteria (+33.5% and +2.6%, respectively, in comparison with the non-inoculated plants grafted onto 'Tomito'). In addition, grafted plants inoculated with *P. graminis* C4D1M showed the highest root dry weight values. Fruit dry weight increased in response to grafting (+55.4%, in comparison with the non-inoculated and non-grafted plants). Nonetheless, the highest values were achieved by grafted plants inoculated with *P. graminis* C4D1M (+66.9%, in comparison with the non-inoculated and non-grafted plants). Finally, grafted plants inoculated with *F. mosseae* + all bacteria showed the highest values of plant dry weights (+54.6%, in comparison with the non-inoculated and non-grafted plants).

Table 6. Treatment effects on morphological no- destructive parameters in field experiment at fruit development. The data are reported as mean ± standard deviation, means followed by the different letters are statistically significant at P <0.05; ns = not significant. FM = 'H3402' grafted onto 'Tomito' and inoculated with *Funneliformis mosseae*, PG = 'H3402' grafted onto 'Tomito' and inoculated with *Paraburkholderia graminis* C4D1M, AB = 'H3402' grafted onto 'Tomito' and inoculated with *Azospirillum brasiliensis* sp 245, CM FM = 'H3402' grafted onto 'Tomito' and inoculated with *F. mosseae*, *P. graminis* C4D1M and *A. brasiliensis* sp 245, CM = 'H3402' grafted onto 'Tomito' and inoculated with *P. graminis* C4D1M and *A. brasiliensis* sp 245, 'H3402' = 'H3402' non-grafted and non-inoculated, H3402 x H3402 = 'H3402' self-grafted and non-inoculated, H3402 x Tomito = 'H3402' grafted onto 'Tomito' and non-inoculated.

Treatments	Number of leaves		Number of fruits		Plant Height (cm)		Stem Diameter (cm)	
FM	69.75 ± 7.09	ab	55.50 ± 10.87	ab	89.75 ± 9.17	ns	1.62 ± 0.17	ns
MICOSAT F UNO	74.50 ± 16.98	ab	**70.00 ± 6.48**	**a**	86.75 ± 7.22	ns	1.82 ± 0.25	ns
PG	**86.00 ± 6.78**	**a**	**71.50 ± 8.38**	**a**	91.75 ± 11.78	ns	1.80 ± 0.21	ns
AB	73.25 ± 15.06	ab	46.00 ± 10.80	ab	76.75 ± 2.98	ns	1.83 ± 0.26	ns
CM FM	**91.00 ± 10.06**	**a**	61.00 ± 10.72	ab	89.25 ± 13.76	ns	1.80 ± 0.14	ns
CM	**82.50 ± 2.88**	**a**	49.75 ± 5.56	ab	98.75 ± 5.61	ns	1.55 ± 0.13	ns
H3402	69.75 ± 7.54	ab	37.00 ± 4.54	b	86.00 ± 6.16	ns	1.75 ± 0.13	ns
H3402 x H3402	57.25 ± 4.85	b	37.50 ± 17.84	b	86.75 ± 12.58	ns	1.67 ± 0.15	ns
H3402 x Tomito	**89.00 ± 7.34**	**a**	65.25 ± 17.46	ab	89.25 ± 4.34	ns	1.75 ± 0.21	ns
F values		**<0.001**		**<0.001**		**0.15**		**0.436**

Table 7. Treatment effects on morphological destructive parameters in field experiment at fruit development. The data are reported as mean ± standard deviation, means followed by the different letters are statistically significant at P<0.05; ns = not significant. FM = 'H3402' grafted onto 'Tomito' and inoculated with *Funneliformis mosseae*, PG = 'H3402' grafted onto 'Tomito' and inoculated with *Paraburkholderia graminis* C4D1M, AB = 'H3402' grafted onto 'Tomito' and inoculated with *Azospirillum brasiliensis* sp 245, CM FM = 'H3402' grafted onto 'Tomito' and inoculated with *F. mosseae*, *P. graminis* C4D1M and *A. brasiliensis* sp 245, CM = 'H3402' grafted onto 'Tomito' and inoculated with *P. graminis* C4D1M and *A. brasiliensis* sp 245, 'H3402' = 'H3402' non-grafted and non-inoculated, H3402 x H3402 = 'H3402' self-grafted and non-inoculated, H3402 x Tomito = 'H3402' grafted onto 'Tomito' and non-inoculated

Treatments	Leaf dry weight (g)		Steam dry weight (g)		Root dry weight (g)		Fruit dry weight (g)		Plant total dry weight (g)	
FM	63.32 ± 15.20	ab	31.70 ± 5.29	abc	11.04 ± 0.97	b	49.71 ± 2.36	abc	155.8 ± 17.31	bcde
MICOSAT F UNO	73.36 ± 18.93	ab	41.43 ± 6.04	ab	16.18 ± 2.69	ab	**60.12 ± 11.78**	**abc**	191.1 ± 30.92	abc
PG	76.81 ± 16.58	ab	36.97 ± 6.39	abc	**20.41 ± 1.83**	**a**	**68.72 ± 8.21**	**a**	202.9 ± 17.13	ab
AB	47.25 ± 12.56	b	41.31 ± 2.19	ab	14.55 ± 3.61	ab	41.09 ± 7.62	bcd	144.2 ± 15.17	cde
CM FM	**98.4 ± 14.44**	**a**	**44.03 ± 5.00**	**a**	17.39 ± 3.74	ab	**61.63 ± 16.08**	**ab**	**221.5 ± 37.23**	**a**
CM	67.68 ± 9.96	ab	30.86 ± 4.87	abc	9.59 ± 4.75	b	73.56 ± 11.16	a	181.7 ± 18.4	abcd
H3402	56.71 ± 10.16	b	27.52 ± 3.77	bc	13.30 ± 3.09	ab	23.4 ± 9.64	d	120.9 ± 16.83	e
H3402 x H3402	59.28 ± 12.19	b	22.67 ± 4.83	c	13.73 ± 1.41	ab	36.21 ± 5.12	cd	131.9 ± 18.25	de
H3402 x Tomito	73.68 ± 12.77	ab	**42.91 ± 9.99**	**a**	15.54 ± 4.30	ab	52.44 ± 9.97	abc	184.6 ± 25.39	abcd
F values	<0.001		<0.001		0.002		<0.001		<0.001	

Measurements at harvest time

The data recorded at harvest time are shown in Figure 1 and Tables 8-10. Although all the treatments increased the marketable yield, the major effect was displayed by microbial inoculations (Figure 1). In fact, grafted plants inoculated with Micosat F UNO, *P. graminis* C4D1M and the bacterial consortium showed the highest marketable yield (+43.1%, +43.5% and +38.5 %, respectively, in comparison with the non-inoculated and non-grafted plants). In addition, the grafted plants inoculated with bacterial consortium showed also the highest leaf area index (LAI) (+39.6%, in comparison with the non-inoculated and non-grafted plants) (Table 8). Considering the physiological parameters, grafting increased the leaf chlorophyll content. On the other hand, the main effects on leaf flavonoid content was highlighted by microbial biostimulant treatments: grafted plants inoculated with *P. graminis* C4D1M showed a reduction (-27.1%) in comparison with non-grafted non-inoculated ones; whereas, when the same plants inoculated with *A. brasiliensis* sp.245 showed an increase (+10.7%) in comparison with the non-grafted non-inoculated plants (Table 8). As far as fruit dry weight is considered (Table 9), we noticed that all the microbial biostimulants had a positive effect and the grafted plants inoculated with *A. brasiliensis* sp. 245 showed the highest values (+32.1%, in comparison with the non-inoculated and non-grafted plants). On the contrary, there were no differences among the non-inoculated non-grafted, self-grafted and grafted onto 'Tomito' plants (Table 10). In addition, the grafted plants inoculated with bacterial consortium presented a striking effect on leaf dry weight (+101.5%, in comparison with non-inoculated and no grafted plants). On the other hand, the main effects on stem dry weight were reported by *P. graminis* C4D1M inoculation (+54.3% and +26.7%, in comparison with the non-inoculated and non-grafted plants and with non-inoculated plants grafted onto 'Tomito', respectively), while the grafted plants inoculated with *F. mosseae* + all bacteria showed the highest root dry weights.

Concerning fruit quality (Table 10), grafting and microbial biostimulants improved the fruit quality. In particular, the grafted plants inoculated with *F. mosseae* produced tomatoes with the highest average fruit weight whereas inoculum with *A. brasiliensis* sp. 245 and the bacterial consortium increased the number of fruits (+42.3% and +40.4%, respectively, in comparison with the non-grafted and non-inoculated plants). Interestingly, all the treatments reduced the incidence of the blossom-end rot physiological disorder, and the inoculation with

A. brasiliensis sp. 245 increased the Brix° and Brix t ha^{-1} (+ 5.29 and + 29.37%, respectively, in comparison with the non-grafted and non-inoculated plants).

