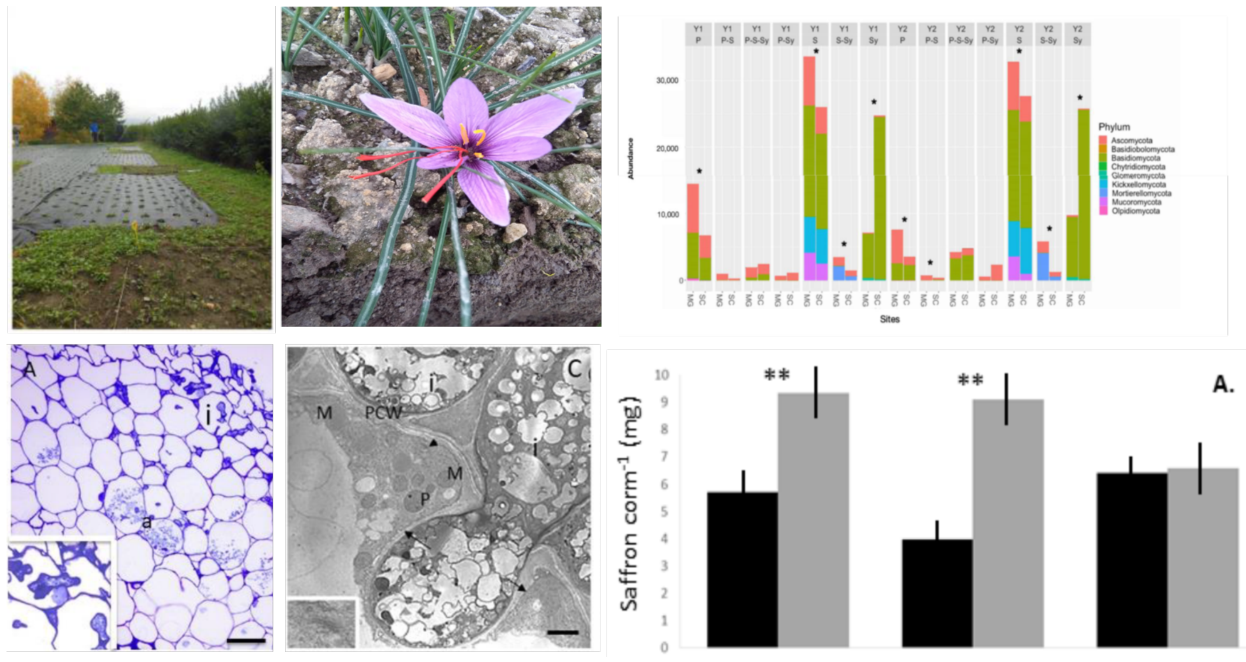
UNIVERSITY OF TURIN

DEPARTMENT OF LIFE SCIENCES AND SYSTEMS BIOLOGY

Doctoral School in Nature Science and Innovative Technology



Doctorate in Biology and Applied Biotechnology - XXXII Cycle

Saffron (*Crocus sativus* L.) cultivation: role of microbial communities on plant growth and biomolecules production

Íris Marisa Maxaieie Victorino

Doctorate Coordinator: Prof Silvia Perotto

Academic year: 2016-2022

Tutors: Dr Valeria Bianciotto

Dr Erica Lumini

2022

From change comes growth...!

Jon Stewart

I hereby declare that the information below is true, complete and correct to the best of my knowledge and belief.

____

Íris Marisa Maxaieie Victorino

Index

Preface 7

Chapter I 9

General introduction 9

1.1. Medicinal and aromatic plants, the new "gold" resource..10

1.2. The saffron 10

- Historical background 10
- Botany and Morphology 11
- Principal bioactive molecules..12
- Uses 13
- Economic value and major cultivation constraints..14

1.3. Natural biofertilizers, a sustainable alternative for horticulture..14

1.4. AMF symbiosis 16

- Fungi kingdom and phylogeny..16
- Applicability in horticulture 18
- Characterization of soil fungal community 19

1.5. MiSeq technology to decipher soil microbial community..20

1.6. Aim 21

1.7. References 23

Chapter II 32

Saffron crop performance and metabolic profile modulation by arbuscular mycorrhizal fungi in soilless cultivation..32

2.1. Preamble 33

2.2. Arbuscular Mycorrhizal Fungi Modulate the Crop Performance and Metabolic Profile of Saffron in Soilless Cultivation 35

- Abstract 35
- Introduction 35
- Materials and Methods 37
  - Plant Material and soilless cultivation..37
  - Determination of Flower Production, Stigma Yield and Corm Growth 37
  - Preparation of the Saffron Extract..38
  - Determination of Saffron Quality by ISO 3632..38
  - Determination of Bioactive Compounds by HPLC..38
  - Phytochemical Characterization 39
  - AMF Evaluation 39
  - Chemicals and Reagents 40
  - Statistical Analysis 40
- Results and Discussion 40

Crop Performance, Quality and Secondary Metabolite Content of Saffron in Soilless Cultivation 40
AMF Colonization 41
Impact of AMF on Saffron in Soilless Cultivation....................44
Crop Performance and Quality Classification....................44
Saffron Metabolic Profiling Comparing to Other Foods....................46
Soilless Cultivation vs. Open Field....................48
♦ Conclusion 49
♦ Acknowledgments 50
♦ References 50

Chapter III **55**

Arbuscular mycorrhizal fungi as natural biofertilizers for saffron on the Marginal Alpine soils: role and potential....................**55**
3.1. Preamble **55**
3.2. Saffron Cultivation in Marginal Alpine Environments: How AMF Inoculation Modulates Yield and Bioactive Compounds **56**
♦ Abstract 58
♦ Introduction 59
♦ Materials and Methods....................60
AMF Inoculation in Pot 60
AMF Inoculation in Open Field....................61
AMF Evaluation 61
Plant Performance and Saffron Yield in Open Field....................61
Saffron Extract Preparation and Quality....................61
Total Phenols 62
Total Anthocyanins 62
Antioxidant Activity 62
Identification and Quantification of Bioactive Compounds....................62
Chemicals and Reagents 63
Statistical Analysis 63
♦ Results 63
Assessment of Saffron Mycorrhization at Pot and Open Field Scale....................63
Impact of AMF Symbiosis on Saffron Productivity and Qualitative Traits in Open Field....................64
♦ Discussion 65
AMF Colonization 65
AMF Modulate Crop Performance and Spice Quality....................66
♦ Conclusions 67
♦ Supplementary information 67
♦ Acknowledgments 68
♦ References 68

Chapter IV **74**

MiSeq Illumina analysis as an additional tool in the labyrinth of studying Italian Alps microbiome......74
4.1. Preamble 75
4.2. Metabarcoding of soil fungal communities associated with alpine field-grown saffron (*Crocus sativus* L.) inoculated with AM fungi 77
♦ Abstract 77
♦ Introduction 78
♦ Materials and Methods 79
Sampling sites 79
Site environmental conditions 80
Soil DNA extraction, PCR amplification and sequencing......81
Bioinformatic and statistical analyses on soil fungal communities......81
♦ Results 82
Composition and structure of fungal soil communities......82
Soil fungal microbiota assembly and AMF inocula impact......84
♦ Discussion 85
♦ Conclusions 87
♦ References 88

Chapter V 96

High-throughput sequencing platform on mycobiome diversity study and fungi identification 96
5.1. Preamble 97
5.2. High throughput sequence-based analysis of Arbuscular Mycorrhizal Fungal communities......99
♦ Abstract 99
♦ Introduction 99
♦ Materials 101
Plant 101
Plant sampling and processing......101
Plant root genomic DNA extraction......102
Soil 102
Soil sampling and processing......102
Soil core genomic DNA extraction: NucleoSpin ⟶ soil kit (Macherey-Nagel) (See Note 1) 102
Soil core genomic DNA extraction: DNeasy PowerSoil ⟶ kit (Qiagen) (See Note 1......102
DNA quantification 103
Gel electrophoresis 103
Nanodrop (ThermoFisher) 103
Qubit™ (Invitrogen) 103
PCR amplification 103
PCR products purification 103
♦ Methods 103

Processing Plant material 104

DNeasy⟶ Plant Mini DNA extraction kit (Qiagen) protocol 105

Processing Soil core samples 106

NucleoSpin⟶ soil DNA extraction kit (Macherey-Nagel) protocol 107

DNeasy PowerSoil⟶ kit soil DNA extraction kit (Qiagen) protocol 108

DNA quantification 109

Gel Electrophoresis 109

Nanodrop quantification....109

Qubit™ (Invitrogen) quantification....109

SSU rDNA amplification 110

First PCR 110

Second (nested) PCR....110

ITS rDNA amplification 111

First PCR 111

Hemi-nested PCR or Direct PCR....112

PCR products purification (Promega) 112

Sequencing 113

Bioinformatics pipeline for analyzing Illumina MiSeq Reads....113

- ♦ Notes 114
- ♦ Acknowledgements 115
- ♦ References 115

Chapter VI 117

Final remarks and Conclusions 118

6.1. Final remarks and Conclusions....118

6.2. References 122

6.3. Supplementary material....124

6.4. Acknowledgements 135

Preface

It was in 2016 when I "sailed" to Italy to pursue my dream of undergoing on a PhD studies. I was so mesmerized by the idea of finally doing what I called "my last degree" and only could think of 'soil biodiversity', 'crop improvement' and 'next generation sequencing'…. terms that centered the research I thought I was going to do. I was a brand new PhD student in Biology and Applied Biotechnologies and I did not yet know those words would have been just a tiny tip of the avalanche about to decay upon almost all my PhD career. In this way a challenging adventure was beginning. I joined the Plant Protection Institute (IPP-CNR) and the Department of Life Sciences and Systems Biology in Turin by the hands of my great tutors Valeria Bianciotto and Erica Lumini, who so tenderly dared to go on board with me. They taught me so much, not only academically speaking but also as human beings, women professionals and mothers. I have made also great ex PhD dear friends Enrico, Veronica, Valentina, ex PhD students as Ludovica, Matteo, Salvo, Alessandro, Jacopo, Lorenzo, Valeria, Martino, Francesco, Gennaro (who shared with me similar passion for food rsrsrsrs) and so many more. I have worked with Andrea Berruti, who patiently transmitted me some of his vast bioinformatic analysis knowledge.

I started by being introduced to alpinean marginal saffron cultivation areas of Valle d'Aosta that sooner became the central thread of my entire thesis and where the Italian research project 'SaffronALP - Lo zafferano di montagna: tecniche sostenibili per una produzione di qualità' - Fondazione Cassa di Risparmio di Torino (RF= 2017.1966) took place. I was introduced to plant physiology team from the Department of Agricultural, Forest and Food Sciences (DISAFA) of UNITO, Valentina Scariot, Matteo Caser, Sonia Demasi along with Dario Donno who gave me the opportunity to navigate for the first time into the high performance liquid chromatography analysis of saffron secondary metabolites. Outcomes from this work are presented in chapter II and III.

Four years later, COVID 19 in the middle with all its limitations, it is now time to draw conclusions from what I actually did which I will be doing by putting together all my results in this thesis. Thanks to Prof Mauro Colombo and Paola Bonfante I was able to meet all these amazing people from which I will be eternally grateful. I was able to perform morphological and molecular analysis by following a detailed fluxogram (and sometimes add some of my own to the pre-existed ones) aiming to have the best material for Illumina MiSeq technology for our fungal biodiversity surveys. This meant many sequences to analyze and many troubles as well which I was able to detangle with the help of Andrea Berruti and Samuele Voyron. The first memories I have were the endless sessions face-to-face with Erica and Valeria in order to understand how to manage new data (horticultural crops, saffron metabolic profiles, ITS primers, next generation

sequencing) which day by day, gave space to the skills that grew up and I was finally able to describe both the arbuscular mycorrhizal and overall fungal diversity present in the alpine soils and inoculation aspects that could interfere with crop performance. Chapters IV and V tell this story. Thanks to the gained knowledge, I also started to analyze soil sequences, coming from a project with Mozambique. To do that I went to Maputo and Nampula (Mozambique) - Department of Biological Science of Eduardo Mondlane University to perform soil sampling and PCR that further contributed to a study that was orally presented in Istanbul, at an international conference. Last but not least, the PhD gave me the great opportunity to attend many international conferences in Italy and to meet many experienced scientists that brought up many additions to my career.

Chapter I

General introduction

1.1. Medicinal and aromatic plants, the new “gold” resource

From ancient times until now, Medicinal and Aromatic Plants (MAPs) are known to be used for several purposes such as medicine, nutrition, cosmetics, coloring and aromatic in herbal teas, food supplements, liquors, insecticides, fungicides, essential oils, perfumes and cleaning products. In recent decades the demand for MAPs has grown causing over-exploitation of wild plant population, the main source of raw material. Medicinal and Aromatic Plants (MAPs) includes a group of plants used in medicine - traditional or conventional, and/or those containing essential oils (Bhattarai and Karki, 2004; Palos, et al., 2005) which are a natural mixture of volatile secondary metabolites responsible for the characteristic aromatic flavor and biological properties present in this type of plants (Burt, 2004; Barbara et al., 2004; Vagionas, et al., 2007). MAPs cultivation not only allows effective land use of wastelands and forests but also employs many rural families from sowing to marketing, representing an income diversification option for farmers. According to WHO (2008) reports, by 2050 trade of medicinal plants will be up to US$ 5 trillion (Allkin, 2017; Maiko 2019), estimating that new modern medicines and 60% of antitumor drugs will be produced from it.

1.2. The saffron

- Historical background

Saffron (*Crocus sativus* L.) origin reports are controversial, showing its first appearance in Kashmir 550 AD followed by Persia from where it was then spread to the East and Mediterranean basin (Zohary and Hoof, 1994). A second theory of its origin postulates that after Kashmir has been conquered by the Persians, Persian saffron corms were planted in Kashmir soils by Persian rulers to fulfill their newly built gardens and parks and later on spread to other continents. As a spice saffron has been cultivated for at least 3500 years in Egypt and Middle East (Kumar, 2008), starting from the wild species *Crocus sativus* subsp. *cartwrightianus* Herb. K.Richt., that supposedly hybridized with *Crocus thomasii* Ten. or *Crocus sativus* subsp. *pallasii* Maw K.Richt. originating the sterile triploid *Crocus sativus* (3n =24) on the late Bronze Age (Kumar, 2008). According to the same author *Crocus cartwrightianus* (2n=16) was spread in meridional and insular Greece while *Crocus pallasii* (2n = 12, 14, 16) was present in the oriental south part of Europe. *Crocus hadriaticus* Herbert (2n = 16) was present in Greece while the *Crocus thomasii* (2n = 16) existed in the south of Europe and Italy. In Great Britain and Spain saffron was introduced by the Romans and the Arabs respectively (The Royal Horticultural Society, 2006), often used as a commercial trade commodity (Landi, 2007). The word saffron or simply zafran derived from the Arabic word *asfar* meaning “yellow” (The Royal Horticultural

Society, 2006) while the word C*rocus* derived from the Greek word *Corycus*, a region in Cilicia in the Eastern Mediterranean (Kumar, 2008).

- Botany and Morphology

From Iridaceous family the *Crocus sativus* commonly known as saffron belongs to the *Crocus* genera and is a bulb-originated perennial stemless herb (Figure 1A) that can reach up to 30 cm in height (Mzabri 2019) with roots (Figure 1B) that can be fibrous and thin, when coming from the base of the mother bulb or contractile, when coming from the base of the lateral buds (Kalesi et al, 2004). The leaves (Figure 1C), 5 to 11 true leaves are very narrow and dark green measuring up to 60 cm in length and in October, the plant blooms striped purple flowers with a honey-like smell (Koocheki et al., 2006). The flowers (Figure 1D) are protected by whitish membranous bracts and the pistil is composed of an inferior ovary from which a thin style, 9 to 10 cm long, arises. The style ends with a single stigma composed of three filaments of intense red color which are the part from where the saffron powder is extracted (Molina et al., 2003, Mzabri 2017).

A

B

C

Figure 1. *Crocus sativus* plant morphology: (A) saffron plants; (B) types of roots in saffron; (C) saffron flower (star) and leaves (arrows).

Saffron is a perennial plant, whose activity slows down in spring in contrast to most flowering plants and as a sterile triploid it propagates by underground vegetative organs known as corms (Nehvi and Yasmin, 2013). Characterized by a biannual life cycle *C. sativus* L., possess three distinct stages: dormant (July-Aug), flowering (Oct-Nov) and vegetative (Jan-May), with sowing of the corms occurring between July-August and depending upon their weight, if more than 8g, flowering may happen between October-November in same year or may go through the annual cycle to attain the minimum required weight to support flowering (Figure 2). Flowering stage is followed by emergence of grass like leaves in vegetative stage wherein daughter

corms/cormlets are born and vegetative phase finally leads to the next dormant growth phase (Nehvi and Yasmin, 2013).

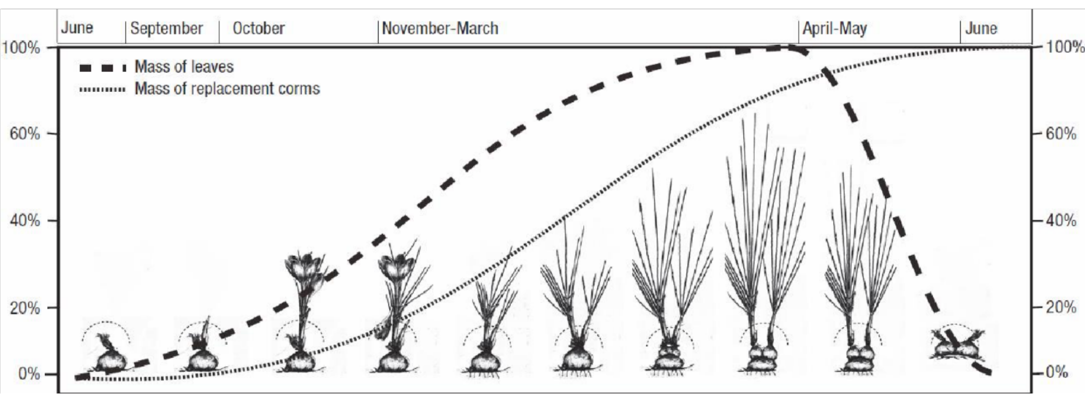


Figure 2. *Crocus sativus* annual life cycle (Modified from Corcoles et al., 2015).

- Principal bioactive molecules

According to Winterhalter and Straubinger (2000) *C. sativus* stigmas chemical analysis has shown to possess more than 150 volatile and non-volatile compounds, but till date only around 50 constituents has been identified being the most important constituents the dye material – crocin, the pharmacologically active, bitter metabolite – picrocrocin and the volatile agent and aroma donor – safranal (Kianbakht, 2009).

Crocin ($C_{44}H_{64}O_{24}$) is a rare water-soluble carotenoid widely applied as a dye, in foods and medicines, due to its high solubility, while picrocrocin ($C_{16}H_{26}O_7$) and safranal ($C_{10}H_{14}O$) are the main factor influencing the bitter taste of saffron and the responsible for the aroma respectively (Moghaddasi, 2010) – Figure 3.

Figure 3. Saffron main biologically active compounds modified from Mzabri et al., 2019.

Safranal however has little or no presence in fresh stigmas, its concentration depends on the conditions applied to saffron drying or conservation process (Ait Oubahou et al., 2009). The concentration of these three constituents combine to determine the saffron spice quality, as defined by the International Organization for Standardization - ISO 3632 updated every three

years (ISO, 2011; Caser et al., 2019a). Additionally, to crocin and picrocrocin non-volatile (zeaxanthin, lycopene, and various α- and β-carotenes) and volatile (mainly terpenes, terpene alcohols, and their esters) compounds can be found in saffron (Liakopulou and Kyriakides, 2002) along with anthocyanins, flavonoids (such as kaempferol), vitamins, amino acids, proteins, starch, mineral matter and gums (Winterhalter and Straubinger, 2000).