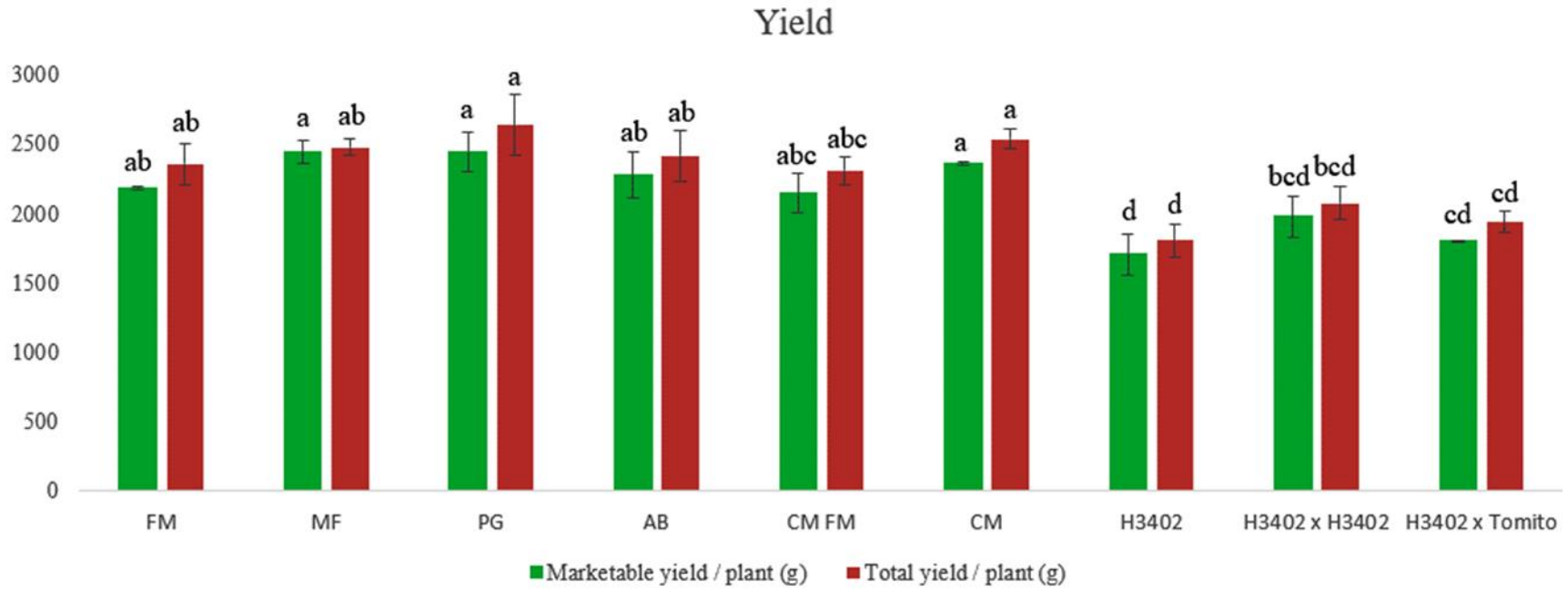

Figure 1. Mean values of marketable yield (green) and total yield (red) in processing tomato plants inoculated with different plant biostimulants and grafted on a cherry genotype. Vertical bars represent significant differences at $P < 0.05$. FM = 'H3402' grafted onto 'Tomito' and inoculated with *Funneliformis mosseae*, PG = 'H3402' grafted onto 'Tomito' and inoculated with *Paraburkholderia graminis* C4D1M, AB = 'H3402' grafted onto 'Tomito' and inoculated with *Azospirillum brasiliensis* sp 245, CM FM = 'H3402' grafted onto 'Tomito' and inoculated with *F. mosseae*, *P. graminis* C4D1M and *A. brasiliensis* sp 245, CM = 'H3402' grafted onto 'Tomito' and inoculated with *P. graminis* C4D1M and *A. brasiliensis* sp 245, 'H3402' = 'H3402' non-grafted and non-inoculated, H3402 x H3402 = 'H3402' self-grafted and non-inoculated, H3402 x Tomito = 'H3402' grafted onto 'Tomito' and non-inoculated

Table 8. Treatment effects on physiological parameters in field experiment at harvest time. The data are reported as mean ± standard deviation, means followed by the different letters are statistically significant at P <0.05; ns = not significant. Chl = index of chlorophyll content in the leaves; Flv = index of flavonoid content in the leaves; Antho = index of anthocyanin content in the leaves; NBI = nitrogen balance index, LAI = Leaf area index. FM = 'H3402' grafted onto 'Tomito' and inoculated with *Funneliformis mosseae*, PG = 'H3402' grafted onto 'Tomito' and inoculated with *Paraburkholderia graminis* C4D1M, AB = 'H3402' grafted onto 'Tomito' and inoculated with *Azospirillum brasiliensis* sp 245, CM FM = 'H3402' grafted onto 'Tomito' and inoculated with *F. mosseae*, *P. graminis* C4D1M and *A. brasiliensis* sp 245, CM = 'H3402' grafted onto 'Tomito' and inoculated with *P. graminis* C4D1M and *A. brasiliensis* sp 245, 'H3402' = 'H3402' non-grafted and non-inoculated, H3402 x H3402 = 'H3402' self-grafted and non-inoculated, H3402 x Tomito = 'H3402' grafted onto 'Tomito' and non-inoculated

Treatments	Chl		Flv		Antho		NBI		LAI	
FM	28.96 ± 3.5	ab	4.06 ± 0.6	bc	0.63 ± 0.01	ab	7.32 ± 2.0	abc	0.99 ± 0.1	abcd
MICOSAT F UNO	**34.10 ± 2.9**	**a**	4.57 ± 0.2	bc	0.69 ± 0.05	ab	7.50 ± 1.1	abc	0.64 ± 0.3	d
PG	**33.12 ± 2.6**	**a**	3.92 ± 0.1	c	0.59 ± 0.07	b	**8.43 ± 0.2**	**a**	1.36 ± 0.4	ab
AB	22.03 ± 1.6	b	**6.01 ± 0.1**	**a**	0.64 ± 0.05	ab	**3.66 ± 0.2**	**c**	1.09 ± 0.2	abcd
CM FM	**33.08 ± 1.1**	**a**	4.78 ± 0.3	abc	0.56 ± 0.01	b	6.95 ± 0.7	abc	1.19 ± 0.2	abc
CM	**33.72 ± 3.4**	**a**	4.42 ± 0.2	bc	0.56 ± 0.02	b	7.66 ± 1.1	ab	1.49 ± 0.5	a
H3402	21.81 ± 3.7	b	5.43 ± 0.6	ab	0.78 ± 0.03	a	3.99 ± 0.2	bc	0.91 + 0.1	bcd
H3402 x H3402	**34.07 ± 2.3**	**a**	4.39 ± 0.8	bc	0.56 ± 0.04	b	**8.05 ± 2.2**	**a**	1.09 ± 0.1	abcd
H3402 x Tomito	**32.27 ± 5.4**	**a**	5.24 ± 0.4	abc	0.77 ± 0.08	a	6.22 ± 1.4	abc	0.66 ± 0.2	cd
F values	<0.001		<0.001		<0.001		<0.001		0.02	

Table 9. Treatment effects on morphological destructive parameters in field experiment at harvest time. The data are reported as mean ± standard deviation, means followed by the different letters are statistically significant at P <0.05; ns = not significant. FM = 'H3402' grafted onto 'Tomito' and inoculated with *Funneliformis mosseae*, PG = 'H3402' grafted onto 'Tomito' and inoculated with *Paraburkholderia graminis* C4D1M, AB = 'H3402' grafted onto 'Tomito' and inoculated with *Azospirillum brasiliensis* sp. 245, CM FM = 'H3402' grafted onto 'Tomito' and inoculated with *F. mosseae, P. graminis* C4D1M and *A. brasiliensis* sp. 245, CM = 'H3402' grafted onto 'Tomito' and inoculated with *P. graminis* C4D1M and *A. brasiliensis* sp. 245, 'H3402' = 'H3402' non-grafted and non-inoculated, H3402 x H3402 = 'H3402' self-grafted and non-inoculated, H3402 x Tomito = 'H3402' grafted onto 'Tomito' and non-inoculated.

Treatments	Fruit dry weight (g plant^{-1})		Leaf dry weight (g plant^{-1})		Strem dry weight (g plant^{-1})		Root dry weight (g plant^{-1})		Plant total dry weight (g plant^{-1})	
FM	123.8 ± 2.2	bc	52.64 ± 0.4	bc	50.11 ± 1.3	b	11.33 ± 1.9	e	237.80 ± 2.6	d
MICOSAT F UNO	137.2 ± 9.2	ab	40.08 ± 4.4	c	44.99 ± 6.4	b	24.18 ± 1.4	ab	246.50 ± 2.8	cd
PG	136.9 ± 4.6	ab	70.65 ± 8.9	ab	**78.47 ± 6.7**	**a**	18.2 ± 2.4	abcde	**304.30 ± 18.5**	**a**
AB	**148.5 ± 0.8**	**a**	60.58 ± 1.1	ab	61.54 ± 5.0	ab	12.45 ± 0.5	de	283.05 ± 6.5	abc
CM FM	140.8 ± 7.1	ab	63.55 ± 3.5	ab	64.11 ± 8.2	ab	**26.69 ± 6.5**	**a**	295.16 ± 4.7	ab
CM	138.1 ± 7.2	ab	**76.92 ± 10.7**	**a**	68.02 ± 4.7	ab	14.11 ± 2.0	cde	**297.15 ± 2.4**	**a**
H3402	112.4 ± 6.2	c	53.84 ± 2.6	bc	50.86 ± 10.3	b	20.99 ± 3.3	abcd	238.06 ± 11.4	d
H3402 x H3402	111.5 ± 11.6	c	60.53 ± 9.9	ab	57.05 ± 11.1	ab	23.07 ± 2.9	abc	252.20 ± 33.4	bcd
H3402 x Tomito	111.5 ± 0.1	c	38.16 ± 3.2	c	61.94 ± 11.4	ab	16.13 ± 1.2	bcde	227.70 ± 9.6	d
F values	<0.001		<0.001		0		<0.001		<0.001	

Table 10. Treatment effects on fruit quality parameters in field experiment at harvest time. The data are reported as mean ± standard deviation, means followed by the different letters are statistically significant at P <0.05; ns = not significant. FM = 'H3402' grafted onto 'Tomito' and inoculated with *Funneliformis mosseae*, PG = 'H3402' grafted onto 'Tomito' and inoculated with *Paraburkholderia graminis* C4D1M, AB = 'H3402' grafted onto 'Tomito' and inoculated with *Azospirillum brasiliensis* sp 245, CM FM = 'H3402' grafted onto 'Tomito' and inoculated with *F. mosseae, P. graminis* C4D1M and *A. brasiliensis* sp 245, CM = 'H3402' grafted onto 'Tomito' and inoculated with *P. graminis* C4D1M and *A. brasiliensis* sp 245, 'H3402' = 'H3402' non-grafted and non-inoculated, H3402 x H3402 = 'H3402' self-grafted and non-inoculated, H3402 x Tomito = 'H3402' grafted onto 'Tomito' and non-inoculated

Treatments	Average fruit weight (g)		Number of fruits (piant^{-1})		BER fruits (no plant^{-1})		pH		BRIX °		BRIX t ha^{-1}	
FM	**68.66 ± 0.5**	**a**	34.35 ± 2.3	bc	0.50 ± 0.00	b	4.17 ± 0.1	ns	5.13 ± 0.2	b	2.80 ± 0.10	ab
MICOSAT F UNO	62.46 ± 1.8	abcd	39.67 ± 2.1	ab	1.00 ± 0.50	b	4.27 ± 0.1	ns	5.47 ± 0.2	ab	3.33 ± 0.04	ab
PG	64.80 ± 0.4	ab	40.70 ± 3.1	ab	0.00 ± 0.00	b	4.29 ± 0.2	ns	5.37 ± 0.5	ab	3.29 ± 0.52	ab
AB	53.06 ± 2.7	e	**45.51 ± 3.1**	**a**	1.17 ± 0.28	b	4.15 ± 0.0	ns	**6.23 ± 0.1**	**a**	**3.54 ± 0.23**	**a**
CM FM	63.60 ± 0.6	abc	36.28 ± 1.2	bc	2.83 ± 0.28	ab	4.26 ± 0.1	ns	5.50 ± 0.2	ab	2.95 ± 0.30	ab
CM	56.54 ± 3.1	de	**44.92 ± 1.1**	**a**	2.33 ± 0.76	ab	4.37 ± 0.1	ns	5.20 ± 0.5	b	3.07 ± 0.30	ab
H3402	56.40 ± 3.6	de	31.98 ± 0.1	c	**5.83 ± 2.25**	**a**	4.29 ± 0.0	ns	5.90 ± 0.2	ab	2.50 ± 0.13	b
H3402 x H3402	57.94 ± 2.9	cde	35.92 ± 3.8	bc	**5.50 ± 3.00**	**a**	4.31 ± 0.0	ns	5.87 ± 0.2	ab	2.91 ± 0.34	ab
H3402 x Tomito	60.80 ± 0.4	bcd	31.85 ± 1.4	c	2.33 ± 0.28	ab	4.23 ± 0.1	ns	6.00 ± 0.2	ab	2.69 ± 0.07	b
F values	<0.001		<0.001		<0.001		0.19		0.004		0.004	

6.4 Discussion

Flowering is a crucial developmental stage for most herbaceous crops and the influencing of the flowering time could be important strategy for either tailoring the crop life cycle, to fit different environments and to reduce the transition from vegetative to reproductive stage (Waseem et al., 2019). In tomato, flowering is controlled by the single flower truss (SFT) gene (Lifschitz and Eshed, 2006). A study conducted by Lu et al. (2019) showed that *Arabidopsis thaliana* rhizosphere microbiota can modulate flowering time by indole-3-acetic acid (IAA) production. Interestingly, in the greenhouse experiment the seedlings inoculated with *A. brasiliensis* showed an early flowering stage. A study performed on wheat, reported that *A. brasilense* can induce the gene of IAA when inoculated on root surface (Rothballer et al., 2005). Therefore, we can hypnotize that *A. brasiliensis* can induce early flowering in processing tomato plants by inducing the IAA gene. On the other hand, in the field experiment the positive effect of *A. brasiliensis* on flowering was not confirmed. Therefore, new studies could be carried out modifying the number of treatments, the timing of the inoculation, the inoculum concentration or testing some adjuvant in order to improve the treatment in open field.

The combined use of grafting and microbial biostimulants increased marketable and total yields of the commercial processing tomato genotype 'H3402'. However, the contribution of the rootstock on the increment of the marketable yield was lower than expected. These results were due to the high incidence of light blight occurred in the open field. In fact, the oomicetes *P. infestans* may lead a decrease of yield tomato (Fontem et al. 1999). On the other hand, the effect of microorganisms on triggering a series of physical and morphological processes that helped the plants to cope the light blight are well known (Montano et al., 2014). Interestingly, the plants grafted onto 'Tomito' and inoculated with *P. graminis* C4D1M, Micosat F UNO and the bacterial consortium achieved the highest marketable yield. The increase of marketable yield was linked both to the increment of number of fruits and an increase of the average fruit weight, however, showing differences between treatments. In fact, *P. graminis* C4D1M influenced both the number and the weight of the fruits, Micosat F UNO influenced mainly the fruit number, while the effect of bacteria consortium was intermediate between *P. graminis* and *A. brasiliensis*. These results are partially in agreement with Candido et al. (2013) that found an increase of number and weight of the fruits in the cherry tomato genotype 'HF1 PX 02325715' inoculated with Micosat F UNO.

The morphological changes correlated to microbial biostimulants inoculations could be attributed to a highest nutrient uptake or increased nutrient availability by nitrogen fixation, mineralization of organic compounds, phosphorus solubilization, and/or production of phytohormone such as IAA (Gouda et al., 2018; Shameer and Prasad, 2018).

In leaf, chlorophyll is a key pigment in the photosynthesis activity as it is responsible for absorbing light energy (Di Martino et al., 2019). Our results showed that all the treatments significantly increased the content of chlorophyll in the greenhouse experiment, while in field experiment the positive effect of rootstock was not observed. The increased chlorophyll content in response to treatment is correlated to the improvement of uptake of nutrient from soil and in particular of nitrogen, the main component influencing this pigment. In addition, a recent study showed that *P. graminis* can produce gramibactin, a siderophore that can bind iron that is an essential element for chlorophyll production (Hermenau et al., 2018).

Leaf area index (LAI) is an important parameter used for monitoring the crop growth as it indicates the capacity of plant canopies to exchange energy and organic matter with environment (Niinemets and Tobias, 2019). In the present study, plants inoculated with *P. graminis* C4D1M showed a significative increment of LAI that should be ascribed to a plant growth promoting effect of rhizobacterium. Our results showed that the inoculation of processing tomato with plant biostimulants significantly increased vegetative growth (plant height, number of leaves, plant dry weight and single organ dry weight) of the processing tomato plants. However, the distribution of the dry matter in the organ depend on treatments. Similar effects of plant biostimulants on crop growth of were reported also in other studies (Rahman et al., 2018; Roesti et al., 2006).

Blossom-end rot (BER) is a physiological disorder that causes important economic losses (Hagassou et al., 2019). Although BER is linked to the concentration of calcium available in the soil solution, other factors are also involved in its occurrence such as reduced nutrient and water uptake and the rapid cell expansion in the distal fruit tissue (de Freitas and Mitcham, 2012; Ho and White, 2005). In our study, all the treatments reduced the number of fruits affected by BER. Previously studies (Boari et al. 2016; Grasselly et al. 2008) reported a lower incidence of BER in some tomato varieties like cherry, cocktail, or round tomato types, indicating that fruit shape influences BER occurrence. However, in our study the fruits were plum, therefore,

other factors were involved such as the use of the cherry rootstock genotype and the interactions among rootstock, scion and plant biostimulant inoculations.

The solid soluble content (°Brix) is an important parameter of commercial quality of tomato juice. In addition, the Brix yield (Brix t ha^{-1}) is a parameter that puts in correlation the harvest and marketable yield with the main quality parameter (°Brix), therefore it is very important in determining the farm income. In the present study, *A. brasiliensis* sp. 245 increased the quality of fruit reaching the highest °Brix and Brix yield. Our results are in accordance with Ordookhani and Zare (2011), who found similar increase of soluble solid content using PGPR (*Pseudomonas putida*, *Azotobacter chroococcum*) and AMF (*F. mosseae*).

6.5 **Conclusion**

Our study indicated that among the investigated microbial biostimulants, *P. graminis* C4D1M, *A. breailiensis* sp. 245 and bacterial consortium positively affected processing tomato growth, fruit yield and quality in sustainable farming systems. Therefore, the use of microbial biostimulants could be a sustainable strategy to reduce the current yield gap between OFS and CFS. In addition, *A. brasiliensis* sp. 245 might be used to induce an early flowering, reducing the growing season of processing tomato. However, microorganism inoculation (timing and number of applications) and formulation (concentration, co-formulants, adjuvants and consortium of the microorganisms) should be improved to make more effective the treatment also in open field, where environmental factors and microorganisms already present in the rhizosphere might influence the activity of the inoculated microorganisms. In general, grafting improved agronomic and fruit quality parameters. In fact, the proposed cherry (‘Tomito’) rootstock influenced morphological and physiological parameters of processing tomato when cultivated in greenhouse, while these effects were reduced by environmental factors when cultivated in open field. Therefore, new studies could be carried out to assess the performances of the rootstock investigated in the present study and its interactions with these microorganisms in different environmental conditions.

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Chapter 7

Nitrogen fertilisers shape the composition of the microbiota of field-grown processing tomato plants

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7.1 Introduction

Limiting the negative impact of agricultural practices on the environment while preserving sustainable crop yield is one of the key challenges facing agriculture in the years to come.

As an essential element for plant nutrition, nitrogen represents a paradigmatic example of such a challenge. Moreover, due to the combined effect of elevated solubility and little retention in soils, the lack of this element is and will be one of the major yield-limiting factors worldwide (Tilman et al., 2011). At the same time, the application of synthetic nitrogen fertilisers is, in many agricultural systems, a low efficiency approach which has been linked with the degradation of natural resources (Elser and Bennett, 2011). One of the strategies adopted to limit the economic and environmental footprint of crop production while maintaining sustainable yield is the "recycling" of, mineral-rich, biodegradable products of the livestock and agricultural sectors.

One example of this approach is the application of the digestate, a by-product the anaerobic digestion of organic waste for the production of biogas (Möller and Müller, 2012) as renewable soil amendment for crop production. The digestate is a mixture of partially-degraded organic matter, microbial biomass and inorganic compounds (Alburquerque et al., 2012). We recently have demonstrated how the digestate can be efficiently used as innovative fertiliser and plant growing media (Ronga et al., 2018b; Ronga et al., 2018a; Ronga et al., 2019), yet the impact of digestate applications on the agroecosystem remains to be fully elucidated.

For instance, the digestate can be a source of phytoavailable nitrogen, in particular ammonium, capable of impacting on organic matter mineralisation and emission of carbon dioxide from the soil profile (Grigatti et al., 2011). Therefore, it is legitimate to hypothesize that such treatments impact on the composition of the microbial communities thriving at the root-soil interface, collectively referred to as the rhizosphere and root microbiota, which play a critical role in mobilisation of mineral elements for plant uptake (Alegria Terrazas et al., 2016). Congruently, several studies indicate that the application of biogas by-product enhances soil microbial activity (Möller, 2015). However, the intertwined relationship among microbiota composition, soil characteristics and amendments as well as host plant species-specificity (Bulgarelli et al., 2013) makes it difficult to infer first principles.

This is particularly true for field-grown crops such as tomato (*Solanum lycopersicum L.*), one of the most cultivated horticultural crops globally with plantations occupying an area of 4.8 million of hectares with a production of 182 million tonnes in 2017 (FAO, 2017). Notably, this species is also an excellent experimental model for basic science investigations: tomato was one of the first crops whose genome was sequenced (Consortium, 2012) and provided a superb platform to test the significance of genome editing for evolutionary studies and plant breeding (Zsögön et al., 2018). Perhaps not surprisingly, tomato is gaining momentum as an experimental system to study host-microbiota interactions in crop plants. Recent investigations have revealed novel insights into the assembly cues of the microbiota associated to this plant (Bergna et al., 2018; Toju et al., 2019) and the contribution of microbes thriving at the tomato root-soil interface to pathogen protection (Chialva et al., 2018; Kwak et al., 2018). However, the composition and functional potential of the tomato microbiota and their interdependency from nitrogen fertilisers remain to be elucidated.

Here we report the metagenomics characterisation of the microbiota thriving at the root-soil interface of field-grown tomato plants. We hypothesize that nitrogen treatments shape and modulate the contribution of the tomato microbiota for crop yield. To test this hypothesis, we focused on processing tomato exposed to different nitrogen fertilisers, either digestate-based or containing a mineral fraction. By using a 16S rRNA amplicon sequencing survey we deciphered how the microhabitat (i.e., either rhizosphere or root) sculpts the tomato microbiota which, in turn, is fine-tuned by the type of fertiliser applied.

7.2 Material and methods

7.2.1 Filed site, plant material and experimental design

A field trial was established in a tomato farm near the city of Ravenna (44°25'40.8"N 12°05'53.3"E), Emilia Romagna Region, Italy, during the 2017 growing season. During the period from transplant to harvest, the minimum and the maximum average temperatures recorded were 17.1°C and 32.8°C, respectively, and the rainfall was 101.7 mm. The soil had a silty loam texture (14% clay, 51% silt, 35% sand), a pH 8.3 (in H_2O), 1.1 g kg^{-1} total N (Kjeldahl method), 7 mg kg^{-1} available P (Olsen method), 129 mg kg^{-1} exchangeable K (Ammonium acetate), and 9 g kg^{-1} organic matter (Walkey-Black method). A schematic illustration of the field trial is reported in Figure 1.