- Uses

Historically saffron has been largely used not only in the kitchen as a spice but also as dye, parfum, phytotherapeutic and commodity for money exchange for Greeks and Romans. Saffron has been used in paintings and textiles by Buddhist monks and due to its biodegradability and compatibility with the environment, lower toxicity and less allergenic properties it is still used as a dye for clothes, oriental carpets, food (butter, pasta, cheeses, and oleomargarine) and cosmetics (Abrishami, 1987; Li et al., 2002; Giaccio, 2004; Zeka et al., 2015, Ahrazem et al., 2018). Today, we find this woody, sweet note in the composition of different perfumes both feminine and masculine, with an original and exotic potential. Saffron, as well as other spices, has always enjoyed a reputation as an aphrodisiac in different Egyptian, Greek, Roman and other civilizations (Akhondzadeh et al., 2008; Dwyer et al., 2011; Akhondzadeh et al., 2013). Saffron was used absorbed in infusion or even in the cutaneous application, mixed with fat or macerated in donkey milk, for youthful properties by Cleopatra (Mzabri 2019), to improve the complexion in traditional Iranian medicine, to refresh the skin of the face by traditional Greek medicine, to treat acne, and more recent has been found to reduce erythema (Dwyer et al., 2011). Few studies have shown that saffron lotion may be a better sunscreen than homosalate (an organic compound used in sunscreens) acting as a natural UV absorbing agent (Tabrizi et al., 2003; Golmohammadzadeh et al., 2010). In cosmetics, saffron has been used with caution due to its high cost when compared to other ingredients (Li et al., 2004). Saffron is rich in antioxidants (Hosseinzadeh et al., 2009) which showed to act in neurodegenerative pathologies such as Alzheimer's disease (Bathaie et al., 2013) and in inhibit the expression of markers of inflammation such as tumor necrosis factor (TNF) and interleukin, acting as anti-cancer agent (Mzabri, 2019). Extracts and tinctures of saffron have been used to treat fever, wounds, lower back pain, abscesses, and gingivitis as well as pain related to the eruption of the first teeth in infants (Premkumar et al., 2003). Aqueous and alcoholic extracts of stigmas and saffron petals have an antinociceptive and anti-inflammatory activity for both acute and chronic pain (Eghdami et al., 2013). Due to the presence of crocetin, saffron indirectly helps to lower cholesterol levels in the blood and thus the severity of atherosclerosis, reducing the risk of a heart attack (Hosseinzadeh and Younesi, 2002; Mzabri 2019). According to Mzabri (2019) the use of saffron

as an antidepressant has a long tradition, ranging from antiquity to modern times. At last but not least, saffron spice is widely used in culinary famous dishes such as Moroccan koftas, Iranian kabab, Indian biryani, Spanish paella, French bouillabaisse, Italian risotto and saffron cake (Mzabri, 2019).

- Economic value and major cultivation constraints

For a long time, saffron cultivation was neglected by researchers and farmers since it was considered a minor crop (Gresta et al., 2008). Only in the last few years' interest in using it as an alternative crop for the diversification of agricultural production and as an important new source of income has increased. *Crocus sativus* (Saffron) is economically important, as it is world's highest priced (~4500 US$ per Kg) medicinal and aromatic plant and, according to ISO 3632 (2011) can be ranked in three major categories of quality (I, II, III) depending on the concentration of the three main metabolites (crocin, picrocrocin and safranal). According to Manzo and colleagues (2015) saffron flower is the most important organ as it is from where the spice is obtained of, however the high number of by-products – leaves and corms, that are usually wasted (Kianmehr, 1981) are now rendering more desirable as recent studies showed their antioxidant effect (Hosseinzadeh and Younesi, 2002; Kanakis et al., 2007; Chen et al., 2008; Mzabri, 2019). For many farms, economic diversification has become the keystone for obtaining an adequate income (Grande, 2011), a fact that is particularly evident in mountain areas.
Saffron culture is accessible, easy to grow and its cultivation is done by families mainly women (The World Bank, 2017). The world estimated saffron production is around 205 million tons per year, being cultivated in Iran, India, Greece, Morocco, Spain, Italy, Turkey, France, Switzerland, Israel, Pakistan, Azerbaijan, China, Egypt, Japan, Afghanistan and Iraq (Rafiq et al., 2016) however its productivity can be seriously threat by biotic stresses as *Macrophomina phaseolina* Tassi goid, *Fusarium oxysporum* Schltdl and *Penicillium verrucosum* Dierckx (Kumar et al., 2008). *Crocus sativus* is a highly labor-intensive crop, especially in flower harvesting and stigma separation. It is traditionally cultivated in small and flat plots in which mechanization is not economically sustainable (Dhar and Mir, 1997) demanding long hand labor hours in order to obtain 1 kg of dried saffron (Koul, 1999).

1.3. Natural biofertilizers, a sustainable alternative for agriculture

Worldwide use of chemical fertilizers in agriculture practices has extensively increased in the last few years, and, despite all efforts done by public awareness in informing farmers of chemical fertilizers harmful effect - deterioration of human health, disruption of ecosystem functioning and degradation of the environment (Pelosi et al. 2013; Bhandari 2014), it's use is predicted to

increase significantly in the future. Predictions of population growth expect numbers to double by 2050 pressuring global agriculture on food demand; however, any increase in the dosage of agrochemicals should be avoided in order to safeguard environment health to future generations. The soil as a complex habitat for many microorganisms is a key component in the soil–plant–microbe interactions and it determines further ahead a successful plant growth and development. Some microorganisms "capable of improving soil fertility and optimize plant growth" commonly referred as *biofertilizers* have gained notoriety on sustainable agriculture and environmental management thanks to remarkable results shown in agroecosystems (Reddy et al. 2014) being for that, also, extensively exploited by agricultural biotechnology. A detailed understanding of their various functions and applications however is still to be revealed.

The plant growth promoting microorganisms (PGPM) are soil and rhizosphere-inhabiting microorganisms that can colonize plant roots and influence plant growth in a positive manner (Antoun and Prevost 2005; Spaepen et al. 2009). According to du Jardin (2015), PGPM can be divided into two main groups: plant growth-promoting rhizobacteria (PGPR), defined in 1978 by Kloepper and Schroth to describe plant growth enhancer soil bacteria capable of colonize plants roots in a mutualistic manner and more recently found, plant growth-promoting fungi (PGPF). As mentioned above the PGPRs are mutualistic endosymbionts of the type *Rhizobium* (Bhattacharyya and Jha, 2012; Gaiero et al., 2013; Berg et al., 2014) considered multifunctional (Philippot et al., 2013; Vacheron et al., 2013) and capable of influence all aspects of plant life - nutrition and growth, morphogenesis and development, response to biotic and abiotic stress, interactions with other organisms in the agroecosystems (Ahmad et al., 2008; Babalola, 2010; Berendsen et al., 2012). Its agricultural uses although is constrained by its own complexity, by the variable responses of the plant cultivars and the receiving environments and by the technical difficulties associated with the formulation of the inoculants, which gives inconsistent results in practice (Arora et al., 2011; Brahmaprakash and Sahu, 2012). Despite this, the world market of PGPR inoculants is growing and they are now referred to as "plant probiotics", i.e. efficient contributors to plant nutrition and immunity (Berendsen et al., 2012).

Plants and fungi have co-evolved since the origin of terrestrial plants and the heterogeneous taxa of mycorrhizal fungi which establish symbioses with over 90 % of all plant species (Bonfante and Genre, 2010) have been deeply investigated as potential biofertilizers. Redundantly is to mention their applicability, the AMF a widespread type of endomycorrhiza associate with crop and horticultural plants, where fungal hyphae of Glomeromycotina species penetrate root cortical cells and form branched structures called arbuscules (Bonfante and Genre, 2010; Behie and Bidochka, 2014). Even though mycorrhizal association can be found in almost all ecosystems with the majority of plant species in agroecosystems and urban green areas, their presence is

not always a certainty, mainly because of human activities. There is an increasing interest for the use of mycorrhizal fungi to promote sustainable agriculture, considering the wide range of benefits that the symbioses bring (Gianinazzi et al., 2010; Berruti et al. 2016; Rouphael and Colla, 2020) and, more recently, AMF tripartite association with plants and rhizobacteria became relevant in practical field situations (Toscano et al., 2019).

Conventional agriculture practices as deep and frequent tillage and high pesticides and inorganic fertilization input had brought changes into soil community structure reducing AMF species diversity (Berruti et al., 2014 and 2016; Agnolucci et al., 2019), but, on the other hand, the transition to organic farming has caused a positive response in AMF activity and diversity, reinforcing the idea that direct inoculation and appropriate management of AMF species pool into target soil, can be a promising sustainable alternative to chemical input (Manoharan et al. 2017; van der Heijden et al. 2008).

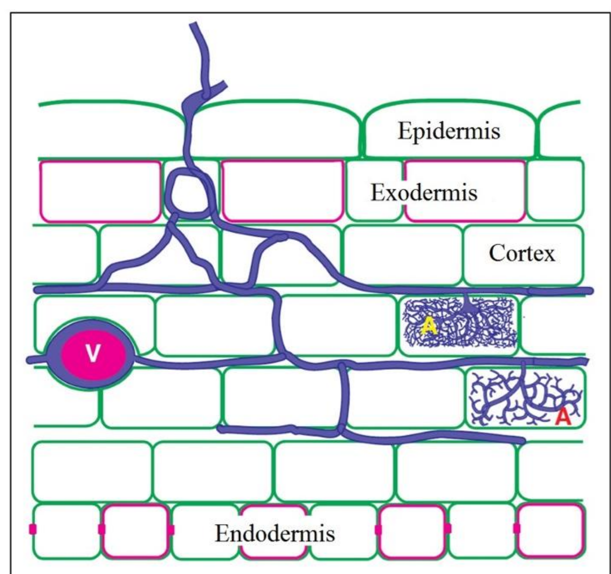
1.4. AMF symbiosis

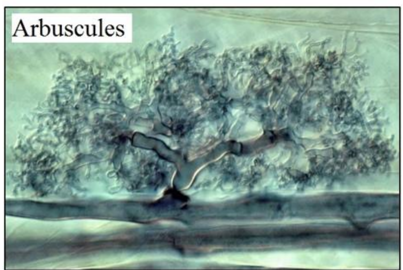
- ♦ Fungi kingdom and phylogeny

Plants do not exist as single entities rather found forming a complex community with microbes and other organisms. Mycorrhizal symbiosis represents one of the major associations within the plant-fungal interactions being known to exist for more than 600 million years ago (Redecker et al. 2000, Strullu-Derrien et al., 2021). It is formed by the widespread obligate symbionts arbuscular mycorrhizal fungi (AMF) which colonize roots of more than 200,000 plant species, including numerous important horticultural crops among the Solanaceae (e.g. tomato, eggplant or petunia), the Alliaceae (e.g. onion, garlic and leek), fruit trees (e.g. grapevine, citrus sp.), ornamentals and herbal plants (e.g. crocus, basil, thyme, rosemary) (Azcón-Aguilar and Barea, 1997, Bianciotto et al., 2018).

AMF belongs to Glomeromycotina (Schüßler et al., 2001; Spatafora et al. 2016; Bruns et al. 2018) with a life cycle divided into an asymbiotic phase occurring in the soil, where spores are asexually formed and a pre-symbiotic phase where ramification of the primary germ tube occurs (Brundrett and Tedersoo. 2018). Once in physical contact with the root surface, the fungus builds up hyphopodia (appressoria) on the root surface and on the plant side, epidermal cells undergo a particular mycorrhiza-specific process forming the so-called pre-penetration apparatus, a transient intracellular structure which is used by the fungus to enter the root (Genre et al., 2005). Then fungal hyphae colonize roots cortical cells - coiled hyphae (Gianinazzi-Pearson and Gianinazzi, 1988) and builds up highly branched hyphal tree-like structures in the apoplast of the plant cells – arbuscules (Figure 4) or form lipid-rich vesicles as storage organs (Walker, 1995). Parallel to root colonization, the fungus can explore the surrounding soil with its

hyphae, interact with other microorganisms and colonize roots of neighboring plants and it is within this connected web that AM fungi hyphae and plant roots exchange nutrients or signals (Ganugi et al., 2019). Lastly, new spores are formed at the extra-radicular mycelium and the life cycle is closed (Ganugi et al., 2019).





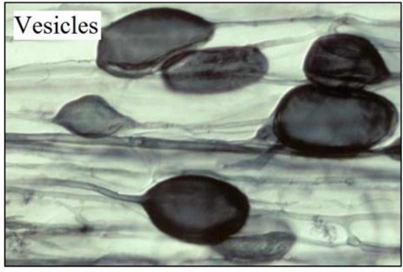


Figure 4. Typical intracellular structures (A – arbuscules and V – vesicle) of arbuscular mycorrhiza produced by *Glomus* species (left). A mature arbuscule (right up) and vesicles (right down) of *Glomus.* Modified from Piliarová et.al. (2019).

Currently, the Glomeromycotina comprise approximately more than 200 known species Błaszkowski et al., 2015) but molecular diversity studies, however, have suggested the existence of 348 to 1,600 Glomeromycotina species (Ohsowski et al., 2014; Crossay et al., 2017). The reasons for the existence of so many possible unknown taxa are: first, they may represent isolates that are difficult to grow (Ohsowski et al., 2014). Second, according to the same author, they may not have been discovered due to a lack of AMF sampling in many earth terrestrial regions and third, only a small number of experts currently address morphological and molecular characterization of this group of fungi. The mutualistic relationship established by AMF has a substantial impact on the nutrition, growth and productivity of host plants AMF by increasing plant mineral nutrients intake, especially phosphorus, zinc, copper and ammonium (Crossay et al., 2017). This is achieved as a consequence of root surface enlargement. AMF confers protection against soil borne pathogens, *Phytophthora*, *Rhizoctonia*, *Pythium*, *Aphanomyces*, *Verticillium*, *Fusarium*, *Macrophomina* (Smith and Read, 2008), increase tolerance against abiotic stresses like drought, capacity to reduce toxic matters in soil (Bianciotto et al., 2018; Ranganathswamy et al., 2019) and increase soil aggregation or soil stabilization. The mechanisms implicated in the protective action of AM symbiosis includes

competition for photosynthates or for colonization/infection sites, induction of changes in the root system morphology/anatomy and induction of changes in mycorrhizosphere populations (Salvioli and Bonfante, 2013).

It is important to mention that in addition to the intrinsic characteristics of both partners (fungus and plant) a wide range of environmental influences and cultural practices modulate the efficiency of AM associations in terms of improving plant performance, such as soil fertility that when low to moderate favors AM formation (Azcón-Aguilar and Barea, 1997; Chaudhary et al., 2014), agricultural practices (Gianinazzi et al., 1990, Mosbah et al., 2018) and substrate type (sand, gravel, peat, expanded clay, pumice, perlite, bark, sawdust, vermiculite or a mixture of these) (Azcón-Aguilar and Barea, 1997; Malicka et al., 2021, Zai et al., 2021).

- Applicability in horticulture

A common practice in horticultural nurseries to achieve high yield and quality is the use of chemical treatments to kill harmful organisms; however, this can also kill the beneficial microorganisms impacting by this on the mycorrhizal association directly, by damaging or killing AMF, and indirectly, by creating unfavorable conditions to AMF (Gosling et al., 2006). The impact of various agricultural practices on soil biodiversity and AMF is still poorly understood and in cultivated soils AMF presence is impoverished, particularly in terms of numbers of species (Rouphael et al., 2015).

Many studies have already described the effects of AMF on the growth and development of horticultural plants which includes: (i) enhanced seedling growth; (ii) reduced phosphate requirements; (iii) increased survival rate and development of micropropagated plantlets; (iv) increased resistance to fungal root pathogens; (v) increased resistance to abiotic stresses; (vi) earlier flowering and fruiting; (vii) increased crop uniformity; (viii) improved rooting; (ix) increased fruit production (Smith and Read, 2008; Davison et al., 2015; Thirkell et al.2017). Menge et al. (1977) first showed evidence of the positive influence of the AM symbiosis on horticultural production where the establishment of citrus plants was achieved by inoculation with an AMF propagule. Since then, experiments have been continuously carried out in vegetable crops and spices, temperate fruit crops, tropical plantation crops and floricultural crops. While many studies have shown mycorrhizal positive effect in improving contents of secondary metabolites (Zeng et al., 2013; Rouphael et al., 2015; Bianciotto et al., 2018), in-depth research is still required to better understand the mechanisms by which AM fungi affect the accumulation of effective ingredients in horticultural plants.

- Characterization of soil fungal community

AMF are asexual organisms making their classification consequently based almost entirely on spore morphology. Spores however are a form of fungal resting stage not representing the active community (Merryweather and Fitter, 1998; Renker et al., 2005; Hempel et al., 2007). Also, their morphology full comprehension is restricted to few experts in this field, due to sparse spore characters, the ability of species to form dimorphic spores, ambiguous or incomplete species description and possible spontaneous changes of the spore characters (e.g., color, size). Because using exclusively morphological characters is known to be difficult the systematic and taxonomy studies has lately been relying on phylogenetic analyses of molecular data (Krüger, 2011) using reliable markers such as the rDNA regions, which are well defined and conserved (Bruns et al.2018). The largest taxon sampling for AMF is provided for the SSU rDNA marker region (Berruti et al., 2017), but it only allows phylogenetic resolution down to genus level (Vasar et al., 2017). Phylogenetic analyses of the ITS and LSU rDNA region in other hand has showed to be capable of distinction to AMF species-level (Öpik and Davison, 2016) but due to the high intraspecific variability of the ITS region, this marker alone is not suited to resolve very closely related species, as for example *Rhizophagus intraradices* N.C. Schenck & G.S. Sm and its close relatives (Stockinger et al., 2009; Stürmer et al., 2018), nevertheless, the ITS region has become the most likely DNA-barcoding region for fungi, potentially in combination with the partial LSU rDNA region. Further molecular markers are available for AMF, such as the genes for the mitochondrial LSU rRNA (Croll et al., 2008; Börstler et al., 2008; Thiéry et al., 2010; Stürmer et al., 2018; Delavaux et al. 2021) , β-tubulin (Msiska and Morton, 2009; Morton and Msiska, 2010a,b), elongation factor 1-α (Sokolski et al., 2010), H+-ATPases (Requena et al., 2003), etc., but they are either inapplicable or data are only available for a few closely related AMF (Krüger, 2011; Stürmer et al., 2018). Techniques including DGGE and T-RFLP for in-field community analyses have been tested and used but as for other techniques the not phylogenetic inclusive primers and the high number of repeated multiple copies makes them problematic to produce reliable results (Calheiros et al., 2019).

According to Berg and colleagues (2020) of the nearly 400 species of Glomeromycotina comprising arbuscular mycorrhizal fungi (AMF) (Schüßler et al. 2001b; Tedersoo et al. 2018; Turrini et al. 2018, et al., Jobim et al., 2019, Wijayawardene et al., 2020), only a small part can be sequence-annotated. The use of molecular markers has expanded in AMF study but some diversity field studies are still based also on spore surveys. Current studies have shown the relevance of combining morphological characterizations with a molecular phylogenetic approach (Ontivero et al. 2020), and more in concrete the need to develop reliable molecular markers as well as data baseline for correct identification of AMF on species level (Berruti et al., 2017, Victorino et al., 2020).

1.5. MiSeq technology to decipher soil microbial community

Soil quality is one of the major threats to sustainable soil management particularly in cultivated land areas in which the soil microorganism's potential can be harnessed providing an eco-friendly and cost-effective method for soil improvement. Microorganisms residing in soil, although numerous, widespread and known to be present and active from more than 1 billion years ago, are still hard to study because many of the mastered techniques involve *in vitro* cultivation or expertise in microbial taxonomy. With the advent of molecular techniques many uncultivable species were discovered and as DNA sequencing technologies emerged and evolved it became clear that the unseen — and perhaps unseeable — microbiota outnumbered the diversity observable through cultivation. According to Zhu and colleagues (2014) sequencing methods started with the single specimens to parallel Sanger sequencing (also known as low throughput sequencing) followed by the so-called high-throughput sequencing — HTS. Sanger DNA sequencing, developed by Dr. Frederick Sanger, was a great achieve in molecular studies rendering him a Nobel prize in Chemistry in the 80's (Zhou et al., 2010); Same authors refers that this method however were limited by the amount of DNA that could be processed at a given time so to address the low throughput, newer sequencing technologies capable of read the sequence of multiple DNA molecules in parallel were developed. Therefore, a number of bioinformatics methods and software have been created to accelerate HTS studies and to reduce sequencing costs dramatically, making it possible to sequence an entire genome for less than 1000 euros (Kulski, 2015).

The Illumina MiSeq platform (http://www.illumina.com/) is the major player among the HTS platforms and the default choice for metabarcoding studies of fungi and other organisms on the basis of fragmented DNA or complementary DNA (Nilsson et al., 2019), offering a much larger sequence outputs than the previous platforms. According to the same author, it uses a paired-end approach covering amplicons of up to ~550 bases in length (MiSeq 2 × 300) and although offering shorter single-end read lengths of up to 300 bases, its read quality is relatively high for most taxa. Many factors can dictate a successful HTS study, namely, experimental design and sample preparation, genetic markers selection, primers and amplification conditions setup program (Nilsson et al., 2019; Agnolucci et al., 2019), and, because choice of primers dictates the microorganisms that may be recovered from the sample additional time analyzing the choice of primers is recommended. Since 2012 commercially available sequencing platforms are various but the Illumina one dominates the sequencing industry (Quail et al., 2012; Kulski, 2015). The MiSeq technology is an interesting tool to monitor and study microbial associations in the rhizosphere and the rapid advances in DNA and RNA sequencing technologies may enable us to

study microbial communities in a more integrative way allowing the exploration of taxonomic profiles and correspondent functional and ecological roles.

1.6. Aim

The general aim of my thesis has mainly been devoted to investigating the potentiality of fungi and in particular Arbuscular Mycorrhizal fungal symbionts to be used as biofertilizers for a sustainable cultivation of Saffron. In this context a combination of morphological and molecular approaches was used in order to identify and select cultivation methods and AMF taxa more useful for Saffron plant growth and secondary metabolites production.

In the meanwhile, the composition and biodiversity of the fungal communities that have best adapted to the complex soil/fungus/plant system of the typical Alpine agro ecosystem where Saffron is cultivated have been evaluated. In particular, genetic diversity of fungi in agricultural and natural soils were assayed by using a targeted-metagenomics approach based on Illumina MiSeq high-throughput technique.

The above topic started from the idea that the soil is an important diversity source to be preserved and in order to do this, knowing what soil contains is essential. For this purpose, we performed comparative analyses over two years on microbial communities associated to Saffron cultivated soils, located in the municipality of Morgex (45°45′35.1′′ N; 7°02′37.3′′ E; 1000 m a.s.l.) and Saint Cristophe (45°45′06.9′′ N; 7°20′37.0′′ E; 700 m a.s.l.) in Italy.

This research has been mainly carried out in the frame of the National Research Project project titled "SaffranALP - Lo zafferano di montagna: tecniche sostenibili per una produzione di qualità", CRT Foundation (2018-2020). One of the environments analyzed by the project is a typical Alpine ecosystem; investigating biodiversity in such environment is particularly interesting for two reasons: firstly, because being it an under-exploited area it may represent a biodiversity hotspot and secondly because it has been an area under agriculture exploitation for many years many environmental parameters (e.g. soil types, soil threats, climatic zones, and land uses) are already monitored for longer periods.

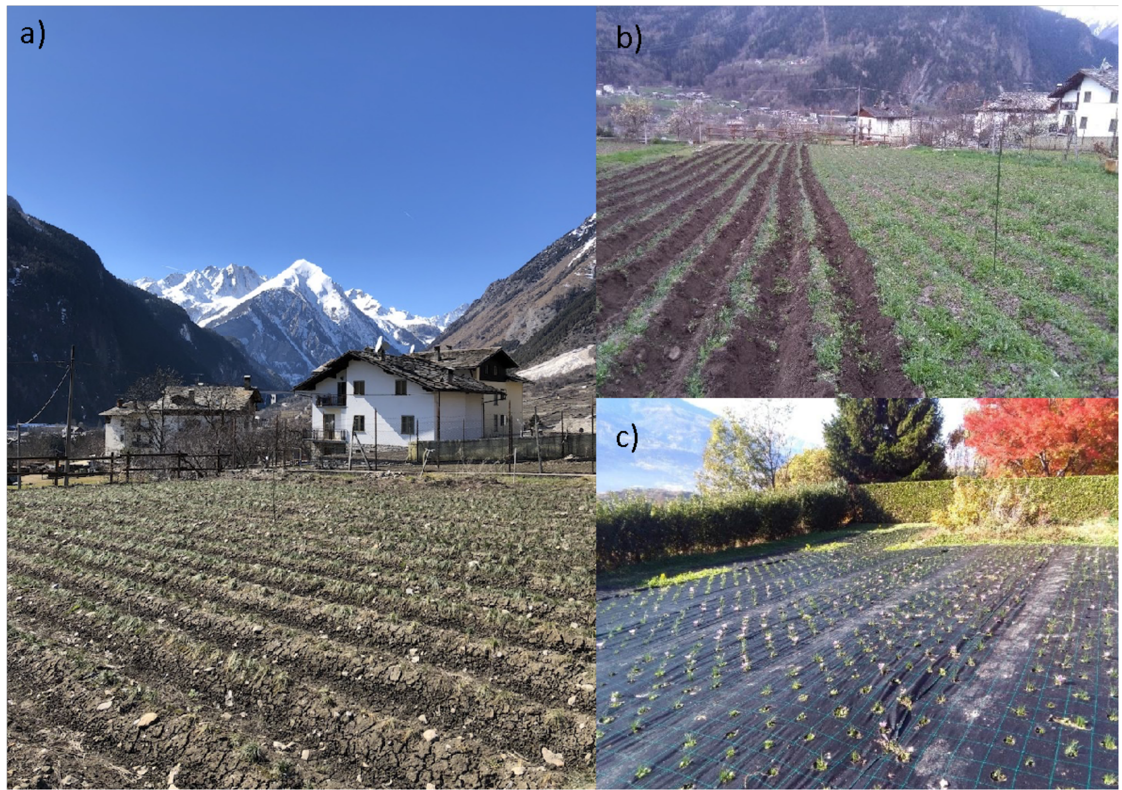


Figure 5. Landscapes from Valle d'Aosta. a), b) and c) the saffron cultivated areas. These environments were the central thread of all performed fungal studies.

In detail this thesis is divided into chapters mirroring the work I did during my PhD. The outline is:

- verify the constitutive association of AMF with saffron roots in sterile pot conditions and to assess the AMF symbiosis in open field conditions and its effects on saffron plant growth, productivity, and bioactive compounds content in Alpine open field conditions (Chapter II);

- evaluate if saffron cultivation in soilless systems and AMF inocula application may improve crop performance, spice yield and quality, and modulate bioactive compounds content over two-year cultivation cycles (Chapter III);

- evaluate by MiSeq analysis the fungal diversity associated to saffron cultivated fields located in Valle d´Aosta (Chapter IV);

- describe a workflow for AMF identification by high-throughput sequencing through Illumina MiSeq platform of two DNA target regions: Small Subunit (SSU) and Internal Transcribed Spacer (ITS) that can apply to both soil and root AMF communities (Chapter V).

1.7. References

- Abrishami, M. 2002. Understanding of Iranian Saffron, 1st ed.; Tous: Tehran, Iran, 1987.
- Agnolucci, M., Avio, L., Pepe, A., Turrini, A., Cristani, C., Bonini, P., Cirino, V., Colosimo, F., Ruzzi, M. and Giovannetti, M. 2019. Bacteria Associated with a Commercial Mycorrhizal Inoculum: Community Composition and Multifunctional Activity as Assessed by Illumina Sequencing and Culture-Dependent Tools. Front. Plant Sci. 9:1956. DOI: 10.3389/fpls.2018.01956.
- Ahmad, I., Pichtel, J., Hayat, S., 2008. Plant-Bacteria Interactions. Strategies and Techniques to Promote Plant Growth. WILEY-VCH Verlag GmbH and Co., KGaA, Weinheim. DOI: 10.1002/9783527621989.
- Ahrazem, O., Argandona, J., Fiore, A., Aguado, C., Lujân, R., Rubio-Moraga, Â., Marro, M., Araujo- Andrade, C., Loza, A.P., Diretto, G., Gómez-Gómez, L. 2018. Transcriptome analysis in tissue sectors with contrasting crocins accumulation provides novel insights into apocarotenoid biosynthesis and regulation during chromoplast biogenesis. Sci. Rep. 8: 2843.
- Ait Oubahou, A. 2009. Bonnes pratiques agronomiques de la conduite technique du safran dans la region de Taliouin -Taznakht. FAO/TCP/MOR/320.
- Akhondzadeh, B., Khondzadeh, S., Ghoreishi, S., Noorbala, A., Rezazadeh, S. 2008. Petal and Stigma of *Crocus sativus* L. in the Treatment of Depression: A Pilot Double—Blind Randomized Trial. J. Med. Plant. Res. 7: 29–36.
- Akhondzadeh, S., Ashrafi, M., Ghaeli, P., Jamshidi, A., Kashani, L., Mansouri, P., Modabbernia, A., Nasehi, A., Raisi, F., Saroukhani, S. 2013. Saffron for treatment of fluoxetine-induced sexual dysfunction in women: Randomized double-blind placebo-controlled study. Hum. Psychopharmacol. 28(1): 54–60. DOI: 10.1002/hup.2282.
- Allkin, B. 2017. Useful plants–medicines. In: Willis KJ, editor. Royal Botanic Gardens, Kew. London: Royal Botanic Gardens, Kew. pp. 22-29.
- Antoun, H. and Prevost, D. 2005. Ecology of plant growth promoting rhizobacteria. In: Siddiqui ZA (ed) PGPR: biocontrol and biofertilization. Springer, Dordrecht, pp 1–38.
- Arora, N., Khare, E., Maheshwari, D. 2011. Plant growth promoting rhizobacteria: constraints in bioformulation, commercialization, and future strategies. In: Maheshwari, D.K. (Ed.), Plant Growth and Health Promoting Bacteria. Springer, Berlin/Heidelberg, pp. 97–116.
- Azcón-Aguilar, C. and Barea, J. 1997. Applying mycorrhiza biotechnology to horticulture: significance and potentials. Scientia Horticulturae 68:1-24. DOI: 10.1016/S0304-4238(96)00954-5.
- Barbara, M., Kathe, W., Schmitt, S., Rotar, I. 2004. Conservation of Eastern European medicinal plants: Arnica montana in Romania. Bulletin of the University of Agricultural Sciences and Veterinary Medicine, Vol 60 - AGRICULTURE 60: 228-230.
- Babalola, O. 2010. Beneficial bacteria of agricultural importance. Biotechnol. Lett. 32, 1559–1570.
- Berendsen, R., Pieterse, C., Bakker, P. 2012. The rhizosphere microbiome and plant health. Trends Plant Sci. 17, 1360–1385.
- Berg, G.; Rybakova, D.; Fischer, D.; Cernava, T.; Vergès, M.C.; Charles, T.; Chen, X.; Cocolin, L.; Eversole, K.; Corral, G.H.; et al. Microbiome definition re-visited: Old concepts and new challenges. Microbiome 2020, 8, 103.
- Behie, S., Bidochka, M. 2014. Nutrient transfer in plant-fungal symbioses. Trends Plant Sci. 19, 734–740.

- Berendsen, R., Pieterse, C., Bakker, P. 2012. The rhizosphere microbiome and plant health. Trends Plant Sci. 17, 1360–1385.
- Berg, G., Grube, M., Schloter, M., Smalla, K. 2014. Unraveling the plant microbiome: looking back and future perspectives. Front. Microbiol. 5, 1–7, Article 148. DOI: 10.3389/fmicb.2014.00148.
- Berrutti, A., Borriello, R., Orgiazzi A., Barbera A., Lumini E. and Bianciotto, V. 2014. Arbuscular Mycorrhizal Fungi and their value for ecosystem management. In Biodiversity – The dynamic balance of the planet, O. Grillo, ed. (InTech), p. 159-191.
- Berruti, A., Lumini, E., Balestrini, R., Bianciotto, V. 2016. Arbuscular mycorrhizal fungi as natural biofertilizers: Let’s benefit from past successes. Front. Microbiol. 6, 1559.
- Berruti, A., Desirò, A., Visentin, S., Zecca, O., Bonfante, P. 2017. ITS fungal barcoding primers versus 18S AMF-specific primers reveal similar AMF-based diversity patterns in roots and soils of three mountain vineyards. Environ Microbiol Rep 9(5):658-667. DOI:10.1111/1758-2229.12574.
- Bhandari, G. 2014. An overview of agrochemicals and their effects on environment in Nepal. Appl Ecol Environ Sci 2:66–73. DOI: 10.12691/aees-2-2-5.
- Bathaie, S., Bolhassani, A., Khavari, A. 2013. Saffron and natural carotenoids: Biochemical activities and anti-tumor effects. Biochim. Biophys. Acta. 1845, 20–30.
- Bhattarai, N. and Karki, M. 2004. Medicinal and aromatic plants - Ethnobotany and conservation status. In: J. Burley, J. Evans and J. Youngquist (Eds.). Encyclopedia of Forest Sciences. Academic Press, London, UK. pp. 523-532. DOI:10.1016/B0-12-145160-7/00255-6.
- Bhattacharyya, P. and Jha, D. 2012. Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. World J. Microbiol. Biotechnol. 28, 1327–1350.
- Bianciotto, V., Victorino, I., Scariot, V., Berruti, A. 2018. Arbuscular mycorrhizal fungi as natural biofertilizers: current role and potential for the horticulture industry. Acta Hortic. 1191: 207-215. Proc. III Int. Symp. on Woody Ornamentals of the Temperate Zone. DOI 10.17660/ActaHortic.2018.1191.29.
- Błaszkowski, J., Chwat, G. and Góralska, A. 2015. Acaulospora ignota and Claroideoglomus hanlinii, two new species of arbuscular mycorrhizal fungi (Glomeromycota) from Brazil and Cuba. Mycological Progress 14. DOI 10.1007/s11557-015-1042-2.
- Brahmaprakash, G., Sahu, P. 2012. Biofertilizers for Sustainability. J. Indian Inst. Sci. 92, 37–62.
- Brundrett, M. and Tedersoo, L. 2018. Evolutionary history of mycorrhizal symbioses and global host plant diversity. New Phytol, 220: 1108-1115. https://doi.org/10.1111/nph.14976.
- Bruns, T., Corradi, N., Redecker, D., Taylor, J. and Öpik, M. 2018. Glomeromycota: what is a species and why should we care? New Phytol, 220: 963-967. https://doi.org/10.1111/nph.14913.
- Börstler, B., Raab, P., Thiery, O., Morton, J., Redecker, D. 2008. Genetic diversity of the arbuscular mycorrhizal fungus Glomus intraradices as determined by mitochondrial large subunit rRNA gene sequences is considerably higher than previously expected. New Phytologist 180: 452–465.
- Bonfante, P. and Genre, A. 2010. Interactions in mycorrhizal symbiosis. Nat. Commun. 1, 1–11.
- Burt, S. 2004. Essential oils: their antibacterial properties and potential applications in foods - a review. Int J Food Microbiol 94: 223-253. DOI:10.1016/j.ijfoodmicro.2004.03.022.
- Calheiros, C., Pereira, S., Franco, A. and Castro P. 2019. Diverse Arbuscular Mycorrhizal Fungi (AMF) Communities Colonize Plants Inhabiting a Constructed Wetland for Wastewater Treatment. Water 11, 1535. DOI:10.3390/w11081535.

- Caser, M., Victorino, I., Demasi, S., Berruti, B., Donno, D., Lumini, E., Bianciotto, V., Scariot, V. 2019a. Saffron cultivation in marginal alpine environments: How AMF inoculation modulates yield and bioactive compounds. Agronomy Journal 9: 12. DOI 10.3390/agronomy9010012.
- Chaudhary, V., O'Dell, T., Rillig, M. and Johnson, N. 2014. Multiscale patterns of arbuscular mycorrhizal fungal abundance and diversity in semiarid shrublands. Fungal Ecology, 12, 32–43. https://doi.org/10.1016/j.funeco.2014.06.003
- Chen, Y., Zhang, H., Tian, X., Zhao, C., Cai, L., Liu, Y. 2008. Antioxidant potential of crocins and ethanol extracts of Gardenia jasminoides ELLIS and *Crocus sativus* L.: A relationship investigation between antioxidant activity and crocin contents. Food Chemistry. 109 (3): 484-92.
- Croll, D., Wille, L., Gamper, H., Mathimaran, N., Lammers, P., Corradi, N., Sanders, I. 2008. Genetic diversity and host plant preferences revealed by simple sequence repeat and mitochondrial markers in a population of the arbuscular mycorrhizal fungus *Glomus intraradices*. New Phytologist 178: 672– 687.
- Crossay, T., Antheaume, C., Redecker, D., Bon, L., Chedri, N., Richert, C., Guentas, L., Cavaloc, Y., Amir, H. 2017. New method for the identification of arbuscular mycorrhizal fungi by proteomic-based biotyping of spores using MALDI-TOF-MS. Sci Rep. 7(1):14306. DOI: 10.1038/s41598-017-14487-6.
- Corcoles, H., Brasa-Ramos, A., Garcia, F., Montero-Riquelme, F. 2015. Phenological Growth stages of saffron plant (*Crocus sativus* L.) according to the BBCH Scale. Spanish Journal of Agricultural Research. 13 (3): 1-6.
- Davison, J., Moora, M., Öpik, M., Adholeya, A., Ainsaar, L., Bâ, A., Burla, S., Diedhiou, A. G., Hiiesalu I., Jairus, T., Johnson, N. C., Kane, A., Koorem, K., Kochar, M., Ndiaye, C., Pärtel, M., Reier, Ü., Ü. Saks, Singh, R., Vasar, M. and Zobel, M. 2015. Global assessment of arbuscular mycorrhizal fungus diversity reveals very low endemism. Science 349 (6251), 970-973. DOI: 10.1126/science.aab1161.
- Delavaux, C., Sturmer, S., Wagner, M., Schütte, U., Morton, J. and Bever, J. 2021. Utility of large subunit for environmental sequencing of arbuscular mycorrhizal fungi: a new reference database and pipeline. New Phytol, 229: 3048-3052. https://doi.org/10.1111/nph.17080.
- Dhar, A. and Mir, G. 1997. Saffron in Kashmir-VI: A review of distribution and production. J. Herbs Spices Med. Pl. 4, 83–90. DOI: 10.1300/J044v04n04_09.
- Dwyer, A., Hawrelak, J., Whitten, D. 2011. Herbal Medicines, other than St. John's Wort, in the treatment of Depression: A systematic Review. Sci. Rev. Altern. Med. 16: 40–49.
- Eghdami, K.; Erfanparast, A.; Farshid, A.A.; Samadi, F.; Tamaddonfard, E. 2013. Comparison of the effects of crocin, safranal and diclofenac on local inflammation and inflammatory pain responses induced by carrageenan in rats. Pharmacol. Rep. 65, 1272–1280.
- Gaiero, J., McCall, C., Thompson, K., Dayu, N., Best, A., Dunfield, K. 2013. Inside the root microbiome: bacterial root endophytes and plant growth promotion. Am. J. Bot. 100, 1738–1750. DOI: 10.3732/ajb.1200572.
- Ganugi, P., Masoni A., Pietramellara, G., Benedettelli S. 2019. A Review of Studies from the Last Twenty Years on Plant–Arbuscular Mycorrhizal Fungi Associations and Their Uses for Wheat Crops. Agronomy 9: 840. DOI:10.3390/agronomy9120840.
- Genre, A., Chabaud, M., Timmers, T., Bonfante, P., Barker, D., 2005. Arbuscular mycorrhizal fungi elicit a novel intracellular apparatus in *Medicago truncatula* root epidermal cells before infection. Plant Cell 17, 3489–3499.