Figure 1. Schematic illustration of the field

We used the tomato cultivar ‘Fokker’, a processing-type genotype with blocky fruit, late fruit ripening and suitable for tomato puree, for the experimentation. Seedlings were provided by Bronte Soc. Coop. Agr. A.R.L. (Mira, Italy). Processing tomato seedlings were transplanted at the end of May when they were 6-week old corresponding to plants at the fourth true leaf stage. Plant density was 3 plants m^{-2}. Plants were transplanted into single row, with a spacing of 0.22 m between plants in each row and 1.50 m between rows.

We established a randomized complete design with three replicates and seven treatments: pelleted digestate (hereafter PE), liquid digestate (LD), slow-acting liquid digestate (SRLD), organo-mineral fertiliser based on digestate (SC), mineral fertiliser (MF), slow-acting mineral fertiliser (SRMF), and no fertilization treatment (NT). The composition of the treatments is summarised in Table 1.

Table 1. Composition and information on fertilisers used in this study. TOC = Total organic carbon; N = Nitrogen; P = Phosphorus; K = Potassium; H2O = water content

Treatment	TOC%	(N)%	(P)%	(K)%	H_2O%	Additional information
Mineral fertiliser (MF)		41.00				Ammonium nitrate (N 26%) and calcium nitrate (N 15%)
Pelleted digestate (PE)	39.70	1.50	2.50	2.00	7.80	(Pulvirenti et al., 2015)
Slow acting liquid digestate (SRLD)	3.74	0.34		0.95		Liquid digestate plus the nitrogen stabilizer Vizura® (BASF, 2 L ha^{-1}),
Liquid digestate (LD)	3.74	0.34		0.95		EC 1.07 dS m^{-1} and pH 8.3
Organo-mineral fertiliser (SC)	10.50	10.00	5.00	15.00	7.00	Produced by SCAM Spa (Modena, Italy), based on solid digestate for the organic fraction
Slow acting mineral fertiliser (SRMF)		15.00	15.40	15.00		NPK Original Gold® (Compo Expert)

For each treatment, we applied a total amount of nitrogen in the ratio 150 N kg ha^{-1} on the basis of soil analysis, crop rotation and crop nutrients required. Nitrogen was supplied at transplanting time with the exception of the mineral fertiliser treatment. For this latter treatment, the amount of total Nitrogen was equally divided and applied in 3 times (transplanting, full flowering and fruit ripening) using ammonium nitrate in the first treatment and calcium nitrate in the second and in the third ones. During the trial, 600 m^3 ha^{-1} of irrigation water was distributed by drip irrigation to each treatment. The other soil and crop management practices were performed according to the production rules of Emilia Romagna Region, Italy. Briefly, weeds control was performed through a single treatment (on 11th June) using products based on metribuzin and propaquizafop. Sulphur and Copper were used to control phytopatogenic fungi while imidacloprid, abamectin and spinosad were used as insecticide.

7.2.2 Yield traits

At harvest we determined the marketable yield (t ha^{-1}), as a weight of fully ripe fruits, and the Brix yield (°Brix t ha^{-1}) as a proxy for fruit quality. The °Brix parameter (the solid soluble content) was determined using the digital refractometer HI 96814 (Hanna, Italy), while the Brix yield (°Brix t ha^{-1}) was calculated by multiplying the hectare marketable yield by °Brix and dividing the result by 100.

7.2.3 Root, rhizosphere and bulk soil sampling and DNA extraction

At transplanting time (May 2017), 5 root specimens per treatment were collected. Upon uprooting, soil particles loosely bound to roots were dislodged by hand shaking and root segments of ~ 6 cm were placed in sterile 50 mL tubes. The samples were stored in a portable cooler (~ 4°C), transported to the laboratory and immediately processed. Root specimens were incubated in 30 mL of PBS (Phosphate buffered saline) and placed on a shaker for 20 minutes in order to separate the soil tightly adhering to plant material, which we operationally defined as “rhizosphere”, from the roots. The first tubes were centrifuged for 20 minutes at 4,000 x g and the rhizosphere pelleted was collected in liquid nitrogen and stored at -80°C. The roots were moved to a new sterile tube containing 30 mL PBS and sonicated by Ultrasonics Sonomatic Cleaner (Langford Ultrasonics, Birmingham, UK) for 10 minutes (intervals of 30 seconds pulse and 30 seconds pause) at 150 W, as previously reported (Schlaeppi et al., 2014) to enrich for endophytic microorganisms. Roots were then washed in the same new buffer and

dried on sterile filter paper. After few minutes, the roots were moved to 50 mL tubes and frozen in liquid nitrogen for storage at -80°C. Three independent soil samples were harvested from unplanted soil in different points of the field, frozen in liquid nitrogen and stored at -80°C. At harvest time (September 2017) the whole plants were harvested, 5 roots per treatment and 3 bulk soil samples were collected, prepared and stored like the previous samples. Frozen root samples were pulverized in a sterile mortar using liquid nitrogen prior DNA preparation. DNA was extracted from all the specimens (i.e., bulk soil, rhizosphere and pulverized roots) using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, USA) following the instruction manual provided by manufacturer. DNA samples were diluted using 50 µL DES water and quantified using the Nanodrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, United States).

7.2.4 PNA blocker and 16S rRNA gene sequencing

Before generating the whole amplicon libraries, a PCR blocker was evaluated in order to minimize the cross-amplification of plastidial-derived sequences in root samples. Different concentrations (0 µM; 0.25 µM and 0.5 µM) of the Peptide Nucleic Acid (PNA) (PNA Bio, Newbury Park, United States), designed for *Arabidopsis thaliana* plastids (Lundberg et al. 2013), were tested using 3 root and 3 bulk random samples.

The sequencing libraries (the first one to test the PNA blocker and the second one to test the microhabitat and fertiliser treatments) were generated using primers specific (515F 5'-GTGCCAGCMGCCGCGGTAA-3' and 806R 5'-GGACTACHVGGGTWTCTAAT-3') for hypervariable V4 region of the 16S rRNA gene. The reverse primers included a 12-mer unique "barcode" sequences (Caporaso et al., 2012) to facilitate the multiplexing of the samples into a unique sequencing run. Individual PCR reactions were performed as previously reported (Robertson-Albertyn et al., 2017), with the exception of the concentration of the Bovine Serum Albumin, added at 10 µg/reaction, and the addition of a Peptide Nucleic Acid (PNA) blocker (in the second library the PNA blocker was applied at a concentration of 0.5 µM/reaction). For each barcoded primers, three technical replicates and a no-template control (NTC) were organised and processed starting from a unique master mix. Five microliters of amplified samples and cognate NTCs were inspected on a 1% (w/v) agarose gel. Two independent sets of triplicated amplicons, displaying the expected amplicon size and lacking detectable contaminations, were combined in a barcode-wise manner and purified using the Agencourt

AMPure XP kit (Beckman Coulter, Brea, United States) with a ratio of 0.7 mL AMPure XP beads per 1 mL of sample. Purified DNA samples were quantified using Picogreen (Thermo Fisher, United Kingdom) and combined in an equimolar ratio into an amplicon pool. This latter material was used for the preparation of a MiSeq run at the Genome Technology facilities of the James Hutton Institute (Invergowrie, UK) as previously reported (Robertson-Albertyn et al., 2017)

7.2.5 OTU table generation and pre-processing

We used QIIME, version 1.9.0 (Caporaso et al., 2010) to process the sequencing output of the MiSeq machine. Briefly, the command join_paired_ends.py was used to decompress and merged (minimum overlap 5bp) forward and reverse read FASTQ files. Next, we removed in silico low-quality sequencing reads and sequencing reads without the barcode information. Then, the reads were assigned to individual samples. In these analyses, the command split_libraries_fastq.py was used imposing a minimum PHRED score of 20. The resulted high-quality reads were assembled into an Operational Taxonomic Unit (OTU) table at 97% sequence identity. We used a 'closed reference' approach for OTU-picking using the command pick_closed_reference_otus.py. We imposed the Green Genes database version 13_5 (DeSantis et al., 2006) as a references database to identify microbial OTUs and prune for chimeric sequences. We used SortMeRNA algorithm for OTU -picking and taxonomy assignment. Finally, OTUs whose representative sequences were classified as either chloroplast or mitochondria, as well as OTUs accruing only one sequencing read over the entire dataset (i.e., singletons), were depleted in silico using the function filter_otus_from_otu_table.py.

7.2.6 Data visualization and statistical analysis

Agronomic traits were analysed by Analysis of variance (ANOVA) using GenStat 17th (VSN International, Hemel Hempstead, UK). Means were compared using Bonferroni's test at the 5% level.

The OTU table produced in QIIME was analysed in R using a custom script developed from Phyloseq (McMurdie and Holmes, 2013).

Initially, the data were filtered removing the samples with less than 1,000 reads and the OTUs with less than 10 reads in at least 5% of the samples. For alpha-diversity calculation,

sequencing reads were rarefied at an even sequencing deep of 18,467 reads per sample retaining 2,439 unique OTUs. The number of observed OTUs and Chao1 index was used as richness estimators, while the Shannon index was used for evaluating the evenness. Upon inspecting distribution of the data using a Shapiro-Wilk test, the means of rhizosphere and root samples at harvest time were compared using a non-parametric Wilcoxon rank sum test. Next, we performed a non-parametric Kruskal–Wallis test independently on rhizosphere and root samples to identify significant effect of the individual treatments on the ecological indices.

For beta-diversity calculation, the original counts (i.e., not rarefied) were transformed to relative abundances and we imposed an abundance threshold to target PCR-reproducible OTUs. The differences among microbial communities of the samples were computed using Bray Curtis index and weighted Unifrac index, with this latter index including phylogenetic information in the analysis (Lozupone and Knight, 2005). A principal coordinates analysis was generated to visualize similarities and dissimilarities of microhabitats and treatments. In order to assess the effects of microhabitats and the treatments on the bacterial community composition a Permutational Multivariate Analysis Of Variance (PERMANOVA) was performed using the distance matrices using a two-pronged approach. First, we assessed the effect of nursery/harvest stage a microhabitat in rhizosphere and root samples. Next, we used the same test to assess the impact of the treatment on rhizosphere and root specimens at harvest stage. In the two approaches, the computed R^2 therefore reflects the proportion of variance explained by the given factor in the group of sample tested.

Finally, original counts data were used to perform a differential analysis to identify individual bacteria differentially enriched in the tested samples using DESeq2 (Love et al., 2014).

The phylogenetic tree was constructed using the representative sequences of the OTUs significantly enriched in rhizosphere and root specimens and annotated with iTOL (Letunic and Bork, 2006).

7.2.7 Data and script availability

The 16S rRNA gene sequences presented in this study are available at the European Nucleotide archive under the study accession number PRJEB32219. The scripts to reproduce the statistical analysis and figures are available at https://github.com/BulgarelliD-Lab/Tomato_nitrogen.

7.3 Results

7.3.1 Fertiliser treatment impacts on yield and quality of processing tomato

At harvest time the two most important parameters such as marketable yield and fruit quality were collected to evaluate the effect of 7 different fertiliser performances on processing tomato (Figure 2). The fertiliser treatments had a significant effect on fruit fresh biomass (ANOVA, Bonferroni's test, $P < 0.001$). Pelleted digestate registered the best performance followed by mineral fertiliser and slow acting liquid digestate. In addition, the different fertilisers influenced significantly also the quality of processing tomato (Figure 2) (ANOVA, Bonferroni's test, $P < 0.001$).

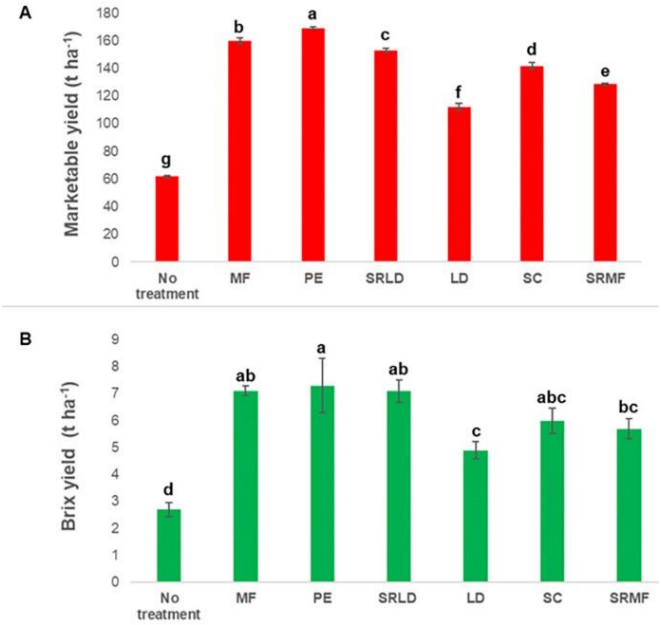

Figure 2. Effect of the nitrogen treatments on tomato yield traits. Mean and standard deviation of (A) marketable yield and (B) Brix yield of tomato plants exposed to the following treatments: LD (Liquid Digestate), SRLD (Slow acting Liquid Digestate), PE (Pelleted Digestate); SC (Organo-mineral fertiliser); MF (Mineral Fertiliser); SRMF (Slow acting Mineral Fertiliser). Different letters denote statistically significant differences between treatments by Analysis of variance (ANOVA). Means were compared using Bonferroni's test at the 5% level ($P < 0.001$).

7.3.2 PNA effects on plasmid DNA amplification

Owing to the potential biases associated with cross-contamination of host-derived sequences in the endophytic compartment of plants (Lundberg et al., 2013), we first assessed

the effectiveness of plastidial PNA (pPNA) in blocking the amplification of plasmid DNA from roots of processing tomato.

We observed that the presence of pPNA at concentration of 0.5 µM in the PCRs allowed us to efficiently deter the PCR primers from the amplification of plastidial DNA. Likewise, the mitochondrial contamination remained below acceptable levels compared to pPNA at concentration of 0.25 µM (Figure 3). Of note, the composition of the microbial community of (root and bulk) samples was not altered by blocker presence (Figure 4 and 5), therefore, pPNA at concentration of 0.5 µM was added to all reactions during the amplification of the sequences.

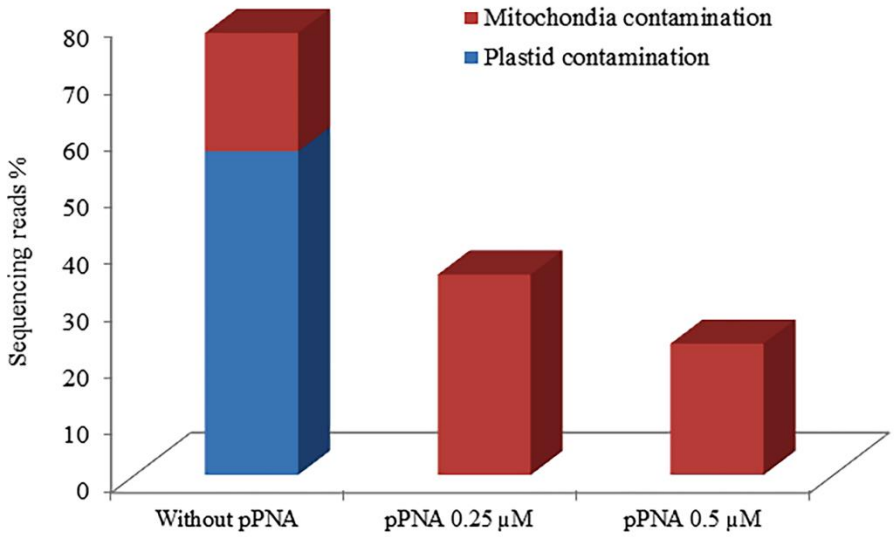

Figure 3. The plastidial PNA (pPNA) blocker effectiveness. Data show the average value of the all samples tested (3 root and 3 bulk soil samples)

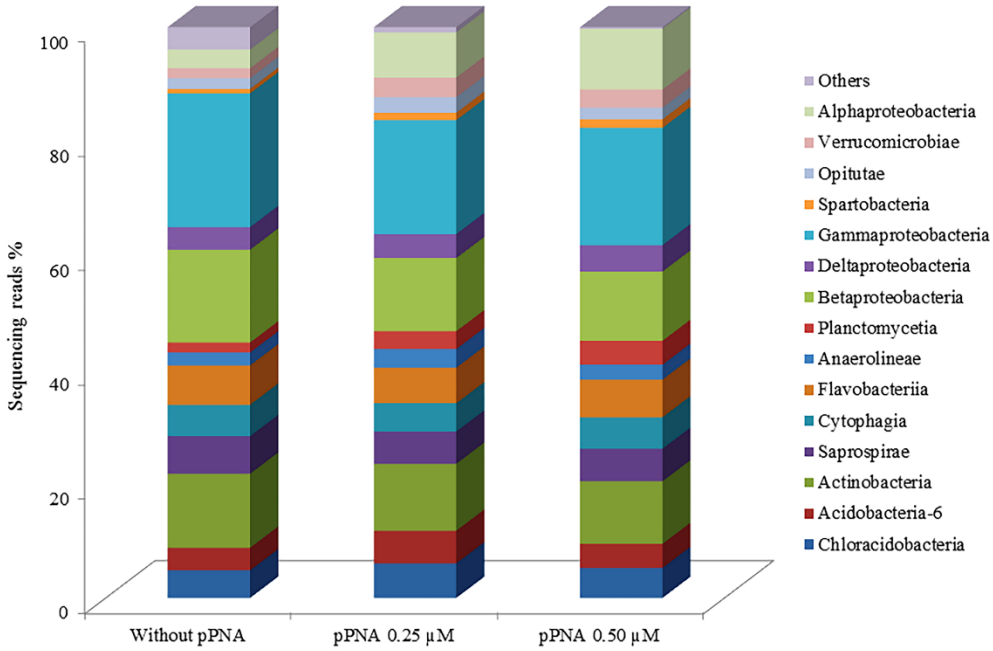

Figure 4. Percentage of bacteria phyla belonging to enriched OTUs (Operational Taxonimic Units) in roots samples using a plastidial PNA (pPNA) blocker in the amplification of sequences

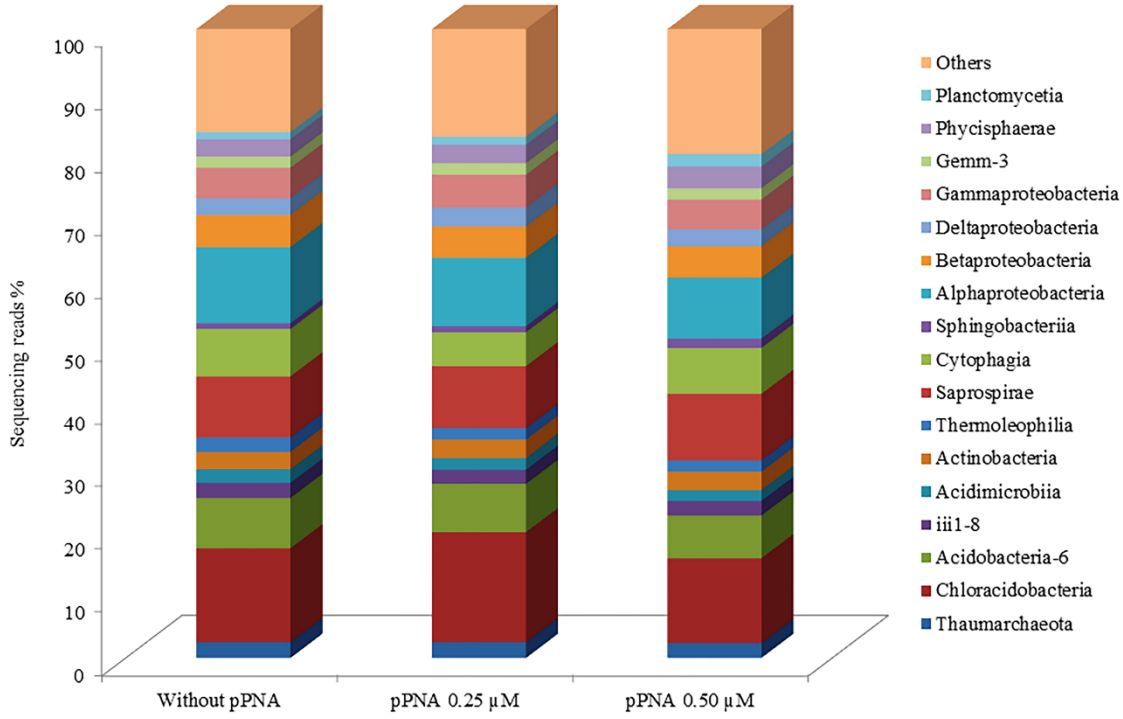

Figure 5. Percentage of bacteria phyla belonging to enriched OTUs (Operational Taxonimic Units) in bulk soil samples using a plastidial PNA (pPNA) blocker in the amplification of sequences

7.3.3 The assembly dynamics of the bacterial microbiota of field-grown processing tomato

To gain insights into the relationships between yield traits and microbiota composition in field-grown processing tomato plants, we generated 5,546,303 high quality 16S rRNA gene sequences for 86 samples generated in this study.

Upon in silico depletion of OTUs classified as Mitochondria we reduced the number of analysable sequences to 4,645,503 with a retaining proportion of 83.7% of the original sequences (mean per samples = 54,017.48 reads; max = 111,213 reads; min = 272 reads). The data were further filtered removing the samples with less than 1,000 reads as well as the OTUs with less than 10 reads in 5% of samples. This allowed us to retain 2,515 unique OTUs accounting for 4,308,580 high quality reads and 85 samples.