- Giaccio, M. 2004. Crocetin from saffron. An active component of an ancient spice. Crit. Rev. Food Sci. Nutr. 44: 155–172.
- Gianinazzi-Pearson, V. and Gianinazzi, S. 1988. Morphological interactions and functional compatibility between symbionts in vesicular arbuscular endomycorrhizal associations. In: Scannerini, S. (Ed.), Cell to Cell Signals in Plant, Animal and Microbial Symbiosis. Springer-Verlag, Berlin, pp. 73–84.
- Gianinazzi, S., Trouvelot, A. and Gianinazzi-Pearson, V. 1990. Role and use of mycorrhizas in horticultural crop production. 27 August- 1September, XXIII International Horticulture Congress, Florence, pp. 25-30.
- Gianinazzi, S., Gollotte, A., Binet, M.-N., van Tuinen, D., Redecker, D., Wipf, D. 2010. Agroecology: the key role of arbuscular mycorrhizas in ecosystem services. Mycorrhiza 20, 519–530.
- Golmohammadzadeh, S., Jaafari, M.R., Hosseinzadeh, H. 2010. Does saffron have antisolar and moisturizing effects? Iran. J. Pharm. Res. 9, 133–140.
- Gosling, P., Hodge, A., Goodlass, G., Bending, G. 2006. Arbuscular mycorrhizal fungi and organic farming. Agric. Ecosyst. Environ. 113, 17–35.
- Grande, J. 2011. New venture creation in the farm sector – Critical resources and capabilities. Journal of Rural Studies 27: 220-233. DOI: 10.1016/j.jrurstud.2011.02.003.
- Gresta, F., Lombardo, G., Siracusa, L., Ruberto, G. 2008. Saffron, an alternative crop for sustainable agricultural systems: a review. Agronomy for Sustainable Development 28(1): 95- 112. DOI: 10.1051/agro: 2007030.
- van der Heijden, M., Bardgett, R., van Straalen, N. 2008. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecol. Lett., 11, pp. 296-310.
- Hempel, S., Renker, C., Buscot, F. 2007. Differences in the species composition of arbuscular mycorrhizal fungi in spore, root and soil communities in a grassland ecosystem. Environmental Microbiology 9: 1930–1938.
- Hosseinzadeh, H.; Younesi, H.M. 2002. Antinociceptive and anti-inflammatory effects of *Crocus sativus* stigma and petal extracts in mice. BMC Pharmacol. 2: 7.
- Hosseinzadeh, H., Molaei, M., Shakeri, M., Rajabi, O., Shamsa, A. 2009. Evaluation of *Crocus sativus* L. (saffron) on male erectile dysfunction: A pilot study. Phytomedicine, 16: 690–693.
- ISO 3632-1:2011. Spices—Saffron (*Crocus sativus* L.*).* Food Products SC 7, Spices, Culinary Herbs and Condiments; International Organization for Standardization: Geneva, Switzerland, 2011.
- du Jardin P. 2015. Plant biostimulants: Definition, concept, main categories and regulation. Scientia Horticulturae 196: 3-14. DOI: 10.1016/j.scienta.2015.09.021.
- Jobim, K., Błaszkowski, J., Niezgoda, P., Kozlowska A., Zubek, S., Mleczko, P., Meczko ,P., Chachula, P., Ishiikawa, N. and Goto, B. 2019. New sporocarpic taxa in the phylum Glomeromycota: *Sclerocarpum amazonicum gen. et sp. nov.* in the family Glomeraceae (Glomerales) and Diversispora sporocarpia sp. nov. in the Diversisporaceae (Diversisporales). Mycol Progress 18, 369–384. https://doi.org/10.1007/s11557-018-01462-2.
- Kalesi, M., Behboodi, B. and Ebrahimzadeh, H. 2004. Development and contraction of contractile roots in *Crocus sativus*. Acta Hortic., 650, 55–58. DOI: 10.17660/ActaHortic.2004.650.4
- Kanakis, C., Tarantilis, P., Tajmir-Riahi, H., Polissiou, M. 2007. Crocetin, dimethylcrocetin, and safranal bind human serum albumin: stability and antioxidative properties. J Agric Food Chem. 55(3):970-7. DOI: 10.1021/JF062638L.

- Kianbakht, S. 2009. A systematic review on pharmacology of saffron and its active constituents. J. Med. Plants, 28, 1–23.
- Kianmehr, M. 1981. Vesicular-arbuscular mycorrhizal spore population and infectivity of Saffron (*Crocus sativus* L.) in Iran. New Phytol. 88: 79-82.
- Koocheki, A., Ganjeali, A. and Abbassil, F. 2006. The effect of duration and condition of incubation, weight of mother corms and photoperiod on corm and shoot characteristics of saffron plant (*Crocus sativus* L.). J Iran Field Crop Res, 4: 20-25.
- Koul, A. 1999. In Saffron present status and propagation, Proceedings of National symposium on Saffron, Regional Research Laboratory, Jammu, India. 21–23.
- Krüger, M. 2011. Molecular phylogeny, taxonomy and evolution of arbuscular mycorrhizal fungi DNA-based characterization and identification of Glomeromycota. Dissertation thesis of LMU.
- Kulski, J. 2015. Next-Generation Sequencing — An Overview of the History, Tools, and "Omic" Applications. pg 5.
- Kumar, R., Singh, V., Devi, K., Sharma, M., Singh, M. and Ahuja, P. 2008. State of Art of Saffron (*Crocus sativus* L.) Agronomy: A Comprehensive Review', Food Reviews International, 25:1, 44 — 85. DOI: 10.1080/87559120802458503.
- Landi, R. 2007. "Lo zafferano: tradizione e qualità" Camera di commercio industria, artigianato e agricoltura di Firenze. CCIAA (Camera di Commercio: Industria, Artigianato e Agricoltura): Firenze, Italy. Volume 4, p. 65.
- Liakopulou, K. and Kyriakides, D. 2002. *Crocus sativus*-Biological Active Constituents. Stud. Nat. Prod. Chem. 26: 293–312.
- Li, C. and Wu, T. 2002. Constituents of pollen of *Crocus sativus* L. and their tyrosinase inhibitory activity. Chem. Pharm. Bull. 50: 1305–1309.
- Li, C., Lee, E. and Wu, T. 2004. Antityrosinase principals and constituents of the petals of *Crocus sativus*. J. Nat. Prod., 67: 437–440.
- Maiko, I., Shinichiro, H. and Lyle E. 2019. Role of Medicinal and Aromatic Plants: Past, Present, and Future, Pharmacognosy – Accessed in October 12th 2020.
- Malicka, M., Magurno, F., Posta, K., Chmura, D. and Piotrowska-Seget, Z. 2021. Differences in the effects of single and mixed species of AMF on the growth and oxidative stress defense in *Lolium perenne* exposed to hydrocarbons. Ecotoxicology and Environmental Safety, Volume 217, 112252, ISSN 0147-6513. https://doi.org/10.1016/j.ecoenv.2021.112252.
- Manoharan, L., Rosenstock, N., Williams, A., Hedlund K. 2017. Agricultural management practices influence AMF diversity and community composition with cascading effects on plant productivity, Applied Soil Ecology, Volume 115, Pages 53-59. DOI: 10.1016/j.apsoil.2017.03.012.
- Manzo, A., Panseri, S., Bertoni, D., Giorgi, A. 2015. Economic and Qualitative Traits of Italian Alps Saffron. J. Mt. Sci. 12(6): 1542-1550. DOI: 10.1007/s11629-015-3448-3.
- Menge, J., Lembright, H. and Johnson, E. 1977. Utilization of mycorrhizal fungi in citrus nurseries. Proc. Int. Sot. Citric, 1: 129-132.
- Merryweather, J. and Fitter, A. 1998. The arbuscular mycorrhizal fungi of Hyacinthoides non–scripta. I. Diversity of fungal taxa. New Phytologist 138: 117–129.

- Moghaddasi, M. 2010. Saffron chemicals and medicine usage. J. Med. Plants Res., 4, 427–430. https://doi.org/10.5897/JMPR09.001.
- Molina, R., García-Luis, A., Valero, M., Navarro, Y. and Guardiola, J. 2003. Extending the harvest period of saffron. Hortic. Act, 650, 219–225.
- Morton, J., Msiska, Z. 2010a. Phylogenies from genetic and morphological characters do not support a revision of Gigasporaceae (Glomeromycota) into four families and five genera. Mycorrhiza 20: 483-496.
- Morton, J., Msiska, Z. 2010b. Ontogeny and phylogeny of a *Scutellospora heterogama* mutant, with implications for morphological recognition of species in Glomeromycota. Fungal Biology 114: 410-420.
- Mosbah, M., Philippe, D. L., and Mohamed, M. 2018. Molecular identification of arbuscular mycorrhizal fungal spores associated to the rhizosphere of *Retama raetam* in Tunisia. Soil Science and Plant Nutrition. 64: 335–341. https://doi.org/10.1080/00380768.2018.1431012.
- Msiska, Z. and Morton, J. 2009. Phylogenetic analysis of the Glomeromycota by partial β-tubulin gene sequences. Mycorrhiza 19: 1432-1890.
- Mzabri, I., Legsayer, M., Chetouani, M., Aamar, A., Kouddane, N., Boukroute, A., Bekkouch, I. and Berrichi, A. 2017. Saffron (*Crocus sativus* L.) yield parameter assessment of abiotic stressed corms stored in Low Temperature Journal of Materials and Environmental Sciences. 8 (10): 3588-3597.
- Mzabri, B., Addi, M. and Berrichi, A. 2019. Traditional and Modern Uses of Saffron (*Crocus sativus*). Cosmetics 6, 63. DOI:10.3390/cosmetics6040063.
- Nehvi, F. and Yasmin, S. 2013. Saffron as a valuable spice: A comprehensive review. Afr. J. Agric. Res. 8 (3): 234–242. DOI: 10.5897/ajbm11.2247.
- Ohsowski, B., Zaitsoff, P., Öpik, M. and Hart, M. 2014. Where the Wild Things Are: looking for uncultured Glomeromycota. New Phytol 204, 171–179.
- Ontivero, E., Voyron, S., Allione, L., Bianco, P., Bianciotto, V., Iriarte, H., Lugo, M., Lumini, E. 2020. Impact of land use history on the arbuscular mycorrhizal fungal diversity in arid soils of Argentinean farming fields. FEMS Microbiology Letters. Volume 367, Issue 14. fnaa114. https://doi.org/10.1093/femsle/fnaa114.
- Öpik, M. and Davison, J. 2016. Uniting species and community-oriented approaches to understand arbuscular mycorrhizal fungal diversity. Fungal Ecol 24:106-113.
- Palos, E., Cabau, R., Gorgues, A. 2005. Commercialization of aromatic and medicinal plants in Catalonia. Spain. Acta Hort (ISHS) 679: 149-156. DOI: 10.17660/ActaHortic.2005.679.18.
- Pelosi, C., Barot, S., Capowiez, Y., Hedde, M. and Vandenbulcke, F. 2013. Pesticides and earthworms. A review. Agron Sustain Dev 34:199–228. DOI: 10.1007/s13593-013-0151-z.
- Philippot, L., Raaijmakers, J., Lemanceau, P., Putten, W. 2013. Going back to the roots: the microbial ecology of the rhizosphere. Nat. Rev. Microbiol. 11, 789–799.
- Piliarová, M., Ondreicková, K., Hudcovicová, M., Mihálik, D., Kraic, J. 2019. Arbuscular mycorrhizal fungi – their life and function in ecosystem. Agriculture (Polnohospodárstvo), vol. 65, no. 1, pp. 3–15.
- Premkumar, K., Abraham, S., Santhiya, S., Ramesh, A. 2003. Protective effects of saffron (*Crocus sativus* L.) on genotoxins induced oxidative stress in Swiss albino mice. Phytother. Res. 17: 614–617.
- Rafiq, L., Shuab, R., Ahamad Malla N., Kumar, A. 2016. Beneficial Effects of Arbuscular Mycorrhizal Fungi on Underground Modified Stem Propagule Plants Journal on New Biological Reports. 5 (1): 41 – 51. DOI: 10.3389/fpls.2018.01270.

- Ranganathswamy, M., Kadam, G. and Jhala Y. 2019. An Insight into Mycorrhiza Involved in Building Soil and Plant Health. In: Soil Fertility Management for sustainable development. India. pg 211.
- Redecker, D., Kodner, R., Graham, L. 2000. Glomalean fungi from the Ordovician. Science 289, 1920–1921.
- Reddy, M., Rodolfo, I., Patricio, S., William, D., William, D., Riyaz, S., Sudini, H., Vijay, K., Armanda, A. and Gopalkrishnan, S. 2014. Recent advances in biofertilizers and biofungicides (PGPR) for sustainable agriculture. Cambridge Scholars Publishing, Newcastle upon Tyne, UK, p 540.
- Renker, C., Blanke, V., Buscot, F. 2005. Diversity of arbuscular mycorrhizal fungi in grassland spontaneously developed on area polluted by a fertilizer plant. Environmental Pollution 135: 255–266.
- Requena, N., Breuninger, M., Franken, P., Ocón, A. 2003. Symbiotic status, phosphate and sucrose regulate the +expression of two plasma membrane H-ATPase genes from the mycorrhizal fungus *Glomus mosseae*. Plant Physiology 132: 1540-1549.
- Rouphael, Y., Franken, P., Schneider, C., Schwarz, D., Giovannetti, M., Agnolucci, M., De Pascale, S., Bonini, P., Colla, G. 2015. Arbuscular mycorrhizal fungi act as biostimulants in horticultural crops. Sci. Hortic. 196, 91-108. DOI:10.1016/j.scienta.2015.09.002.
- Rouphael, Y. and Colla, G. 2020. Biostimulants in Agriculture. Frontiers in Plant Science. Vol. 11. Pg. 40. DOI: 10.3389/fpls.2020.00040.
- The Royal Horticultural Society. Plants of current interest. 2006. http://212.78.71.150/gardens/wisley/archive/wisleypcisept.asp. Accessed in November 13th 2021.
- Salvioli A. and Bonfante P. 2013. Systems biology and "omics" tools: A cooperation for next generation mycorrhizal studies. Plant Science. 203–204.
- Schüßler, A., Schwarzott, D., Walker, C. 2001. A new fungal phylum, the Glomeromycota: phylogeny and evolution. Mycological Research 105: 1413-1421. DOI: 10.1017\S0953756201005196.
- Sokolski, S., Dalpé, Y., Séguin, S., Khasa, D., Lévesque, C., Piché, Y. 2010. Conspecificity of DAOM197198, the model arbuscular mycorrhizal fungus, with *Glomus irregulare*: molecular evidence with three protein-encoding genes. Botany 88: 829–838.
- Spaepen, S., Vanderleyden, J. and Okon, Y. 2009. Plant growth-promoting actions of rhizobacteria. Adv Bot Res 51:283–320.
- Spatafora, J., Chang, Y., Benny, G., Lazarus, K., Smith, M., Berbee, L., Bonito, G., Corradi, N., Grigoriev, I., Gryganskyi, A., James, T., O'Donnell, K., Roberson, R., Taylor, T., Uehling, J., Vilgalys, R., White, M., and Stajich, J. 2016. A phylum-level phylogenetic classification of zygomycete fungi based on genome-scale data. Mycologia 108:1028–1046. https://doi.org/10.3852/16-042.
- Stockinger, H., Walker, C., Schüßler, A. 2009. 'Glomus intraradices DAOM197198', a model fungus in arbuscular mycorrhiza research, is not Glomus intraradices. New Phytologist. 183(4): 1176-1187. DOI: 10.1111/j.1469-8137.2009.02874.x.
- Strullu-Derrien, C., Selosse, M., Kenrick, P. and Martin, F. 2018. The origin and evolution of mycorrhizal symbioses: from palaeomycology to phylogenomics. New Phytologist 220: 1012–1030. DOI: 10.1111/nph.15076.
- Stürmer, S., Bever J., Morton, J. 2018. Biogeography of arbuscular mycorrhizal fungi (Glomeromycota): a phylogenetic perspective on species distribution patterns. Mycorrhiza 28: 587-603.
- Tabrizi, S., Mortazavi, S. and Kamalinejad, M. 2003. An in vitro evaluation of various *Rosa damascena* flower extracts as a natural antisolar agent. Int. J. Cosmet. Sci. 25: 259–265.

- Tedersoo, L., Sanchez-Ramirez, S., Koljalg, U., Bahram, M., Doring, M., Schigel, D., May, T., Ryberg, M., Abarenkov, K. 2018. High-level classification of the fungi and a tool for evolutionary ecological analyses. Fungal Divers 90(1): 135–159. https://doi.org/10.1007/s13225-018-0401-0.
- Thiéry, O., Börstler, B., Ineichen, K., Redecker, D. 2010. Evolutionary dynamics of introns and homing endonuclease ORFs in a region of the large subunit of the mitochondrial rRNA in Glomus species (arbuscular mycorrhizal fungi, Glomeromycota). Molecular Phylogenetics and Evolution 55: 599-610.
- Thirkell, T., Charters, M., Elliott, A., Sait, S. and Field, K. 2017, Are mycorrhizal fungi our sustainable saviors? Considerations for achieving food security. J Ecol, 105: 921-929. https://doi.org/10.1111/1365-2745.12788.
- Toscano S., Massa D., Romano D., Bulgari R. 2019. Biostimulant applications in low input horticultural cultivation systems. Italus Hortus 25 (2): 27-36. DOI: 10.26353/j.itahort/2018.1.2736.
- Turrini, A., Saran, M., Giovannetti, M., Oehl, F. 2018. *Rhizoglomus venetianum*, a new arbuscular mycorrhizal fungal species from a heavy metal-contaminated site, downtown Venice in Italy. Mycol Prog. https://doi.org/10.1007/s11557-018-1437-y.
- Vacheron, J., Desbrosse, G., Bouffaud, M.-L., Touraine, B., Moënne-Loccoz, Y., Muller, D., Legendre, L., Wisniewski-Dye, P., rigent-combaret, C., 2013. Plant growth-promoting rhizobacteria and root system functioning. Front. Plant Sci. 4, 1–19, Article 356.
- Vagionas, K., Ngassapa, O., Runyoro, D., Graikou, K., Gortzi, O., Chinou, I. 2007. Chemical analysis of edible aromatic plants growing in Tanzania. Food Chem 105: 1711-1717.
- Vasar, M., Andreson, R., Davison, J., Jairus, T., Moora, M., Remm, M., Young, J., Zobel, M., Öpik, M. 2017. Increased sequencing depth does not increase captured diversity of arbuscular mycorrhizal fungi. 27:761-773. DOI: 10.1007/s00572-017-0791-y.
- Victorino, Í.M.M.; Berruti, A.; Orgiazzi, A.; Voyron, S.; Bianciotto, V.; Lumini, E. High-throughput DNA sequence-based analysis of AMF communities. In Arbuscular Mycorrhizal Fungi. Methods in Molecular Biology; Ferrol, N., Lanfranco, L., Eds.; Humana: New York, NY, USA, 2020; Volume 2146.
- Walker, C., 1995. AM or VAM: what’s in a word? In: Varma, A., Hock, B. (Eds.), Mycorrhiza: Structure, Function, Molecular Biology and Biotechnology. Springer Verlag, Berlin, Deutschland, pp. 25–26.
- WHO. 2008. Traditional medicine. World Health Organization Fact Sheet no 134. http://www.who.int/mediacentre/factsheets/fs134/en/index.html. Accessed 27 Feb 2021.
- Wijayawardene, N. N., Hyde, K. D., Dai, D. Q., Tang, L. Z., Aptroot, A., CastañedaRuiz, R. F., et al. (2020). Outline of Fungi and fungi-like taxa. Mycosphere 11, 1060–1456. doi: 10.5943/mycosphere/11/1/8.
- Winterhalter, P. and Straubinger, M. Saffron-renewed interest in an ancient spice. Food Rev. Int. 2000, 16, 39–59. DOI: 10.1081/fri-100100281.
- The World Bank. 2015. http://www.worldbank.org/en/news/feature/2015/01/20/saffron-major-source-income-alternative-poppy. Saffron: A major source of income and an alternative to poppy. Accessed on the September 14th 2017.
- Zai, X., Fan, J., Hao, Z., Liu, X., Zhang, W. 2021. Effect of co-inoculation with arbuscular mycorrhizal fungi and phosphate solubilizing fungi on nutrient uptake and photosynthesis of beach palm under salt stress environment. Sci Rep 11, 5761. DOI:10.1038/s41598-021-84284-9.
- Zeka, K., Ruparelia, K.C., Continenza, M.A., Stagos, D., Vegliò, F., Arroo, R. 2015. Petals of *Crocus sativus* L. as a potential source of the antioxidants crocin and kaempferol. Fitoterapia. 107: 128–134.