Then, we computed alpha-diversity calculations on a dataset rarefied at 18,467 reads per sample and alpha-diversity was investigated considering two microhabitats (root and rhizosphere) and the seven fertilisers treatments. OTUs richness was assessed by Chao1 index and observed OTUs while the OTUs evenness was assessed by Shannon index. This analysis revealed a significant effect of the microhabitat on the characteristics of the microbiota thriving at the tomato root-soil interface: regardless of the treatment, the root microhabitat emerged as less diverse and even compared to the rhizosphere one (Wilcoxon rank sum, $p < 001$, Figure 6). This observation suggests that root microhabitat represents a gated community compared to the surrounding soil environment. Conversely, the treatment impacted only the number of OTUs observed in the rhizosphere compartment (Kruskal-Wallis non parametric analysis of variance followed by Dunn's post-hoc $p < 0.05$) (Figure 6).

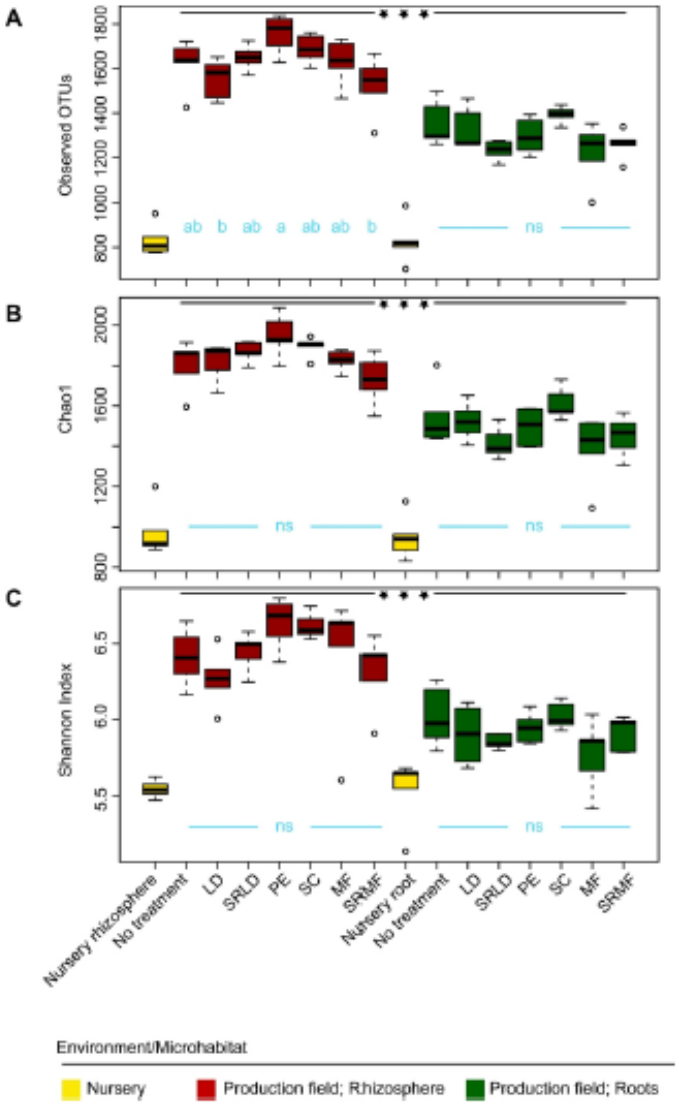

Figure 6. The tomato root microbiota is a gated community. Average (A) number of observed OTUs, (B) Chao 1 index and (C) Shannon index computed on the indicated rhizosphere and root specimens. Abbreviations LD (Liquid Digestate), SRLD (Slow acting Liquid Digestate), PE (Pelleted Digestate); SC (Organo-mineral fertiliser); MF (Mineral Fertiliser); SRMF (Slow acting Mineral Fertiliser). Asterisks denote statistically significant differences between microhabitat by non-parametric Wilcoxon rank sum test ($P < 0.01$). Different blue letters within individual microhabitats denote statistically significant differences between treatment means by Kruskal-Wallis non parametric analysis of variance followed by Dunn's post-hoc test ($P < 0.05$); ns, no significant differences observed.

Congruently, beta-diversity analysis computed on the non-rarefied dataset using both weighted Unifrac and Bray-Curtis indicated a microhabitat-dependent microbiota diversification. In particular, the weighted Unifrac matrix visualised using a Principal Coordinated Analysis revealed such a microhabitat effect on samples processed at harvest time along the axis accounting for the major variation. Interestingly, younger nursery samples

displayed a similar degree of diversification, although their communities were separated from the harvest samples on the axis accounting for the second source of variation (Figure 7).

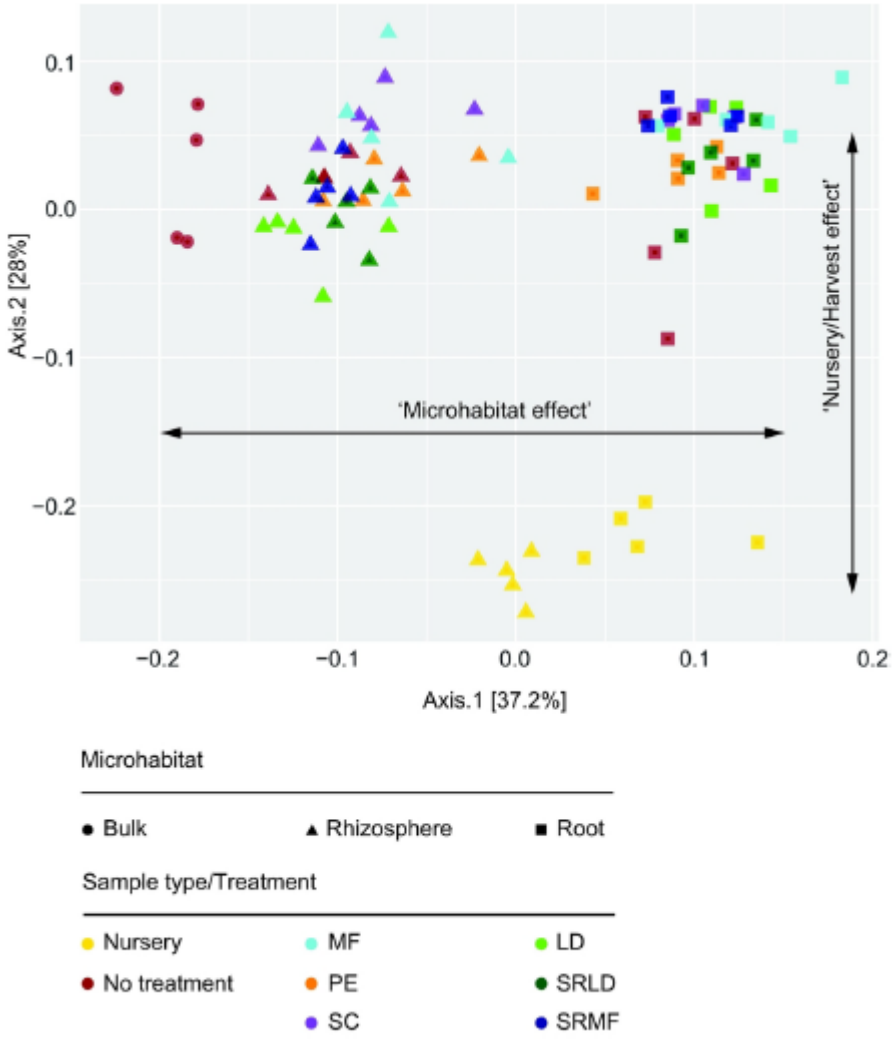

Figure 7. The tomato rhizosphere and root microbiota host compositionally different communities. PCoA calculated using a weighted UniFrac matrix among the indicated microhabitat and treatments.

These data were supported by a permutational analysis of variance which attributed a R^2 of 30% to the microhabitat, a R^2 of 28% to the 'Nursery/Harvest effect' and a R^2 of 2% to their interactions (Adonis test, 5,000 permutations, $p < 0.01$). The analysis conducted on rhizosphere and root samples at harvest stage revealed that, congruently with the observed diversification along the axis accounting for the major variation, the microhabitat remained the major driver of the tomato communities (R^2 47%, Adonis test, 5,000 permutations, $p < 0.01$) while the individual fertiliser treatments impacted these plant-associated microbial assemblages

to a lesser, but significant, extent (R^2 13%, Adonis test, 5,000 permutations, p <0.01). This suggest that, rather than on richness per se, the fertiliser treatment impact on abundances and phylogenetic assignments of members of the tomato microbiota. Remarkably, the Bray-Curtis matrix produced a congruent results, although the temporal effect (i.e., nursery vs. harvest time) explained slightly more variation (~ 29%; Figure 8) than microhabitat diversification manifested along the second axis of variation (~ 26%; Figure 8). Crucially, also in this case the observed diversification was supported by a permutational analysis of variance which attributed a R^2 of 23% to the microhabitat, a R^2 of 29% to the 'Nursery/Harvest effect' and a R^2 of 3% to their interactions (Adonis test, 5,000 permutations, p <0.01).

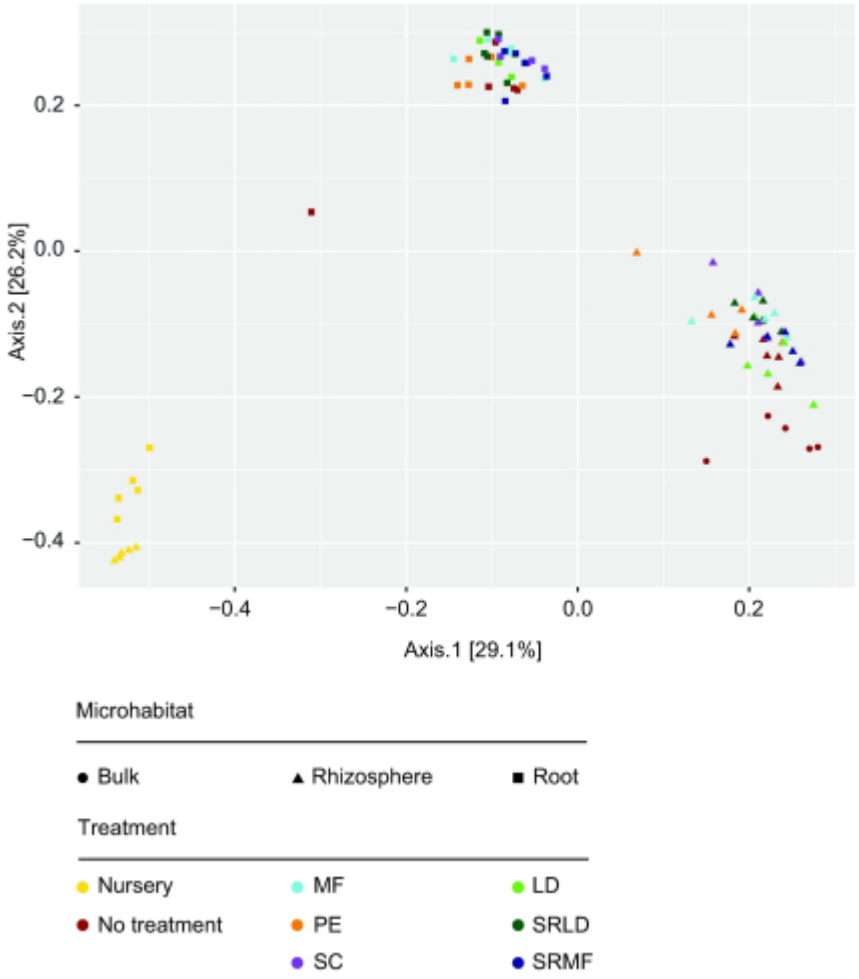

Figure 8. PCoA calculated using a Bray-Curtis matrix calculated on the OTUs clustered at 97% identity among the indicated microhabitat and treatments. Abbreviations: LD (Liquid Digestate), SRLD (Slow acting Liquid Digestate), PE (Pelleted Digestate); SC (Organo-mineral fertiliser); MF (Mineral Fertiliser); SRMF (Slow acting Mineral Fertiliser).