- Zeng Y., Guo L., Chen B., Hao, Z., Wang J., Huang L., Yang G., Cui X., Yang L., Wu Z., Chen M., Zhang Y. 2013. Arbuscular mycorrhizal symbiosis and active ingredients of medicinal plants: current research status and prospectives. Mycorrhiza 23: 253–265. DOI 10.1007/s00572-013-0484-0.
- Zhou, X., Ren, L., Li Y., Zhang, M., Yu, Y. and Yu, J. 2010. The next-generation sequencing technology: a technology review and future perspective. Sci China Life Sci, 53(1): 13-25.
- Zohary, D. and Hopf, M. 1999. Saffron cultivation: past, present and future prospects. In Saffron: *Crocus sativus* L.; Negbi, M.; Ed.; Harwood Academic Publishers: Amsterdam, The Netherlands, pp 1–18.

Sites

Site http://www.illumina.com. Accessed on 04th June 2020.

Chapter II

Saffron crop performance and metabolic profile modulation by arbuscular mycorrhizal fungi in soilless cultivation

Preamble

Saffron (*Crocus sativus*) is a mainly open field worldwide cultivated crop whose spice yield, quality based on ISO category, antioxidant activity, and bioactive compound contents vary greatly according to agronomic and climatic factors. Better general plant performance is expected in systems where they can grow without the use of soil as a rooting medium, supplied with inorganic nutrients via water irrigation and no competition with pathogens (Putra and Juliando, 2015 from 2019_Caser et al Agronomy published). For saffron crop however limited and controversial studies using soilless cultivation had been reported, and, from the one´s reported, the substrate type, mixture content and chamber temperatures has been mentioned as the most influencing factors. Arbuscular mycorrhizal fungi are well known to symbiotically interact with plants enhancing nutritional status of both soil and plant. They can associate with a wide variety of plants forming vesicles, arbuscules, and hyphae in roots, and also spores and hyphae in the rhizosphere determining improvement in plant growth and molecules production (Jami et al., 2020; Avio et al., 2018; Pellegrino et al., 2014). Experimental trials on horticulture plants inoculated with AMF alone or in combination with other microorganisms reported increased leaf area, enhanced dietary quality of crops and increased levels of secondary metabolites as benefits of the AMF use (Toscano et al., 2018). The use of AMF in saffron production has already been investigated reporting, in most cases, an increase in corm growth, spice yield, and the nutraceutical compound content [12,40–42 from2019_Caser et al Agronomy published), however proper saffron AMF inocula application effect in soilless conditions remains unclear. In the paper below we presented the results obtained in saffron bulb inoculation with single and multispecies commercial inocula in a soilless system.

The objective of this research was to:

- evaluate saffron cultivation in soilless systems, where plants can grow without having to deal with stress caused by pathogens or nutrients deficit.
- evaluate saffron plant performance and metabolite profile if inoculated with two types of inoculums: a single AMF species (*Rhizophagus intraradices*) or a mixture of *R. intraradices* and *Funneliformis mosseae*.

We were able to visualize AMF symbiosis with saffron roots on light and transmission electron microscopy and to confirm improved crop performance (replacement corms produced and polyphenols content). Higher antioxidant activity was seen on the single species inocula plant

treatment and the soilless systems proved to be a good candidate in saffron cultivation without compromising spice quality.

Article

Arbuscular Mycorrhizal Fungi Modulate the Crop Performance and Metabolic Profile of Saffron in Soilless Cultivation

Matteo Caser [1,†,*], Sonia Demasi [1,†], Íris Marisa Maxaieie Victorino [2,3,4], Dario Donno [1], Antonella Faccio [2], Erica Lumini [2], Valeria Bianciotto [2] and Valentina Scariot [1,2]

1. Department of Agricultural, Forest and Food Sciences, University of Torino, Largo Paolo Braccini 2, 10095 Grugliasco (TO), Italy; sonia.demasi@unito.it (S.D.); dario.donno@unito.it (D.D.); valentina.scariot@unito.it (V.S.)
2. Institute for Sustainable Plant Protection, National Research Council, Viale Mattioli 25, 10125 Torino, Italy; irismarisamaxaieie.victorino@unito.it (Í.M.M.V.); antonella.faccio@ipsp.cnr.it (A.F.); erica.lumini@ipsp.cnr.it (E.L.); valeria.bianciotto@ipsp.cnr.it (V.B.)
3. Department of Life Sciences and Systems Biology, University of Turin, Viale Mattioli 25, 10125 Torino, Italy
4. Biological Science Department, Science Faculty, Eduardo Mondlane University (UEM), Av. Julius Nyerere-Campus Universitário, Maputo 3453, Mozambique

* Correspondence: matteo.caser@unito.it; Tel.: +39-011-670-9835

† Both authors contributed equally to this work.

Received: 7 March 2019; Accepted: 7 May 2019; Published: 8 May 2019

Abstract: Saffron (*Crocus sativus* L.) is cultivated worldwide. Its stigmas represent the highest-priced spice and contain bioactive compounds beneficial for human health. Saffron cultivation commonly occurs in open field, and spice yield can vary greatly, from 0.15 to 1.5 g m^{-2}, based on several agronomic and climatic factors. In this study, we evaluated saffron cultivation in soilless systems, where plants can benefit from a wealth of nutrients without competition with pathogens or stresses related to nutrient-soil interaction. In addition, as plant nutrient and water uptake can be enhanced by the symbiosis with arbuscular mycorrhizal fungi (AMF), we also tested two inocula: a single species (*Rhizophagus intraradices*) or a mixture of *R. intraradices* and *Funneliformis mosseae*. After one cultivation cycle, we evaluated the spice yield, quality (ISO category), antioxidant activity, and bioactive compound contents of saffron produced in soilless systems and the effect of the applied AMF inocula. Spice yield in soilless systems (0.55 g m^{-2}) was on average with that produced in open field, while presented a superior content of several health-promoting compounds, such as polyphenols, anthocyanins, vitamin C, and elevated antioxidant activity. The AMF symbiosis with saffron roots was verified by light and transmission electron microscopy. Inoculated corms showed larger replacement corms (+30% ca.). Corms inoculated with *R. intraradices* performed better than those inoculated with the mix in terms of spice quality (+90% ca.) and antioxidant activity (+88% ca.). Conversely, the mixture of *R. intraradices* and *F. mosseae* increased the polyphenol content (+343% ca.). Thus, soilless systems appeared as an effective alternative cultivation strategy for the production of high quality saffron. Further benefits can be obtained by the application of targeted AMF-based biostimulants.

Keywords: biostimulants; *Crocus sativus*; *Funneliformis mosseae*; glasshouse; protected cultivation; *Rhizophagus intraradices*; substrate

1. Introduction

Crocus sativus L. (saffron) is a flowering plant belonging to the Iridaceae family [1], grown for its red scarlet stigmas that represent the world's highest-priced spice. The market price for high quality

saffron can reach 15,000–20,000 € kg^{-1} [2]. This species is widely cultivated in several countries, such as Iran, Italy, Spain, Morocco, France, Greece, China, India and Mexico [3], with an annual spice production that exceeds 220,000 kg [4].The importance and notoriety of saffron, used since ancient times as a dye, ingredient for the preparation of spirits, and condiment for food, is due to the substances contained in the spice, primarily crocins, picrocrocin and safranal [5,6]. These compounds confer the saffron's unique colour, taste, and aroma, and can also have positive biological effects. Saffron active constituents, such as carotenoids (i.e., crocins), polyphenols, and vitamins showed significant antioxidant activity [7–12]. Furthermore, saffron extracts exhibit anti-carcinogenic, anti-depressive, anti-hyperglycemic, hypoglycemic, and memory-enhancing effects [3,13]. *Crocus sativus* is a highly hand labour-intensive crop, mainly during flower harvesting and stigma separation. It is traditionally cultivated in small and flat plots, wherein mechanisation is not economically sustainable due to the harvest type and short flowering period [5,8]. Five hundred hand labour hours are needed to obtain 1 kg of dried saffron [4,5]. Saffron cultivation can be carried out on an annual or multi-year cycle [14,15]. Annual cultivation guarantees the effective control of plant diseases with a more accurate corm selection. On the contrary, in a multi-year cycle (e.g., 3–4 years in Spain, 4–5 years in Italy, and 6–8 years in India and Greece) [14], corm multiplication and the size of replacement corms in the ground can decrease drastically over the third year [15]. Environment and cultivation management affect flower induction in *C. sativus* [5,16–18]. In Mediterranean environments, flower induction occurs from early spring to mid-summer, while flower emergence occurs from early- to late-autumn. Differences in the time required for flower initiation have mostly been attributed to the corm size [19]. To produce flowers, the *C. sativus* corm diameter needs to be greater than 1 cm [20]. As the corm increases, flowering increases [16,21] and occurs in advance [22]. Commercially, a 2.5–3.5 cm diameter corm appears to be the most common size used to have full flowering already during the first cultivation cycle [23]. To increase saffron yield and quality, and to reduce production costs, flowering modulation through cultivation in soilless systems has been proposed [6,19,24]. In this cultivation system, plants are grown without the use of soil as a rooting medium and are supplied of inorganic nutrients via the irrigation water [25], and thus can benefit of a wealth of nutrients without competition with pathogens or stresses related to nutrient-soil interaction [26]. However, at present, only limited and controversial reports of saffron soilless cultivation under protected conditions are present in the literature. Molina et al. [18] reported that, in a glasshouse, temperatures may be responsible for production differences in terms of flower induction and flowering duration. Maggio et al. [19] showed that, in southern Italy, cultivation in a cold glasshouse on vermiculite and perlite-based substrates positively affected the yield and number of replacement corms. Similarly, Helal Beigi et al. [27] found that cocopeat and perlite substrates enhanced corm dry weight. While Souret and Weathers [28] and Mollafilabi et al. [24] concluded that soilless cultivation in experiments carried out in France and Iran, respectively significantly decreased the spice yield, in comparison to open field cultivation.

Plant performance in soilless systems may be improved through use of biostimulants, i.e., any natural substance or microorganism applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of its nutrients content [29], with a consequent decrease of chemicals and increase of sustainability of the production system [30]. Soil microorganisms such as arbuscular mycorrhizal fungi (AMF) are collecting growing interest as biostimulants. They can form mutualistic symbiosis with about 80% of land plant species, including several crops [31]. Across the interface between the plant and the fungus, carbohydrates and mineral nutrients (i.e., N, P, Zn and B) are exchanged [32]. Thus, AMF can alleviate the limitation in plant growth caused by an inadequate nutrient supply and can improve tolerance to biotic and abiotic stress [33]. Additionally, there is evidence to indicate that AMF symbiosis may have a positive impact on crop quality [34]. Increased yield of essential oils, terpenes and polyphenols, and enhanced antioxidant activity were induced by AMF symbiosis in several medicinal and aromatic plants (MAPs) [12,35–38]. This higher concentration of bioactive molecules makes AMF-hosted plants generally more attractive for the pharmaceutical and food industries [39].

The positive effects of AMF on corm growth, spice yield, and the nutraceutical compound content of C. *sativus* have already been reported in open field trials [12,40–42]. However, so far little is known of the proper saffron AMF inocula application and effects in soilless conditions, where plants are cultivated in pots filled with sterilised substrates that are free of AM fungal propagules or highly reduced in AMF diversity [43]. In the meta-analysis performed by Berruti et al. [31], it has been observed that the fungal colonization gain in inoculated plants was significantly more frequent in the greenhouses than in the open-field conditions, even if the effectiveness of AMF inoculation on shoot biomass and yield was equally successful.

Thus, in the literature, saffron cultivation on soilless systems has been proposed for spice production, but no comparison with open field has been reported. While, the effects of AMF-based biostimulants have been investigated only in open fields. To evaluate if saffron cultivation in soilless systems and AMF application may improve crop performance, spice yield and quality, and modulate bioactive compounds content, we cultivated saffron on soilless systems, applying two AMF inocula, and we compared results with those obtained in a previous open field-based trial [12].

2. Materials and Methods

2.1. Plant Material and Soilless Cultivation

Crocus sativus corms with horizontal diameters of 2.5–3.5 cm, provided by the Azienda agricola "Les épices Vda" di Alessandro Putzolu (Chatillon, AO, Italy), were planted during the last 10 days of August 2017 in the experimental heated glasshouse of the Department of Agricultural Forest and Food Sciences (DISAFA) of the University of Torino (Italy, 45°06′23.21″N Lat, 7°57′82.83″E Long; 300 m a.s.l.). Corms were cultivated in pots (4 L, 14 cm diameter and 17 cm height; two corms per pot; density of 91 corms m^{-2}) filled with sterile quartz sand (2 L per pot; bulk density of 1.2 kg m^{-3}) on a layer of sterilised expanded clay (1 L per pot; bulk density of 300 kg m^{-3}) for a total weight of about 1.5 kg. During the flowering period, the average temperatures were 22 °C during the day and 14 °C during the night.

Two inocula (MycAgro Lab, Breteniére, FR) were used in this experiment: one composed of a single fungus *Rhizophagus intraradices* (Ri) and one composed of *R. intraradices* and *Funneliformis mosseae* (Ri+Fm). Both inocula consisted of AMF spores and inorganic substrate (calcined clay, vermiculite and zeolite). Inocula treatments were compared to a control without any formulation (AMF-). Ten grams of each inoculum were inserted into each vase. The treatment was placed under each corm in order to guarantee contact between the inoculum and the roots, therefore, favouring mutualistic symbiosis. Corms were not treated for fungal pathogens and cultivation lasted one cycle (August 2017–April 2018).

A complete randomised block design was used, with a total of 48 pots in two experimental plot units (24 pots per unit) and three treatments (8 pots per treatment). Irrigation water (pH 7.4, EC 505 µS cm) was added weekly (250 mL per pot) with a drip system. The corms were fertilised by fertigation (N:K 13:46; VIGORFLOR, AL.FE. srl, MN, Italy) every 2 weeks starting from the emergence of the spate, in quantities of 1.5 g L^{-1} of water.

2.2. Determination of Flower Production, Stigma Yield and Corm Growth

At flowering (October and November 2017), the number of flowers produced daily per corm and the yield of spice (i.e., stigmas dried at 40 °C for 8 h in an oven) were measured. The spice yield was calculated by weighting the mg of saffron produced per pot (area equal to 196 cm^2) and comparing the values to g of spice per square meter (m^2). At the end of the vegetative period (April 2018), corms were lifted, rid of topsoil, cleaned and de-tunicated, then the number, size and weight of replacement corms were determined.

2.3. Preparation of the Saffron Extract

The saffron aqueous extracts were prepared according to Caser et al. [12]. Briefly, 50 mg of powdered saffron were suspended into 5 mL of deionised water. After stirring (1000 rpm) for 1 hour at room temperature (circa 21 °C) in the dark, the solution was filtered with polytetrafluoroethylene (PTFE, VWR International, Milano, Italy) filters with a 25 mm diameter and 0.45 µm pore size. The saffron extract was then diluted 1:10 with deionised water to obtain the working solution. Each sample was prepared in triplicate.

2.4. Determination of Saffron Quality by ISO 3632

Saffron aqueous extracts were analysed with a spectrophotometer (Ultrospec 2100 Pro, Ultrospec 2100 pro, Amersham Biosciences, Uppsala, Sweden) to determine the content of picrocrocin, safranal, and crocin to have the information on the bitterness, the flavouring strength, and the colouring strength [44]. Data were related to the dry matter percentage and expressed as the absorbance of a 1% aqueous solution of dried saffron at 257, 330 and 440 nm respectively, using a 1 cm pathway quartz cell [A1% 1 cm (λ max)] and calculated according to the following formula [45]:

$$A1\%1cm\ (\lambda\ max) = D \times 10000/m \times (100 - wMV) \quad (1)$$

where D is the specific absorbance; m is the mass of the evaluated solution in grams; and wMV is the moisture expressed as a percentage mass fraction of the sample.

Moisture content (wMV) was determined using the following formula:

$$wMV = (m0 - m1) \times (100/m0)\% \quad (2)$$

where m0 is the mass, in grams, of the saffron portion before drying; and m1 is the mass, in grams, of the dry residue after incubation, performed in an oven for 16 h at 103 ± 2 °C.

All analytical steps were conducted in the dark to prevent analyte degradation.

2.5. Determination of Bioactive Compounds by HPLC

Bioactive compounds were determined by means of four high performance liquid chromatography-diode array detection (HPLC–DAD) methods (Table 1; [46]) using an Agilent 1200 High-Performance Liquid Chromatograph coupled to an Agilent UV-Vis diode array detector (Agilent Technologies, Santa Clara, CA, USA). Phytochemical separation was achieved with a Kinetex C18 column (4.6 × 150 mm, 5 µm, Phenomenex, Torrance, CA, USA) using several mobile phases for compound identification and recording UV spectra at different wavelengths, based on HPLC methods, as previously tested and validated [47], with some modifications. UV spectra were recorded at 330 nm (α), 280 nm (β), 310 and 441 nm (γ), and 261 and 348 nm (δ). All single compounds were identified by a comparison and combination of their retention times and UV spectra with those of authentic standards under the same chromatographic conditions.

Table 1. Characteristics of the HPLC methods applied to analyse the bioactive compounds present in the studied saffron samples.

HPLC Method	Class	Standard	Stationary Phase	Mobile Phase	Flow ($mL\ min^{-1}$)	Time (min)
α	Cinnamic acids Flavanols	Caffeic acid Chlorogenic acid Coumaric acid Cerulic acid Hyperoside Isoquercitrin Quercetin Wuercitrin Rutin	KINETEX–C18 column (4.6 × 150 mm, 5 μm)	A: 10mM KH_2PO_4/H_3PO_4, pH = 2.8 B: CH_3CN	1.5	20 + 2 (CT)
β	Benzoic acids Catechins	Ellagic acid Gallic acid Catechin Epicatechin		A: $H_2O/CH_3OH/HCOOH$ (5:95:0.1 v/v/v), pH = 2.5 B: $CH_3OH/HCOOH$ (100:0.1 v/v)	0.6	23 + 2 (CT)
γ	Carotenoids	Crocin I Crocin II Safranal		A: H_2O B: CH_3CN	0.6	35 – 10 (CT)
δ	Vitamin C	Ascorbic acid Dehydroascorbic acid		A: 5 mM $C_{16}H_{33}N(CH_3)_3Br$/50 mM KH_2PO_4, pH=2.5 B: CH_3OH	0.9	10 + 5 (CT)

CT = conditioning time; Method α—gradient analysis: 5% B to 21% B in 17 min + 21% B in 3 min + 2 min of conditioning time—wavelength: 330 nm; Method β—gradient analysis: 3% B to 85% B in 22 min + 85% B in 1 min + 2 min of conditioning time—wavelength: 280 nm; Method γ—gradient analysis: 5% B to 95% B in 30 min + 95% B to 5% B in 5 min – 10 min of conditioning time—wavelengths: 310 nm + 441 nm; Method δ—isocratic analysis: 10 min + 5 min of conditioning time—wavelengths: 261 nm + 348 nm.

2.6. *Phytochemical Characterisation*

The phytochemical characterisation of each sample was performed as previously described by Caser et al. [48,49]. Briefly, the total anthocyanin content (TAC) was determined using the pH-differential method. Saffron solution was added to pH 1 and pH 4.5 buffer solutions. The absorbance of samples was determined at 515 and 700 nm after 15 min of equilibration. The results were expressed as milligrams of cyanidin 3-O-glucoside (C3G) per 100 grams of dry weight (mg_{C3G} $100g^{-1}$ DW). The total phenol content (TPC) was measured using the Folin–Ciocalteau phenolic method at 765 nm. The results were expressed as mg of gallic acid equivalents (GAE) per 100 g of dry weight (DW; mg_{GAE} $100g^{-1}$ DW). The antioxidant activity (AOA) was determined at 595 nm using the ferric reducing antioxidant power (FRAP) method and at 734 nm using the 2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid; ABTS) method. Results were expressed as millimoles of ferrous iron (Fe^{2+}) equivalents per kilogram of dry weight (mmol Fe^{2+} kg^{-1} DW) and as μmol of Trolox equivalents per gram of dry weight (μmol TE g^{-1} DW), respectively. All analyses were performed in three replicates and the absorbances were read using a spectrophotometer (Ultrospec 2100 Pro, Ultrospec 2100 pro, Amersham Biosciences, Uppsala, Sweden).