7.3.3 Differential bacterial enrichments define microhabitat and treatment "signatures" on the field grown tomato microbiota

To gain insights into individual members of the tomato microbiota responsible for the observed diversification we implemented a series of pair-wise comparison among microhabitats and treatments at harvest stage. We took a two-pronged approach. First, we identified bacterial underpinning the microhabitat effect i.e., the selective enrichment of bacteria in the roots and the rhizosphere microhabitats amended with no fertiliser. Next, we assessed the effect of the fertiliser treatment on roots and rhizosphere bacterial composition by comparison with bacteria enriched in untreated samples.

This allowed us to identify 170 bacterial OTUs whose abundance was significantly enriched in and differentiated between rhizosphere specimens and unplanted soil samples (Wald test, $p < 0.01$, FDR corrected). Similarly, we identified 374 bacterial OTUs whose abundance was significantly enriched in and differentiated between root specimens and unplanted soil samples (Wald test, $p < 0.01$, FDR corrected). Of these differentially enriched bacteria, 96 OTUs represented a set of tomato-competent OTUs capable of colonising both the rhizosphere and root environments. When we then looked into the taxonomic affiliations of this tomato-competent microbiota, we discovered that the field-grown tomato microbiota is dominated by members of Actinobacteria, Bacteroidetes, Alpha-, Beta-, Gamma- and Deltaproteobacteria as well as Verrucomicrobia (Figure 9). Strikingly, the taxonomic investigation revealed a bias for Actinobacteria in the root compartment, possibly reflecting an adaptive advantage of member of this phylum in colonising the endophytic environment.

Interestingly, each fertiliser treatment had a distinct impact on these tomato-enriched microbiota. The pelleted digestate (PE) and the slow-acting mineral fertiliser (SRMF) yielded the highest number of uniquely enriched OTUs regardless of the microhabitat investigated, albeit with a distinct pattern: the SRMF had a more pronounced effect on the rhizosphere communities while the PE impacted more on the bacteria thriving in association with root tissues. (Wald test, $p < 0.01$, FDR corrected; Figure 10).

Interestingly, when we inspected the taxonomic composition of the bacteria differentially impacted by the fertiliser treatment we observed an increase of the number of OTUs belonging to phylum of Actinobacteria. In particular, PE had 12 OTUs out of 80 and 14

OTUs out of 105, in root and rhizosphere, respectively, belonging to phylum Actinobacteria. While, MF had 15 OTUs out of 38 and 22 OTUs out of 49 in root and rhizosphere, respectively, belonging to phylum Actinobacteria. Within this phylum we observed the presence of OTUs classified as *Streptomyces* spp., *Agromyces* sp., *Microbispora* sp. and *Actinoplanes* spp.

Together these data suggested that the enrichment of specific bacteria underpins the observed microhabitat effect whose magnitude is fine-tuned by the applied fertiliser.

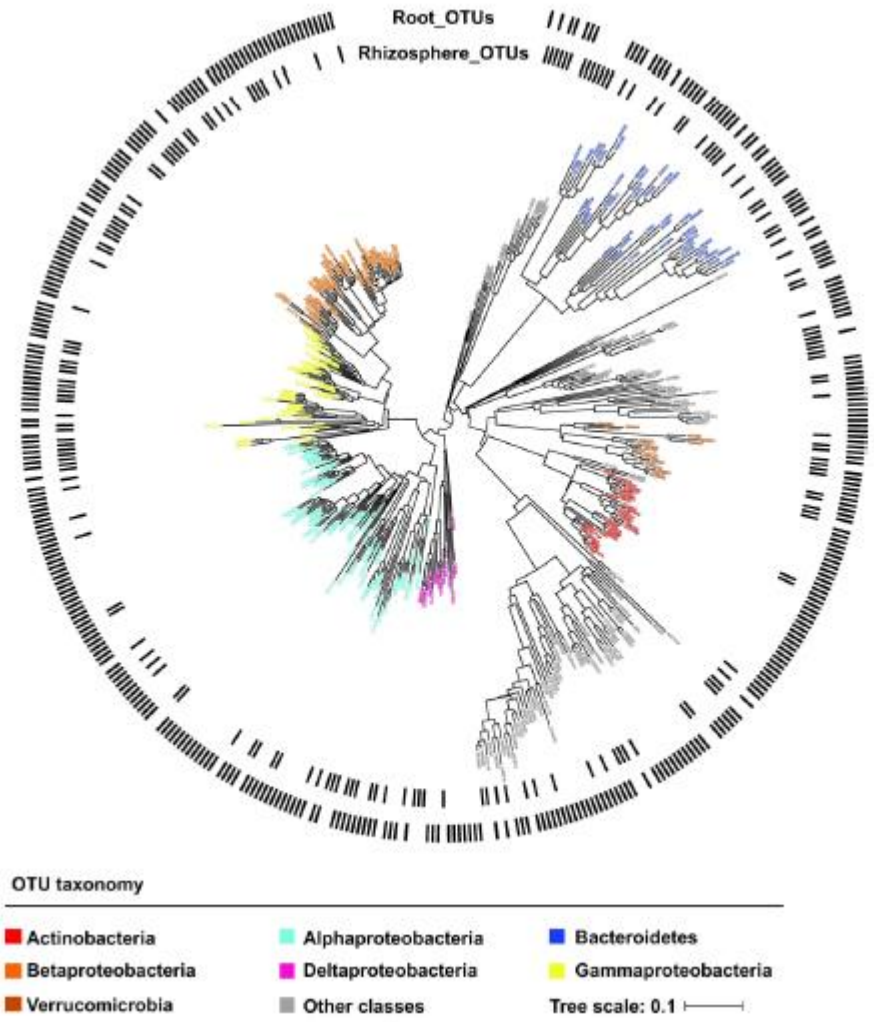

Figure 9. The enrichment of Actinobacteria is a distinctive feature of the tomato root microbiota. Phylogenetic relationships of the OTUs enriched in rhizosphere and root compartment. Individual external nodes represent one of the OTUs enriched in either (or both) rhizosphere or root samples in no treatment conditions (Wald test, P value < 0.01, FDR corrected) whose colour reflects their taxonomic affiliation at Phylum level. A black bar in the outer rings depicts whether that given OTUs was identified in the rhizosphere- or root-enriched

sub-communities, respectively. Phylogenetic tree constructed using OTUs 16S rRNA gene representative sequences.

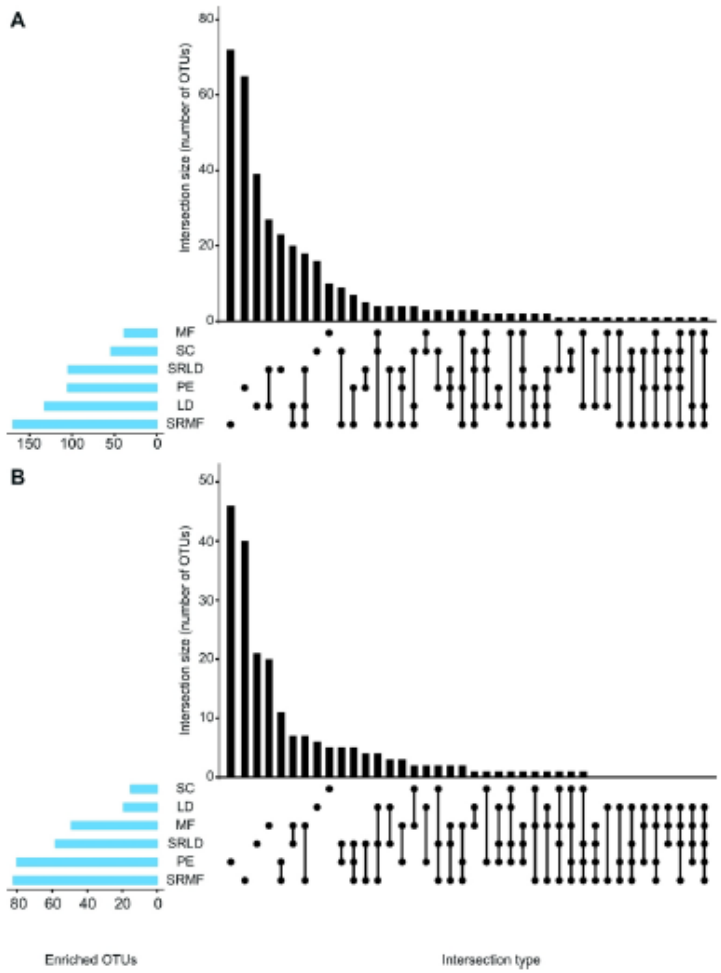

Figure 10. Nitrogen fertiliser modulates bacterial enrichment in the tomato rhizosphere and root compartments. Number of OTUs significantly enriched (Wald test, P value < 0.01, FDR corrected) in the indicated treatment versus untreated controls in (A) rhizosphere and (B) roots. In each panel, blue bars denote the total number of enriched OTUs for a given treatment, the black bars denote the magnitude of the enrichment in either the individual treatment or among two or more treatments highlighted by the interconnected dots underneath the panels. Abbreviations LD (Liquid Digestate), SRLD (Slow acting Liquid Digestate), PE (Pelleted Digestate); SC (Organo-mineral fertiliser); MF (Mineral Fertiliser); SRMF (Slow acting Mineral Fertiliser).

7.4 Discussion

This study revealed that all nitrogen treatments led to an increase of tomato production in comparison with the no fertilization treatment (fold change between 0.8 and 1.73) confirming that, in the tested conditions, nitrogen limits the yield potential of processing tomato crops as observed in previous studies (Ronga et al., 2015; Ronga et al., 2017). Yet, despite the same amount of nitrogen was applied in each treatment (i.e., 150 kg ha^{-1}), all the treatments were statistically different from each other. A prediction of this observation is that, under the tested conditions, the nature of the fertilisers, rather than the amount of nitrogen per se, affect the yield and the fruit quality of tomato plants. These observations and the putative contribution to fertiliser use efficiency of the microbial communities thriving at the root-soil interface (Alegria Terrazas et al., 2016), motivated us to investigate relationships between yield traits and the composition of the tomato rhizosphere and root microbiota under field conditions.