2.7. *AMF Evaluation*

On the base of saffron highly mycorrhization level (70 to 90% mycorrhizal intensity) previously reported [12], we randomly selected saffron roots in April 2018. Then, the root segments were processed for observation in light and under transmission electron microscopy. Root segments were excised under a stereomicroscope and quickly fixed in 2.5% glutaraldehyde in 0.1 M cacodilate buffer (pH 7.2) for 2 hours at room temperature and overnight at 4 °C. The samples were then post-fixed in 1% OsO4 in the same buffer and dehydrated in an ascending series of ethanol to 100%, incubated in two changes of absolute acetone and infiltrated in Epon-Araldite resin [50]. The resin was polimerised for 24 h at 60 °C. Semi-thin (1 μm) sections were then stained with 1% toluidine blue and ultra-thin (70 nm) sections were counter-stained with uranyl acetate and lead citrate [51], and used for electron microscopy analyses under a Philips CM10 transmission electron microscope.

2.8. Chemicals and Reagents

Sodium carbonate, Folin–Ciocalteu phenol reagent, sodium acetate, citric acid, hydrochloric acid, iron (III) chloride hexahydrate, 2,4,6-tripyridyl-S-triazine (TPTZ) and 1,2-phenylenediamine dihydrochloride (OPDA) were purchased from Sigma Aldrich (St. Louis, MO, USA), whereas acetic acid was purchased from Fluka Biochemika (Buchs, Switzerland). Ethylenediaminetetraacetic acid (EDTA) disodium salt was purchased from AMRESCO (Solon, OH, USA), whereas sodium fluoride was purchased from Riedel-de Haen (Seelze, Germany). Ethanol, acetone, sodium citrate and lead nitrate were purchased from Fluka Biochemika. Analytic HPLC grade solvents, methanol and formic acid were purchased from Sigma Aldrich and Fluka Biochemika, respectively; potassium dihydrogen phosphate, ammonium dihydrogen phosphate and phosphoric acid were also purchased from Sigma Aldrich. Milli-Q ultrapure water was produced by Sartorius Stedium Biotech mod. Arium (Sartorius, Goettingen, Germany). Cetyltrimethylammonium bromide (cetrimide) was purchased from Extrasynthése (Genay, France), whereas 1,2-phenylenediamine dihydrochloride (OPDA) was purchased from Sigma Aldrich. All polyphenolic and terpenic standards were purchased from Sigma Aldrich. The organic acids were purchased from Fluka Biochemika, whereas ascorbic acid and dehydroascorbic acid were purchased from Extrasynthése. All chemicals specific for electron and optical microscopy were purchased from Electron Microscopy Sciences (Newark, PA, USA), i.e., glutharaldeyde, cacodylate buffer, osmium tetroxide, epon/araldite resin, toluidine "O" and uranyl acetate.

2.9. Statistical Analysis

An arcsin transformation was performed on all percentage incidence data before statistical analysis in order to improve the homogeneity of the variance (Levene test). All the analysed data were checked for the normality of variance. For all the analysed parameters, mean differences were computed using a one-way ANOVA with a Tukey *post hoc* test ($p \leq 0.05$). Mean comparisons between data obtained in soilless and those from the first growing season of a previous work conducted in open field [12] cultivations were performed using an independent samples t-test. All analyses were performed using SPSS 24.0 Inc. software (SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

3.1. Crop Performance, Quality and Secondary Metabolite Content of Saffron in Soilless Cultivation

Soilless cultivation in a glasshouse has been recently proposed as an alternative method to open field cultivation for saffron. Maggio et al. [19] and Gresta et al. [6] reported that, by controlling growth conditions, flowering could be modulated, extended and considerably increased, compared with open field cultivation. In the present study, under protected conditions, flowering had the same duration (ca. 22 days) compared to cultivation of the same corms planted on the same days in a northwestern Italian open field [12], but the saffron flowering moved forward about 20 days (from 5 October 2017 to 23–30 October 2017), in agreement with Gresta et al. [6]. Since, for the flower emergence, corms required to be transferred from 23–27 °C to 17 °C [18], the most likely reason for this results is related to the fact that, in a glasshouse, the lowering of seasonal temperatures takes place more slowly than in an open field. In addition to the temperature lowering, Gresta et al. [52] indicated the soil water content as another environmental component that can trigger flowering. However, as in these two studies object of comparison, the cultivation occurred in different substrates (quartz sand vs soil), it appears not possible to make speculations.

Saffron yield can vary from 0.15 to 1.5 g m^{-2}, based on planting density, plantation age (from one to six year crop cycles), and climatic conditions during the crop season [1]. In this study, an average of 0.55 g m^{-2} was obtained, indicating a profitable production already during the first year. This yield was similar to what obtained cultivating the same corms at a density of 39 corms m^{-2} in a northwestern Italian open field [12] and superior to that obtained in south Italy under similar glasshouse conditions by Gresta et al. [6] (corm density equal to 40 corms m^{-2}; 0.46 g m^{-2}) with corms

coming from Sardinia (Italy). With similar corm density to our work, Cavusoglu and Erkel [53] and Maggio et al. [19] obtained much higher yields (0.88 g m^{-2} and 2.34 g m^{-2}, respectively) in glasshouses located in Turkey and south Italy. In Iranian open fields, at a corm density similar to our study, Mollafilabi et al. [24] and Koocheki and Seyyedi [54] obtained an average spice yield of 0.48 g m^{-2}. As affirmed by Gresta et al. [52], to trigger saffron flowering, a not yet fully understood combination of temperature and soil water content is needed.

In addition to the spice yield, another economically important attribute of saffron is the number of replacement corms. The obtained values (2.63 replacement corms $corm^{-1}$) are lower of those obtained by Maggio et al. [19] in soilless cultivation in a cold glasshouse in south Italy, by using peat and perlite (1:1) substrates, where corms produced from 3.0 to 4.5 replacement corms per corm. In addition to a different substrate, these authors also incubated corms in the dark for 83 days before planting. Thus, the combination of these two factors could have guarantee a superior result. Comparing to open field experiments that used corms with similar size to our study, results were in agreement with those from our trial in northwest Italy [12], and the trials performed by Turhan et al. [55] in Turkey (2.32 replacement corms $corm^{-1}$), while superior to those obtained by Koocheki and Seyyedi [54] in Iranian fields (1.32 replacement corms $corm^{-1}$).

Guidelines for the analyses of the main compounds that contribute to the sensory profile of saffron have been established by ISO 3632 regulations [44]. These regulations define procedures to determine these compounds by spectrophotometric analyses and have established the limits by which saffron quality is classified into three different categories (first, second and third). Specifically, the saffron produced under soilless conditions belongs to the highest quality, i.e., first category, for all the studied parameters.

The evaluation of antioxidant activity is generally considered as an important method to evaluate the nutraceutical properties of food, as indicated in other previous studies [30]. Apart from crocins, Karimi et al. [56] and Asdaq and Inamdar [57] highlighted that phenols and flavonols are responsible for the antioxidant potential of saffron. Overall, the saffron produced in soilless systems showed a very high TPC (4445.4 mg_{GAE} $100g^{-1}$ DW), more than the saffron cultivated in other sites in the Alps (range between 1340 and 2355 mg_{GAE} $100g^{-1}$ DW) [12], Lebanon (160 mg_{GAE} $100g^{-1}$ DW) [58], and India (828 mg_{GAE} $100g^{-1}$ DW) [8]. In terms of antioxidant activity, FRAP values were superior to those of Iranian and Italian samples (circa 570 and 1250 mmol Fe^{2+} kg^{-1}) [12,56] and ABTS values were comparable to those found in Italian and Greek saffron by Caser et al. [12] and Ordoudi et al. [59].

3.2. AMF Colonisation

In our study, the presence of AMF and their colonisation of saffron roots were confirmed by observations using light microscopy (Figure 1) and transmission electron microscopy (TEM; Figure 2) on semi-thin and thin sections, respectively. Observations on semi-thin sections, stained in blue, show that the saffron roots are mycorrhised when inoculated with both inocula (Figure 1A–C), confirming the mycorrhizal intensity described in Caser et al. [12]. At the level of the cortical root parenchyma, the typical mycorrhizal arbuscular fungal structures have been highlighted (insets Figure 1A,C). Figure 1 shows the presence of intercellular and intracellular hyphae (Figure 1C) and arbuscules (Figure 1A,B). No fungal structures were found in the roots of the control treatments (Figure 1D).

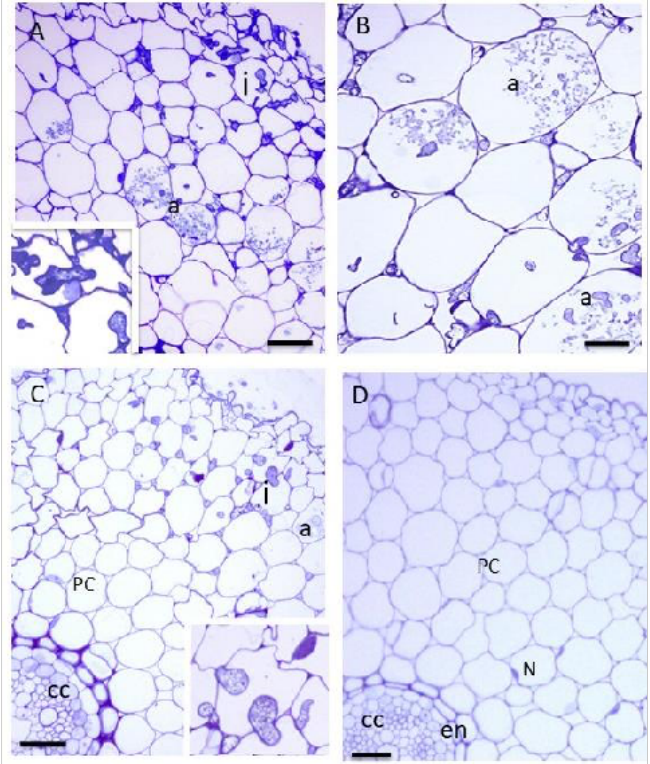


Figure 1. Light microscope images of semi-thin sections of *Crocus sativus* roots inoculated with *Rhizophagus intraradices* and *Funneliformis mosseae* (Ri+Fm, **A**), *R. intraradices* alone (Ri, **B** and **C**) or the control (arbuscular mycorrhizal fungi (AMF)-, **D**), stained with toluidine blue. At the level of the cortical cells, note the presence of intercellular and intracellular hyphae (i) and arbuscules (a). Magnification in insets A and C shows details of the intracellular hyphae. Cortical parenchyma (PC) cells with nucleus (N) are indicated. No fungal structure is present between and inside the root cells in AMF-roots (D). Note the central cylinder (cc) and the endodermide (en). Bars are 20 µm in A, C and D, and 10 µm in B.

Here, the host plasma membrane invaginates and proliferates around all the developing intracellular fungal structures, and cell wall material is laid down between this membrane and the fungal cell surface. The exchange of molecules between the fungal and plant cytoplasm takes place both through their plasma membranes and their cell walls; a functional compartment, known as the symbiotic interface, is thus defined. At the electron microscope level, as seen in Figure 2A,C (arrows), this new apoplastic space, based on membrane proliferation, is evident around the intracellular and arbusculated hyphae of the AMF penetrated inside the saffron root cortical cells. On the basis of TEM observations, we can conclude that the mycorrhizae, formed between saffron roots and the two species of AM fungi in the inocula used in pot experiments, are alive and functionally active.

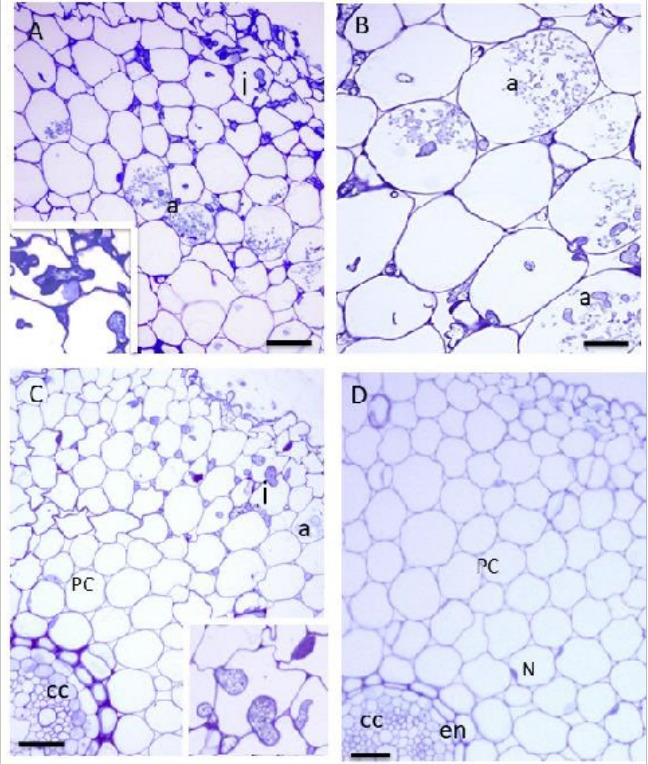


Figure 1. Light microscope images of semi-thin sections of *Crocus sativus* roots inoculated with *Rhizophagus intraradices* and *Funneliformis mosseae* (Ri+Fm, **A**), *R. intraradices* alone (Ri, **B** and **C**) or the control (arbuscular mycorrhizal fungi (AMF)-, **D**), stained with toluidine blue. At the level of the cortical cells, note the presence of intercellular and intracellular hyphae (i) and arbuscules (a). Magnification in insets A and C shows details of the intracellular hyphae. Cortical parenchyma (PC) cells with nucleus (N) are indicated. No fungal structure is present between and inside the root cells in AMF-roots (D). Note the central cylinder (cc) and the endodermide (en). Bars are 20 µm in A, C and D, and 10 µm in B.

Here, the host plasma membrane invaginates and proliferates around all the developing intracellular fungal structures, and cell wall material is laid down between this membrane and the fungal cell surface. The exchange of molecules between the fungal and plant cytoplasm takes place both through their plasma membranes and their cell walls; a functional compartment, known as the symbiotic interface, is thus defined. At the electron microscope level, as seen in Figure 2A,C (arrows), this new apoplastic space, based on membrane proliferation, is evident around the intracellular and arbusculated hyphae of the AMF penetrated inside the saffron root cortical cells. On the basis of TEM observations, we can conclude that the mycorrhizae, formed between saffron roots and the two species of AM fungi in the inocula used in pot experiments, are alive and functionally active.

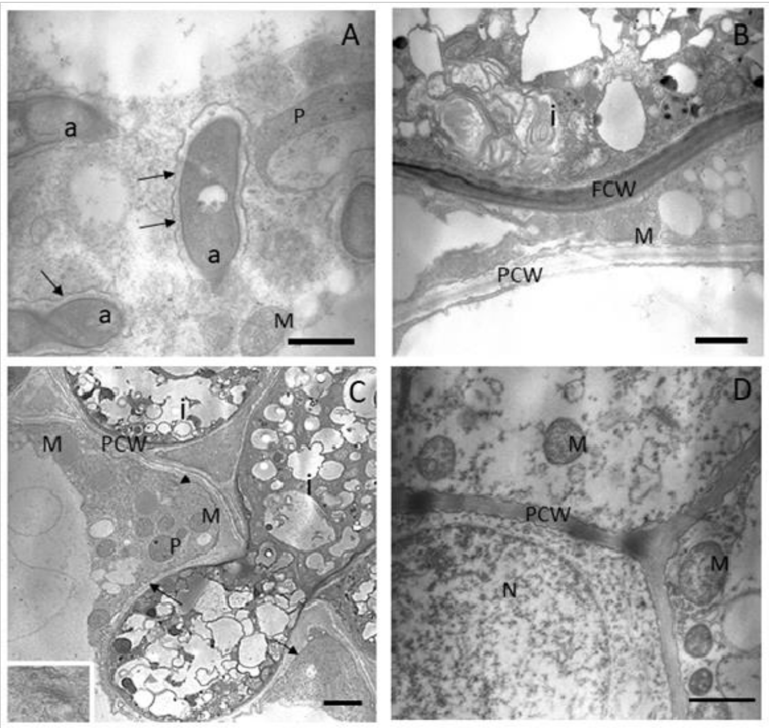


Figure 2. Transmission electron microscopy images of thin sections of saffron roots colonised by *Rhizophagus intraradices* and *Funneliformis mosseae* (Ri−Fm, **A**), *R. intraradices* alone (Ri, **B** and **C**) or the control (AMF-, **D**). In details, a: fungal arbuscule; N: nucleus; M: mithocondria; P: plastids; i: fungal hyphae; PCW: plant cell wall; FCW: fungal cell wall; arrow: plant plasmamembrane; arrowhead and inset: Golgi apparatus. The bar is 1 μm in A, B, C and D.

3.3. *Impact of AMF on Saffron in Soilless Cultivation*

3.3.1. Crop Performance and Quality Classification

In the present study, slight differences in flowering time and production were detected between treated corms (Figure 3 and Table 2). Both applied inocula (Ri and Ri+Fm) anticipated saffron flowering time of one week, compared to untreated corms (AMF-; 23 October vs. 30 October), whereas the flowering peaks and end of flowering occurred in about the same number of days (6–9 November and 11–13 November, respectively).

No significant differences were observed between the treatments in terms of the number of flowers corm^{-1} and the obtained mg of spice flower^{-1} (Table 2). Very few reports about the effective role of AMF in saffron flowering and yield are available in the literature, and only under open field conditions. Aimo et al. [40] and Caser et al. [12] indicated a positive role of AMF on the saffron productive performance, with an increase in flower production (+68% and +138%, respectively, compared to the untreated corms) using AMF species belonging to the genus *Glomus*.

Table 2. Effects of AMF inoculum composed of *Rhizophagus intraradices* alone (Ri), *R. intraradices* and *Funneliformis mosseae* (Ri+Fm) or the control (AMF-) on yield performances (flower corm^{-1} and saffron flower^{-1}), growth (number of replacement corms corm^{-1}; replacement corm size and weight variation between the end and beginning of the trial) and mean absorbance values for picrocrocin, safranal and crocin of saffron samples obtained during glasshouse cultivation.

	Yield		Replacement corm			Quality category (ISO3632 [44])		
Treatment	Flower corm^{-1} (n)	Saffron flower^{-1} (mg)	Size (%)	Corm^{-1} (n)	Weight (%)	Picrocrocin ($A^{1\%}_{1cm}$ (λ257)	Safranal ($A^{1\%}_{1cm}$ (λ330)	Crocin ($A^{1\%}_{1cm}$ (λ440)
Ri	0.84 ± 0.62	6.8 ± 1.3	45.8 ± 4.6a	2.71 ± 1.53	7.8 ± 5.6	143.8 + 4.6(I)$^{\beta}$a	61.0 ± 5.3(I)a	422.6 ± 4.1(I)a
Ri+Fm	0.66 ± 0.60	6.0 ± 1.4	54.6 ± 6.2a	2.25 ± 0.95	8.6 ± 3.8	124.3 ± 3.9(I)c	30.7 ± 3.4(I)c	164.2 ± 3.8(III)c
AMF-	0.97 + 0.53	6.6 + 0.4	33.1 + 6.8b	2.63 + 1.06	12.6 + 5.1	135.9 + 3.4(I)b	54.3 + 6.7(I)b	324.7 + 5.9(I)b
p	ns	ns	***	ns	ns	***	***	***

Mean values with the same letter are not statistically different at $p \leq 0.05$, according to a Tukey *post hoc* test. The statistical relevance of 'Between-Subjects Effects' tests (***$p < 0.001$, ns = not significant). $^{\beta}$ The quality category (ISO3632) is indicated in brackets. The limits for the first (I) quality category are: picrocrocin>70; safranal 20–50; crocins>200. ISO3632 limits for the second (II) quality category are: picrocrocin >55; safranal 20–50; crocins >170. ISO3632 limits for the third (III) category are: picrocrocin>40; safranal 20–50; crocins>120.