7.4.1 The tomato rhizosphere and root microbiota are gated communities

First, we characterised the rhizosphere and root microbiota of processing tomato with no treatment. Both alpha and beta diversity discriminated between the communities of seedlings and adult plants. Despite these differences, which could be attributed to both abiotic, e.g., time of residence in soil (Dombrowski et al., 2017), and biotic factors, e.g., developmental-conditioned rhizodeposits (Chaparro et al., 2014), it is striking to note how tomato plants displayed a rhizosphere and root compartmentalisation regardless of the developmental stage. This is congruent with the observation that in rice, the assembly and structural diversification of the microbiota is a rapid process which reaches a steady-state level within a few weeks from germination (Edwards et al., 2015). Closer inspection of the rhizosphere and root profiles at harvest stage indicates that these plant-associated communities are phylogenetically related to those of unplanted soil, suggesting that the initial tomato microbiota is further modulated by the growing conditions.

Despite this apparent relatedness, the selective enrichment of individual bacterial members of the microbiota discriminates between rhizosphere and root communities for mature plants from unplanted soil profiles (Figure 9). These enrichment displayed a bias for members of the phyla Actinobacteria, Bacteroidetes, Proteobacteria (including the classes Alpha-, Beta-, Delta- and Gammaproteobacteria) as well as Verrucomicrobia. Members of these taxa have been routinely reported in studies focussing on plant-competent bacteria under both laboratory

and field conditions (Bulgarelli et al., 2013; Walters et al., 2018), suggesting that the experimental approach followed in this study can be considered representative for field-grown processing tomato.

However, we noticed a differential selective pressure on the bacteria thriving either in the rhizosphere or in the root tissue: this latter environment produced more distinct profiles, i.e. more differentially enriched bacteria compared to unplanted soil, than the ones retrieved from the soil surrounding the roots. This indicates that the diversification of the tomato-inhabiting microbial communities from the surrounding soil biota initiates in the rhizosphere and progresses through the root tissue, where it produces a more pronounced microbiota diversification compared to unplanted specimens. This observation is reminiscent of the recruitment patterns of other crops such as barley (Bulgarelli et al., 2015) but it is in striking contrast with studies conducted with both model (Bulgarelli et al., 2012) and field-grown (Rathore et al., 2017) Brassicaceae, whose 'rhizosphere effect' appear negligible.

We further noticed that the "root effect" on the microbiota was exerted also at phylogenetic level with a bias for the enrichment Actinobacteria. This observation is in apparent contrast with results gathered from the recent seed-to-seed characterisation of the tomato microbiota which revealed that, albeit averaging 8% of the sequencing reads across microhabitats, members of this phylum did not significantly discriminate root from rhizosphere specimens (Bergna et al., 2018). However, it is worth mentioning that these two studies differed in terms of both soil type and plant genotype used.

Together, our results suggest that both species- and soil-specific traits govern the assembly of the tomato microbiota in field-grown crops.

7.4.2 Nitrogen source impacts on the structural and functional composition of the tomato microbiota

Next, we investigated the impact of the type of nitrogen fertiliser on the tomato microbiota and we demonstrated that each treatment produced "distinct signatures", represented by specific selective enrichment, on both the rhizosphere and root communities. Despite microhabitat-associated variation, the effect of the application of pelleted digestate (PE) resulted in the most distinct microbial profile in the root compartment and the second largest number of specifically enriched OTUs in the rhizosphere Of note, the slow-acting mineral

fertiliser (SRMF) follow a "complementary" pattern: its application yielded the greatest and the second greatest number of differentially enriched OTUs compared to untreated samples in the rhizosphere and root profiles, respectively. Remarkably, these two treatments had a discernible effect also on crop yield, with the PE treatment producing the best performance among the various fertilisers. Our data are congruent with studies conducted on wheat which observed a structural diversification of the soil and plant-associated communities exposed to either mineral or organic fertilisers (Kavamura et al., 2018). Yet, the numerical shift in terms of OTUs differentially enriched per se cannot explain the potential impact of these communities on crop yield: owing to the fact that the SRMF treatment, which is associated to a significant reduction in yield traits (compared to PE) is capable of triggering a comparable OTU enrichment.

We therefore focused our attention on the taxonomical composition of the rhizosphere and root communities. In particular, we noticed that the proliferation of Actinobacteria in the root compartment was retained in the various treatments. The enriched Actinobacteria included *Streptomyces* spp., *Agromyces* sp., *Microbispora* sp. and *Actinoplanes* spp.. *Streptomyces* spp. are well-known bacteria able to produce a wide diversity of bioactive compounds able to promote plant growth and health (de Jesus Sousa and Olivares, 2016). On the other hand, members of the genus Streptomyces are responsible of economically relevant plant diseases, most notably common scab of potato caused by *S. scabies* (Loria et al., 2006).

Our investigation suggests that the bacterial microbiota of field-grown processing tomato is the product of a selective process that progressively differentiates between rhizosphere and root microhabitats. This process initiates as early as plants are in a nursery stage and it is then more marked when plants reached the harvest stage. This selection a) acts both on the relative abundances and phylogenetic assignments of members of the tomato microbiota, b) is modulated, at least in part, by the nitrogen fertiliser provided which, in turn, c) triggers different microbial metabolic specialisations within tomato roots.

It is important to mention that the nitrogen fertiliser may also represent a microbial inoculant per se, in particular in the case of organic-based amendments. For instance, a comparative study of 29 different full-scale anaerobic digestion installations revealed that Firmicutes, followed by Bacteroidetes and Proteobacteria, dominated the resulting microbial communities (De Vrieze et al., 2015). Considering the plant-associated profiles observed in this study, in particular the enrichment of Actinobacteria in the root communities, it is legitimate to

hypothesize that the input digestate bacteria may act as in inoculum for a part of the tomato microbiota, which is further fine-tuned by the exposure to soil microbes. Future studies, integrating the microbial profiling of the input fertiliser treatment, will be required to accurately elucidate microbial dynamics associated with the application mineral (i.e., germ-fee) and organic fertilisers.

7.5 Conclusions

Our experiments represent an example of how cultivation-independent approaches can be efficiently deployed to investigate the plant microbiota under field conditions. Although this type of investigation is not novel per se in tomato (Toju et al., 2019), our results revealed fundamentally novel insights into plant's adaptation to nitrogen fertilisers and the implication for crop yield. Similar to what has recently been postulated for tomato pathogen protection (Kwak et al., 2018), our results predicts that the use of field-derived, sequencing data will allow scientists to identify "signatures" of the plant microbiota that can be targeted to enhance plant performance. This approach, which we define as lab-in-the-field, will be key towards the rationalisation of nitrogen (and other treatments) application in agriculture and we anticipate will pave the way for the effective exploitation of the plant microbiota for agricultural purposes (Schlaeppi and Bulgarelli, 2015; Toju et al., 2018).

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Chapter 8

Conclusions and perspectives

In the research carried out during this PhD project, we focused our attention on processing tomato (*Solanum lycopersicum* L.), that represents a worldwide model for the horticultural crop species. Five main experiments were set-up in order to study the effects of beneficial soil microorganisms (plant growth promoting rhizobacteria – PGPR and arbuscular mycorrhizal fungi-AMF) and the grafting/rootstocks on: i) processing tomato tolerance to abiotic stresses; ii) yield and fruit quality in organic cropping systems. In addition, the influence of different types of nitrogen on processing tomato microbiota were studied in order to improve nitrogen application in agriculture and to foresee an effective exploitation of the plant microbiota for agricultural purposes.

As shown in the Chapters 3 and 4, our results suggested that *Funneliformis mosseae* seemed to be the most effective in reducing the damage caused by chilling stress and in the mitigation of drought effects on morphological and physiological traits. In addition, processing tomato responses are dependent on specific interactions among genotype, beneficial microorganisms and abiotic stress. This confirmed the hypothesis that the development of *ad hoc* formulates, based on AMF consortia, taking into account the environmental conditions and the plant-AMF interactions, is of crucial importance, to obtain the best outcomes in terms of plant resilience/tolerance to adverse conditions.

As demonstrated in Chapter 6, among the assessed microorganisms, *Paraburkholderia graminis* C4D1M, *Azospirillum brasiliensis* sp. 245 and their consortium were the most effective on processing tomato yield, growth and quality in organic open field. In addition, our findings revealed as *A. brasiliensis* sp. 245 could be used to achieve an early flowering, putatively reducing the growing season (i.e. increasing the adaptability) of processing tomato. However, as a future prospective, some technical aspects - like the microorganism inoculation (timing and number of applications) and the formulation (concentration, co-formulants, adjuvants and consortium of the microorganisms) - should be improved to make effectiveness the treatment also in open-field conditions, where environmental factors and microorganisms, already present in the rhizosphere, might influence the activity of the inoculated microorganisms. For these reasons, samples of roots and leaves of the studied seedlings and plants were sampled in liquid nitrogen and stored in -80°C for future studies (e.g. to identify genes activated by *A. brasiliensis* sp. 245 and to understand the mechanisms of action for the improvement of their effectiveness).

Although grafting is a widespread agronomic practice in horticulture, it is not usually used for herbaceous crop as processing tomato due to its high cost of production *per* seedling and to the relatively low final price of processing tomato fruits. Nevertheless, our results demonstrated (Chapter 5 and 6) that grafting might be an interesting opportunity to improve fruit yield and quality of processing tomatoes when grown in organic cropping system. However, the effectiveness of the cherry ('Tomito') rootstock should be better investigated in open-field trials.

Finally, when we investigated the impact of different nitrogen fertilizers on the tomato microbiota (Chapter 7), we demonstrated that each investigated treatment displayed "distinct signatures", represented by specific microbiota composition, on both the rhizosphere and root communities of processing tomato. In addition, this approach could be a key towards the rationalization of nitrogen application in agriculture and will pave the way for the effective exploitation of the processing tomato microbiota for agricultural purposes. In fact, as a future prospective, the enriched identified bacteria should be isolated and studied in order to evaluate their effectiveness as artificial inoculations.

In conclusion, all the acquired knowledge could be transferred to nursery growers and seed/fertilizer companies in order to produce either "strengthened" processing tomato seedlings grafted and inoculated with microorganisms or *ad hoc* fertilizers enriched with microorganisms.

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Publications

Manuscript published on international peer-review journal

1) Ronga D., Caradonia F., Setti L., Hagassou D., Giaretta Azevedo C.V., Milc J., Pedrazzi S., Allesina G., Arru L., Francia E., 2019. Effects of innovative biofertilizers on yield of processing tomato cultivated in organic cropping systems in Northern Italy. Acta Horticulturae, 1233, 129-136. 10.17660/ActaHortic.2019.1233.19.

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