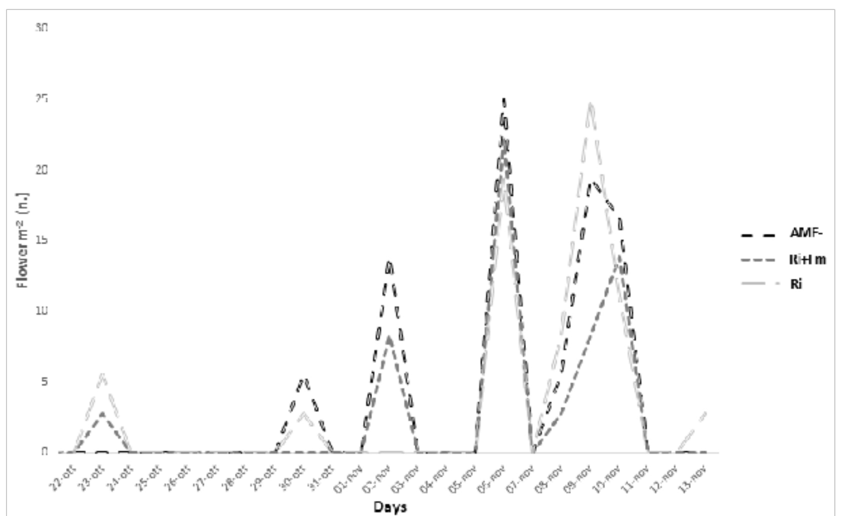


Figure 3. Effects of AMF inoculum composed of *Rhizophagus intraradices* alone (Ri), *R. intraradices* and *Funneliformis mosseae* (Ri+Fm) or the control (AMF-) on the flowering calendar of *Crocus sativus* corms and the daily number of picked flowers m^{-2} during soilless cultivation.

Both of the AMF inocula increased the size of replacement corms in comparison to untreated corms (Table 2), suggesting a positive effect on flower production for the following cultivation cycle, in agreement with Aimo et al. [40] and Mohebi-Anabat et al. [39]. Corm size is indeed a major factor in bulbous plants to determine the flowering capacity and production of new replacement corms [5,42].

Saffron quality greatly depends on the growing conditions [12,60]. In the present study, among the AMF inocula, *R. intraradices* alone significantly increased the content of picrocrocin (bitterness), safranal (flavouring strength) and crocins (colouring strength), in comparison to the other treatments. On the contrary, Ri+Fm significantly reduced the content of these molecules and, thus the quality of the spice, in particular by lowering the crocin content to the third category of ISO 3632. To the best of our knowledge, this is the first report indicating the effect of AMF on the quality (ISO) of saffron obtained by soilless cultivation. The positive role of Ri on the increase of the saffron quality, especially on the content of picrocrocin, was highlighted also in northwestern Italian open field [12]. Thus, the corm inoculation with Ri could further increase the already high quality saffron produced in the Italian Alps [45,61].

3.3.2. Saffron Metabolic Profiling Comparing to Other Foods

In addition to the peculiar organoleptic characteristics, the stigmas of the *C. sativus* flower contain many secondary metabolites with demonstrated pharmacological effects [3,11,62–64]. The identification and quantification of bioactive compounds in saffron and the evaluation of their biological activities are important to gauge their potential efficacy in food and pharmaceutical industries [65]. The range of all chemicals can vary greatly as a result of growing conditions, such as in response to the application of biostimulants [63]. Inoculation with AMF is known to alter the production of secondary metabolites in MAPs, both in roots, shoots, and flowers, even if is not consistent among plant organs [66]. The effects of AMF inocula on the biosynthesis of secondary metabolites in saffron are presented in Table 3. This more in-depth analysis confirmed the results obtained by assessing the spice quality according to ISO3632 guidelines. The single species inoculum Ri significantly increased the content of crocins (crocin I and II), whereas the mix Ri+Fm decreased it; these findings are in agreement with those obtained by Caser et al. [12] under field conditions in a temperate mountain area (north-west Italy), where the saffron obtained by corms inoculated with Ri resulted in superior quality (i.e., quality compared to

the ISO standards). Regarding antioxidant activity (AOA), inoculation with Ri resulted in superior values in both used methods (FRAP and ABTS). The AMF inoculum composed of Ri+Fm significantly increased the contents of isoquercitrin and the total phenolic (TPC) compared to Ri, while of ellagic acid in comparison to Ri and AMF-. Differences in results according to the AMF inoculum composition were also observed in other plant species cultivated on different substrates. Among the reviewed studies, it has been found that the single inoculation of *R. intraradices* tend to be more successful for bioactive compounds increase than inoculation experiments with more than one species applied at the same time. In *Echinacea purpurea* Moench. [67] cultivated in a sand and soil (1:1) substrate, *R. intraradices* alone increased more the content of polyphenols than the mixed inoculum, while in *Cynara cardunculus* L. cultivated in sandy soil [68] and *Lactuca sativa* L. cultivated in a mixture of peat, sandy loam soil and calcinated clay (1:1:1) [69] *R. intraradices* enhanced more the antioxidant activity. However, it has not been observed any effect on the accumulation of polyphenols in *Ocimum basilicum* L. cultivated in a sterilised sand and soil (3:1) substrate [70] and in *Salvia officinalis* L. in sand, soil, and expanded clay (1:1:1) [71,72].

Table 3. Bioactive compounds, anthocyanins, total polyphenol content and antioxidant activity (ferric reducing antioxidant power (FRAP) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS); antioxidant activity (AOA)) of the saffron produced via glasshouse cultivation with AMF inocula composed of *Rhizophagus intraradices* alone (Ri), *R. intraradices* and *Funneliformis mosseae* (Ri+Fm) or a control (AMF-).

Class	Compound (mg $100g^{-1}$ DW)	Ri	Ri+Fm	AMF-	*p*
Cinnamic acids	Coumaric acid	23.1 ± 3.5	23.7 ± 2.6	23.7 ± 3.1	ns
Flavonols	Isoquercitrin	1.9 ± 0.3b	2.6 ± 0.2a	2.3 ± 0.3ab	**
	Quercitrin	17.8 ± 4.6	11.6 ± 4.1	19.1 ± 3.6	ns
Benzoic acids	Gallic acid	4.5 ± 1.5	5.1 ± 1.3	4.9 ± 1.4	ns
	Ellagic acid	1.9 ± 0.5b	3.2 ± 0.3a	1.0 ± 0.4b	**
Catechins	Catechin	1.9 ± 0.4	1.6 ± 0.3	1.8 ± 0.3	ns
	Epicatechin	9.8 ± 2.9	5.9 ± 2.1	9.6 ± 2.5	ns
Carotenoids	Safranal	4.0 ± 0.9	4.0 ± 1.2	4.0 ± 0.7	ns
	Crocin I	104.2 ± 8.6a	22.1 ± 6.5c	55.5 ± 8.4b	***
	Crocin II	42.7 ± 9.6a	16.4 ± 3.8b	38.7 ± 12.9ab	**
Vitamin C	Dehydroascorbic acid	28.8 ± 6.5	30.2 ± 1.1	31.8 ± 6.9	ns
	Ascorbic acid	31.1 ± 9.5	36.3 ± 6.7	41.7 ± 4.8	ns
	Total vitamin C	59.9 ± 10.2	66.5 ± 5.9	73.6 ± 8.4	ns
TAC	Anthocyanin (mg_{C3G} $100g^{-1}$ DW)	640.7 ± 84.6b	146.4 ± 29.8c	1654.5 ± 68.4a	*
	Methods				
TPC	Folin–Ciocalteu (mg_{GAE} $100g^{-1}$ DW)	816.5±152.7b	3619.0±400.2a	4445.4±430.2a	***
AOA	FRAP (mmol Fe^{2+} kg^{-1} DW)	3133.9±1524.3a	1383.0±589.7ab	379.7±128.4b	**
	ABTS (μmol_{TE} g^{-1} DW)	5.4±0.8a	3.6±0.4c	4.5±0.7ab	**

Mean values with the same letter are not statistically different at $p \leq 0.05$, according to a Tukey *post hoc* test. The statistical relevance of 'Between-Subjects Effects' tests (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns – not significant).

Karimi et al. [56] and Rahaiee et al. [63] indicated that the antioxidant capacities of saffron might be due to the presence of total phenolics and flavonoids. Based on the obtained results, the content of the bioactive compounds detected in saffron could be compared to other commonly eaten fruits with highly advantageous nutritive properties. Saffron had a higher total phenol content (TPC) and antioxidant activity (AOA) than fresh *Ribes nigrum* L. berries (circa +1000% and +493%, respectively), and fresh (circa +2000% and +1800%, respectively) and dried (circa +900% and +1650%, respectively) *Lycium* spp. fruits [65,73], analysed with the same method. Since saffron showed an antioxidant activity superior than 500 mg_{GAE} $100g^{-1}$ it could be also listed within the health beneficial fruits such as *Rubus glaucus* Benth. and *Prunus serotina* var. Capuli as suggested by Vasco et al. [74]. Its content of vitamin C was similar to what found in *Actinidia deliciosa* (A.Chev.) C.F.Liang & A.R.Ferguson and *Citrus sinensis* (L.) Osb., and even higher than in *Lycium* spp. (+150%) and *Vaccinium* spp. (+380%). Also, the coumaric acid content was superior (+85%) than in *Morus nigra* L. fruits [75] while lower than in *Lycium* spp. fruits, that showed also higher content of gallic acid, ellagic acid, catechin, and epicatechin

was generally lower in saffron (on average circa −75%, −70%, −92%, and −95%, respectively) [73,75]. Lastly, the content of anthocyanins, that are suggested to have neuroprotective properties [76], was up to 11654.5 mg_{C3G} $100g^{-1}$ DW, i.e., a value very high in comparison to fresh fruit extracts from *Morus nigra*, *Rubus idaeus* L., and *Fragaria ananassa* D. (80.0, 33.7, and 35.2 mg_{C3G} $100g^{-1}$, respectively) [75].

3.3.3. Soilless Cultivation vs. Open Field

Saffron root colonisation by AMF could be affected by the cultivation conditions related to the substrate composition, root temperature or the presence of antagonistic fungi naturally occurring in the soil [31,40,41,76]. In our recent studies, AM fungal colonisation was noted in *C. sativus* roots inoculated with Ri and Ri+Fm, both in soilless (Figures 1 and 2) and in open field conditions [12]. Figures 4 and 5 report the comparisons of the results obtained by these studies. Compared to open field, in soilless conditions not-inoculated corms (AMF-) showed similar spice yields but with higher quality while, referring to AMF treatments, Ri-inoculated corms produced less spice but with a higher quality, whereas Ri+Fm inoculated corms produced less spice, with a lower quality (i.e., reduction in crocin content).

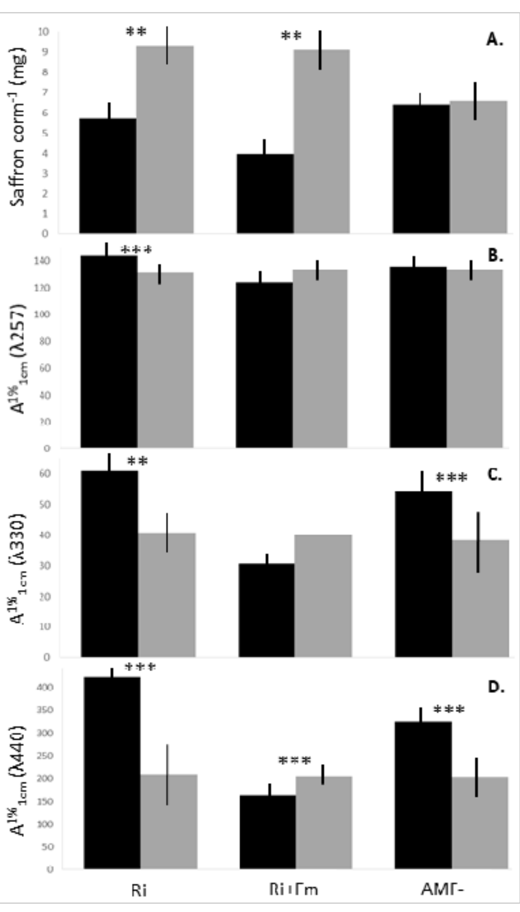


Figure 4. Effects of AMF inoculum consisting of *Rhizophagus intraradices* alone (Ri), *R. intraradices* and *Funneliformis mosseae* (Ri+Fm) or a control (AMF-) on (**A.**) mg of saffron $corm^{-1}$, (**B.**) picrocrocin, (**C.**) safranal, and (**D.**) crocin of *Crocus sativus* corms cultivated in soilless (black bars) and open field (grey bars, [12]) conditions. Mean comparisons of each treatment in the two cultivation types were performed using an independent samples t-test.

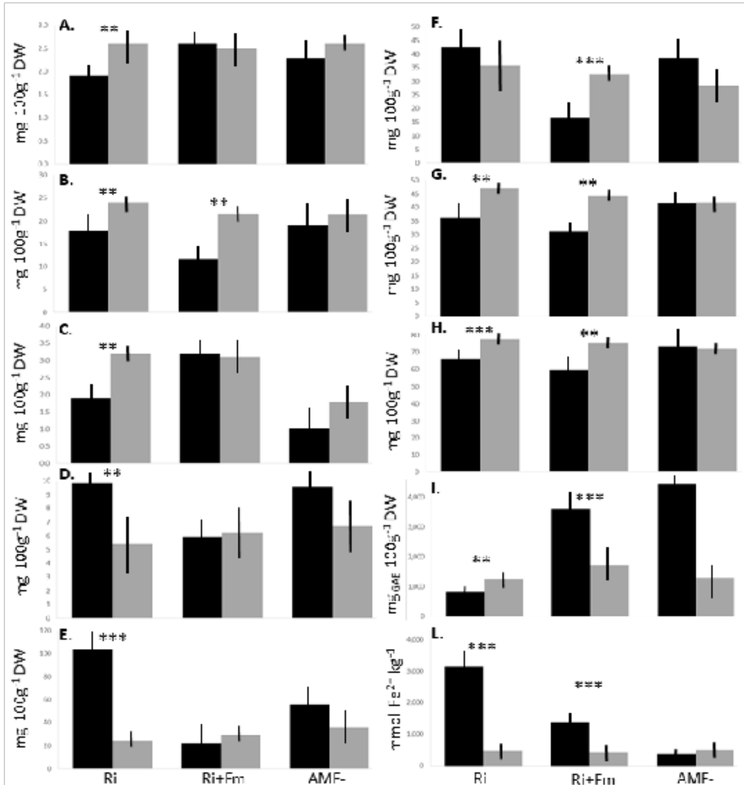


Figure 5. Effects of AMF inoculum consisting of *Rhizophagus intraradices* alone (Ri), *R. intraradices* and *Funneliformis mosseae* (Ri+Fm) or a control (AMF-) on the content of (**A.**) isoquercitrin, (**B.**) quercitrin, (**C.**) ellagic acid, (**D.**) epicatechin, (**E.**) crocin I, (**F.**) crocin II, (**G.**) ascorbic acid, (**H.**) vitamin C. (**I.**) total polyphenol content (TPC), and (**L.**) antioxidant activity (FRAP assay) of saffron produced in soilless (black bars) and open field (grey bars, [12]) conditions. Mean comparisons of each treatment in the two cultivation types were performed using an independent samples t-test.

With respect to the nutraceutical compounds, the comparisons are presented in Figure 5. No differences were reported between the untreated corms (AMF-), whereas the application of Ri in the soilless condition induced an increase in the contents of epicatechin, crocin I, and antioxidant activity (+80%, +435%, and +675%, respectively), while a decrease in the contents of isoquercitrin, quercitrin, ellagic acid, ascorbic acid, vitamin C, and TPC. Fewer differences were induced by Ri+Fm, which positively stimulated both the total phenolic content and antioxidant activity (+210% and +325%, respectively), but caused a decrease in quercitrin, crocin II, ascorbic acid, and vitamin C.

4. Conclusions

Soilless cultivation in a glasshouse appeared as an effective strategy for the cultivation of saffron with a first-year cultivation spice yield that is comparable with open field production sites. Moreover, the high quality saffron produced via soilless cultivation presented an elevated content of several health-promoting compounds with highly advantageous nutritive properties, such as polyphenols and elevated antioxidant activity. Further studies are needed to define better the methodologies to modulate time and duration of flowering, to improve yield, and to efficiently schedule harvest practices.

Arbuscular mycorrhizal-based products have received great interest in agriculture for their potential to improve crop productivity, nutritional quality, as well as resistance to plant pathogens and

numerous environmental stresses. The literature highlights that AMF must be chosen by evaluating different aspects, such as the inoculum type, host plants, and the environmental and growing conditions.

Here, AMF successfully colonised *C. sativus* roots; their effects varied on the basis of inoculum type and cultivation conditions. Among the studied AMF inocula, *R. intraradices* appeared to give more benefits to *C. sativus* than the mix of *R. intraradices* and *F. mosseae*. Specifically, the *R. intraradices* inoculation appeared successful in open field to increase spice yields while in soilless systems to increase the spice quality.

Thus, soilless systems appeared as an effective alternative cultivation strategy for the production of high quality saffron. Further benefits can be obtained by the application of targeted AMF-based biostimulants. A cost-benefit analysis should be performed to assess the economic sustainability.

Author Contributions: M.C., E.L., V.B. and V.S. contributed to the experimental design. M.C., S.D., Í.M.M.V., D.D., A.F. and V.S. acquired and interpreted the data. M.C. drafted the manuscript. V.B. and V.S. conceived the study, coordinated the work and critically revised the manuscript.

Funding: This research was funded by the project titled 'SaffronALP—Lo zafferano di montagna: tecniche sostenibili per una produzione di qualità' - Fondazione Cassa di Risparmio di Torino (RF=2017.1966) and by the program Interreg V-A Francia Italia Alcotra "Attività innovative per lo sviluppo della filiera transfrontaliera del fiore edule–Antea' n. 1139.

Acknowledgments: The authors acknowledge Dario Sacco for statistical assistance and Alessandro Putzolu for technical assistance.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Gresta, F.; Santonoceto, C.; Avola, G. Crop rotation as an effective strategy for saffron (*Crocus sativus* L.) cultivation. *Sci. Hortic.* **2016**, *211*, 34–39. [CrossRef]
2. Maleki, F.; Kazemi, H.; Siahmarguee, A.; Kamkar, B. Development of a land use suitability model for saffron (*Crocus sativus* L.) cultivation by multi criteria evaluation and spatial analysis. *Eco Eng.* **2017**, *106*, 140–153. [CrossRef]
3. Giorgi, A.; Scheurer, T. Alpine resources: Assets for a promising future-conclusions from the forum alpinum. *Mount. Res. Dev.* **2015**, *35*, 414–415. [CrossRef]
4. Husaini, A.; Shafiq, A.; Parvaze, S.; Rather, G.; Parray, A.; Shikari, A.; Javid, M. Bioinformatics for saffron (*Crocus sativus* L.) improvement. *Comm. Biomet. Crop. Sci.* **2009**, *4*, 3–8.
5. Gresta, F.; Lombardo, G.; Siracusa, L.; Ruberto, G. Saffron an alternative crop for sustainable agricultural systems. A review. *Agron. Sustain. Dev.* **2008**, *28*, 95–112. [CrossRef]
6. Gresta, F.; Avola, G. Hypothesis on the relation between meteorological data and flowering peaks in saffron (*Crocus sativus* L.) crop. *Acta Hortic.* **2017**, *1184*, 189–194. [CrossRef]
7. Elgazar, A.; Rezq, A.; Bukhari, H. Anti-Hyperglycemic Effect of Saffron Extract in Alloxan-Induced Diabetic Rats. *Europ. J. Biol. Sci.* **2013**, *5*, 14–22.
8. Baba, S.; Mohiudin, T.; Basu, S.; Swarnkar, M.; Malik, A.; Abbas, N.; Singh, A.; Ashraf, N. Comprehensive transcriptome analysis of *Crocus sativus* for discovery and expression of genes involved in apocarotenoid biosynthesis. *BMC Genom.* **2015**, *16*, 698. [CrossRef] [PubMed]
9. Lahmass, I.; Touria, L.; Delporte, C.; Sikdar, S.; Antwerpen, P.; Saalaoui, E.; Mégalizzi, V. The waste of saffron crop, a cheap source of bioactive compounds. *J. Funct. Foods* **2017**, *35*, 341–351. [CrossRef]
10. Cusano, E.; Consonni, R.; Petrakis, E.A.; Astraka, K.; Cagliani, L.R.; Polissiou, M.G. Integrated analytical methodology to investigate bioactive compounds in *Crocus sativus* L. flowers. *Phytochem. Anal. Phytochem. Analysis* **2018**, *29*, 1–11. [CrossRef]
11. Menghini, L.; Leporini, L.; Vecchiotti, G.; Locatelli, M.; Carradori, S.; Ferrante, C.; Zengin, G.; Recinella, L.; Chiavaroli, A.; Leone, S.; et al. *Crocus sativus* L. stigmas and byproducts: Qualitative fingerprint, antioxidant potentials and enzyme inhibitory activities. *Food Res. Int.* **2018**, *109*, 91–98. [CrossRef]
12. Caser, M.; Maxaiele Victorino, Í.M.; Demasi, S.; Berruti, A.; Donno, D.; Lumini, E.; Bianciotto, V.; Scariot, V. Saffron cultivation in marginal Alpine environments: How AMF inoculation modulates yield and bioactive compounds. *Agronomy* **2019**, *9*, 12. [CrossRef]

13. Talaei, A.; Moghadam, H.; Tabassi, S.; Mohajeri, S. Crocin, the main active saffron constituent, as an adjunctive treatment in major depressive disorder: A randomized, double-blind, placebo-controlled, pilot clinical trial. *J. Affect. Disord.* **2015**, *174*, 51–56. [CrossRef]
14. Landi, R. *Lo zafferano: Tradizione e qualità. Camera di commercio: Industria, Artigianato e Agricoltura di Firenze*; CCIAA (Camera di Commercio: Industria, Artigianato e Agricoltura): Firenze, Italy, 2007; Volume 4, p. 65.
15. Lachaud, C. *La Bible du Safranier. Tout savoir sur le Crocus sativus et sur le Safran*, 1st ed.; La Paradisière: Correze, France, 2012; ISBN 978-2-7466-4412-0.
16. De Juan, J.A.; Córcoles, H.L.; Muñoz, R.M.; Picornell, M.R. Yield and yield components of saffron under different cropping systems. *Ind. Crops Prod.* **2009**, *30*, 212–219. [CrossRef]
17. Yarami, N.; Kamgar-Haghighi, A.A.; Sepaskhah, A.R.; Zand-Parsa, S. Determination of the potential evapotranspiration and crop coefficient for saffron using a water-balance lysimeter. *Arch. Agr. Soil Sci.* **2011**, *57*, 727–740. [CrossRef]
18. Molina, R.; Valero, M.; Navarro, Y.; Guardiola, J.; Garcia Luis, A. Temperature effects on flower formation in saffron (*Crocus sativus* L.). *Sci. Hortic.* **2005**, *103*, 361–379. [CrossRef]
19. Maggio, A.; Raimondi, G.; Martino, A.; De Pascale, S. Soilless cultivation of saffron in mediterranean environment. *Acta Hortic.* **2006**, *718*, 515–522. [CrossRef]
20. Benschop, M. *Crocus*; De Hertogh, A., Le Nard, M., Eds.; The Physiology of Flower Bulbs, Elsevier Science Publishers: Amsterdam, The Netherlands, 1993; pp. 257–272.
21. De Mastro, G.; Ruta, C. Relation between corm size and saffron (*Crocus sativus* L.) flowering. *Acta Hortic.* **1993**, *334*, 512–517. [CrossRef]
22. Douglas, M.H.; Smallfield, B.M.; Wallace, A.R.; McGimpsey, J.A. Saffron (*Crocus sativus* L.): The effect of mother corm size on progeny multiplication, flower and stigma production. *Sci. Hortic.* **2014**, *166*, 50–58. [CrossRef]
23. Kumar, R. Calibration and validation of regression model for non-destructive leaf area estimation of saffron (*Crocus sativus* L.). *Sci. Hortic.* **2009**, *122*, 142–145. [CrossRef]
24. Mollafilabi, A.; Koocheki, A.; Moeinerad, H.; Kooshki, L. Effect of plant density and weight of corm on yield and yield components of saffron (*Crocus sativus* L.) under soil, hydroponic and plastic tunnel cultivation. *Acta Hortic.* **2013**, *997*, 51–58. [CrossRef]
25. Putra, P.A.; Yuliando, H. Soilless culture system to support water use efficiency and product quality: A review. *Agric. Agricult. Sci. Proc.* **2015**, *3*, 283–288. [CrossRef]
26. Augé, R.M.; Toler, H.D.; Saxton, A.M. Arbuscular mycorrhizal symbiosis alters stomatal conductance of host plants more under drought than under amply watered conditions: A meta-analysis. *Mycorrhiza* **2015**, *25*, 13–24. [CrossRef] [PubMed]
27. Helal Beigi, Y.; Khoshgoftar, M.; Shamsi, A.; Zamani, N. Possibility of saffron corm growth in different bed of cultivation in soilless system. In *Proceedings of 1st Congress of Hydroponic in Greenhouse Production*; Isfahan University of Technology: Isfahan, Iran, 2009.
28. Sourell, F.; Weathers, P. The growth of saffron (*Crocus sativus L.*) in aeroponics and hydroponics. *J. Herb. Spices Med. Plants* 2000, *7*, 113–127.
29. Du Jardin, P. Plant biostimulants: Definition, concept, main categories and regulation. *Sci. Hortic.* **2015**, *196*, 3–14. [CrossRef]
30. Rouphael, Y.; Franken, P.; Schneider, C.; Schwarz, D.; Giovannetti, M.; Agnolucci, M.; De Pascale, S.; Bonini, P.; Colla, G. Arbuscular mycorrhizal fungi acta s biostimulants in horticultural crops. *Sci. Hortic.* **2015**, *196*, 91–108. [CrossRef]
31. Berruti, A.; Lumini, E.; Balestrini, R.; Bianciotto, V. Arbuscular mycorrhizal fungi as natural biofertilizers: Let's benefit from past successes. *Front. Microbiol.* **2016**, *6*, 1559. [CrossRef]
32. Smith, S.E.; Read, D.J. *Mycorrhizal Symbiosis*; Academic Press: London, UK, 1997; Volume 40, p. 154.
33. Todeschini, V.; Lahmidi, N.A.; Mazzucco, E.; Marsano, F.; Gosetti, F.; Robotti, E.; Bona, E.; Massa, N.; Bonneau, L.; Marengo, E.; et al. Impact of beneficial microorganisms on strawberry growth, fruit production, nutritional quality, and volatilome. *Front. Plant. Plant Sci.* **2018**, *9*, 1611. [CrossRef] [PubMed]
34. Pellegrino, E.; Bedini, S. Enhancing ecosystem services in sustainable agriculture: Biofertilization and biofortification of chickpea (*Cicer arietinum* L.) by arbuscular mycorrhizal fungi. *Soil Biol. Biochem.* **2014**, *68*, 429–439. [CrossRef]

35. Zeng, Y.; Guo, L.; Chen, B.; Hao, Z.; Wang, J.; Huang, L.; Yang, G.; Cui, X.; Yang, L.; Wu, Z.; et al. Arbuscular mycorrhizal symbiosis and active ingredients of medicinal plants: Current research status and prospectives. *Mycorrhiza* **2013**, *23*, 253–265. [CrossRef] [PubMed]
36. Giovannetti, M.; Avio, L.; Sbrana, C. *Improvement of nutraceutical value of food by plant symbionts*; Ramawat, K.G., Merillon, J.M., Eds.; Natural Products, Phytochemistry, Botany and Metabolism of Alkaloids, Phenolics and Terpenes; Springer: Berlin/Heidelberg, Germany, 2013; pp. 2641–2662.
37. Swamy, M.K.; Akhtar, M.S.; Sinniah, U.R. *Response of PGPR and AM fungi toward growth and secondary metabolite production in medicinal and aromatic plants*; Hakeem, K., Akhtar, M., Eds.; Plant, Soil and Microbes; Springer: Heidelberg, Germany, 2016.
38. Avio, L.; Turrini, A.; Giovannetti, M.; Sbrana, C. Designing the ideotype mycorrhizal symbionts for the production of healthy food. *Front. Plant Sci.* **2018**, *9*, 1089. [CrossRef] [PubMed]
39. De Sousa Oliveira, M.; da Silva Campos, M.; de Albuquerque, U.P.; da Silva, F.S.B. Arbuscular mycorrhizal fungi (AMF) affects biomolecules content in *Myracrodruon urundeuva* seedlings. *Ind. Crops Prod.* **2013**, *50*, 244–247. [CrossRef]
40. Aimo, S.; Gosetti, F.; D'Agostino, G.; Gamalero, E.; Gianotti, V.; Bottaro, M.; Gennaro, M.; Berta, G. Use of arbuscular mycorrhizal fungi and beneficial soil bacteria to improve yield and quality of saffron (*Crocus sativus* L.). *Acta Hortic.* **2010**, *850*, 159–162. [CrossRef]
41. Lone, R.; Shuab, R.; Koul, K. AMF association and their effect on metabolite mobilization, mineral nutrition and nitrogen assimilating enzymes in saffron (*Crocus sativus*) plant. *J. Plant Nutr.* **2016**, *39*, 1852–1862. [CrossRef]
42. Shajari, M.A.; Moghaddam, P.R.; Ghorbani, R.; Koocheki, A. Increasing saffron (*Crocus sativus* L.) corm size through the mycorrhizal inoculation, humic acid application and irrigation managements. *J. Plant Nutr.* **2018**, *41*, 1047–1064. [CrossRef]
43. Mohebi-Anabat, M.; Riahi, H.; Zanganeh, S.; Sadeghnezhad, E. Effects of arbuscular mycorrhizal inoculation on the growth, photosynthetic pigments and soluble sugar of *Crocus sativus* L. (saffron) in autoclaved soil. *Int. J. Agron. Agric. Res.* **2015**, *6*, 296–304.
44. ISO 3632-1:2011. *Spices—Saffron (Crocus sativus L.)*; Food products SC 7, Spices, culinary herbs and condiments; International Organization for Standardization: Geneva, Switzerland, September 2011.
45. Giorgi, A.; Pentimalli, D.; Giupponi, L.; Panseri, S. Quality traits of saffron (*Crocus sativus* L.) produced in the Italian Alps. *Open Agriculture* **2017**, *2*, 52–57. [CrossRef]
46. Donno, D.; Mellano, M.G.; Prgomet, Z.; Beccaro, G.L. Advances in *Ribes* x *nidigrolaria* Rud. Bauer & A. Bauer fruits as potential source of natural molecules: A preliminary study on physico-chemical traits of an underutilized berry. *Sci. Hortic.* **2018**, *237*, 20–27.
47. Donno, D.; Mellano, M.G.; Prgomet, Z.; Cerutti, A.K.; Beccaro, G.L. Phytochemical characterization and antioxidant activity evaluation of Mediterranean medlar fruit (*Crataegus azarolus* L.): Preliminary study of underutilized genetic resources as a potential source of health-promoting compound for food supplements. *J. Food Nutrit. Res.* **2017**, *56*, 18–31.
48. Caser, M.; D'Angiolillo, F.; Chitarra, W.; Lovisolo, C.; Ruffoni, B.; Pistelli, L.; Pistelli, L.; Scariot, V. Ecophysiological and phytochemical responses of *Salvia sinaloensis* Fern. to drought stress. *Plant Growth Regul.* **2018**, *84*, 383–394. [CrossRef]
49. Caser, M.; Chitarra, W.; D'Angiolillo, F.; Perrone, I.; Demasi, S.; Lovisolo, C.; Pistelli, L.; Pistelli, L.; Scariot, V. Drought stress adaptation modulates plant secondary metabolite production in *Salvia dolomitica* Codd. *Ind. Crop Prod.* **2019**, *129*, 85–96. [CrossRef]
50. Hoch, H.C. *Freeze substitution of fungi*; Aldrich, H.C., Todd, W.J., Eds.; Ultrastructure techniques of microorganisms; Plenum Press: New York, NY, USA, 1986; pp. 183–211.
51. Reynolds, E.S. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell. Biol.* **1963**, *17*, 208–212. [CrossRef]
52. Gresta, F.; Avola, G.; Lombardo, G.M.; Siracusa, L.; Ruberto, G. Analysis of flowering, stigmas yield and qualitative traits of saffron (*Crocus sativus* L.) as affected by environmental conditions. *Sci. Hortic.* **2009**, *119*, 320–324.
53. Cavusoglu, A.; Erkel, F. The effect of different planting areas and corm size on yield and harvest period of saffron (*Crocus sativus* L.) in Kocaeli province. *J. Akdeniz Univ. Faculty Agric.* **2005**, *18*, 179–184.

54. Koocheki, A.; Seyyedi, S. How irrigation rounds and mother corm size control saffron yield, quality, daughter corms behavior and phosphorus uptake. *Sci. Hortic.* **2016**, *213*, 132–143. [CrossRef]
55. Turhan, H.; Kahriman, F.; Egesel, C.; Kemal Gul, M. The effects of different growing media on flowering and corm formation of saffron (*Crocus sativus* L.). *Afr. J. Biotech.* **2007**, *6*, 2328–2332.
56. Karimi, E.; Oskoueian, E.; Hendra, R.; Jaafar, H. Evaluation of Crocus sativus L. stigma phenolic and flavonoid compounds and its antioxidant activity. *Molecules* **2010**, *15*, 6244–6256. [CrossRef]
57. Asdaq, S.; Inamdar, M. Potential of garlic and its active constituent, S-allyl cysteine, as antihypertensive and cardioprotective in presence of captopril. *Phytomedicine* **2010**, *17*, 1016–1026. [CrossRef] [PubMed]
58. Makhlouf, H.; Saksouk, M.; Habib, J.; Chahine, R. Determination of antioxidant activity of saffron taken from the flower of *Crocus sativus* grown in Lebanon. *Afr. J. Biotech.* **2011**, *10*, 8093–8100.
59. Ordoudi, S.; Befani, C.; Nenadis, N.; Koliakos, G.; Tsimidou, M. Further Examination of Antiradical Properties of *Crocus sativus* Stigmas Extract Rich in Crocins. *J. Agric. Food Chem.* **2009**, *57*, 3080–3086. [CrossRef]
60. Siracusa, L.; Gresta, F.; Avola, G.; Lombardo, G.M.; Ruberto, G. Influence of corm provenance and environmental condition on yield and apocarotenoid profiles in saffron (*Crocus sativus* L.). *J. Food Comp. Anal.* **2010**, *23*, 394–400. [CrossRef]
61. Manzo, A.; Panseri, S.; Bertoni, D.; Giorgi, A. Economic and qualitative traits of Italian Alps saffron. *J. Mt. Sci.* **2015**, *12*, 1542–1550. [CrossRef]
62. Sánchez-Vioque, R.; Rodriguez-Conde, M.F.; Reina-Ureña, J.V.; Escolano-Tercero, M.A.; Herraiz-Peñalver, D.; Santana-Méridas, O. In vitro antioxidant and metal chelating properties of corm, tepal and leaf from saffron (*Crocus sativus* L.). *Ind. Crop Prod.* **2012**, *39*, 149–153. [CrossRef]
63. Rahaiee, S.; Moini, S.; Hashemi, M.; Shojaosadati, S. Evaluation of antioxidant activities of bioactive compounds and various extracts obtained from saffron (*Crocus sativus* L.): A review. *J. Food Sci. Tech.* **2015**, *52*, 1881–1888. [CrossRef] [PubMed]
64. Shahi, T.; Assadpour, E.; Jafari, S. Main chemical compounds and pharmacological activities of stigmas and tepals of 'red gold'; saffron. *Trends Food Sci. Technol.* **2016**, *58*, 69–78. [CrossRef]
65. Donno, D.; Mellano, M.G.; Raimondo, E.; Cerutti, A.K.; Prgomet, Z.; Beccaro, G.L. Influence of applied drying methods on phytochemical composition in fresh and fried goji fruits by HPLC fingerprint. *Eur. Food Res. Technol.* **2016**, *242*, 1961–1974. [CrossRef]
66. Heidari, M.; Karami, V. Effects of different mycorrhiza species on grain yield, nutrient uptake and oil content of sunflower under water stress. *J. Saudi Soc. Agric. Sci.* **2014**, *13*, 9–13. [CrossRef]
67. Araim, G.; Saleem, A.; Arnason, J.T.; Charest, C. Root colonization by arbuscular mycorrhizal (AM) fungus increases growth and secondary metabolism of purple coneflower, *Echinacea purpurea* (L.) Moench. *J. Agric. Food Chem.* **2009**, *57*, 2255–2258. [CrossRef]
68. Ceccarelli, N.; Curadi, M.; Martelloni, L.; Sbrana, C.; Picciarelli, P.; Giovannetti, M. Mycorrhizal colonization impacts on phenolic content and antioxidant properties of artichoke leaves and flower heads two years after field transplant. *Plant Soil* **2010**, *335*, 311–323. [CrossRef]
69. Avio, L.; Sbrana, C.; Giovannetti, M.; Frassinetti, S. Arbuscular mycorrhizal fungi affect total phenolics content and antioxidant activity in leaves of oak leaf lettuce varieties. *Sci. Hortic.* **2017**, *224*, 265–271. [CrossRef]
70. Toussaint, J.P.; Smith, F.A.; Smith, S.E. Arbuscular mycorrhizal fungi can induce the production of phytochemicals in sweet basil irrespective of phosphorus nutrition. *Mycorrhiza* **2007**, *17*, 291–297. [CrossRef]
71. Nell, M.; Vötsch, M.; Vierheilig, H.; Steinkellner, S.; Zitterl-Eglseer, K.; Franz, C.; Novak, J. Effect of phosphorus uptake on growth and secondary metabolites of garden (*Salvia officinalis* L.). *J. Sci. Food Agric.* **2009**, *89*, 1090–1096. [CrossRef]
72. Geneva, M.P.; Stancheva, I.V.; Boychinova, M.M.; Mincheva, N.H.; Yonova, P.A. Effects of foliar fertilization and arbuscular mycorrhizal colonization on *Salvia officinalis* L. growth, antioxidant capacity, and essential oil composition. *J. Sci. Food Agric.* **2010**, *90*, 696–702.
73. Donno, D.; Beccaro, G.; Mellano, M.; Cerutti, A.; Bounous, G. Goji berry fruit (*Lycium* spp.): Antioxidant compound fingerprint and bioactivity evaluation. *J. Funct. Foods* **2015**, *18*, 1070–1085. [CrossRef]
74. Vasco, C.; Ruales, J.; Kamal-Eldin, A. Total phenolic compounds and antioxidant capacities of majpr fruits from Ecuador. *Food Chem.* **2008**, *111*, 816–823. [CrossRef]

75. Donno, D.; Cerutti, A.K.; Prgomet, I.; Mellano, M.G.; Beccaro, G.L. Foodomics for mulberry fruit (Morus spp.): Analytical fingerprint as antioxidants' and health properties' determination tool. *Food Res. Int.* **2015**, *69*, 179–188. [CrossRef]
76. Ma, H.; Johnson, S.L.; Liu, W.; DaSilva, N.A.; Meschwitz, S.; Dain, J.A.; Seeram, N.P. Evaluation of polyphenol anthocyanin-enriched extracts of blackberry, black raspberry, blueberry, cranberry, res raspberry, and strawberry for free radical scavenging, reactive carbonyl species trapping, anti-glycation, anti-β-amyloid aggregation, and microglial neuroprotective effects. *Int. J. Mol. Sci.* **2018**, *19*, 461.

© 2019 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

Chapter III

Arbuscular mycorrhizal fungi as natural biofertilizers for saffron on the Marginal Alpine soils: role and potential

Preamble

Soil–plant–microbe interactions can be complex and directly influence plant health and productivity, being the microbial activity in the rhizosphere a major factor in determining the availability of nutrients to plants. Groups of beneficial rhizosphere microorganisms present are countless; some of them develop from fairly loose associations with the root, like the so called rhizobial bacteria whilst others engage in well-developed symbiotic interactions in which particular organs are formed - arbuscular mycorrhizal fungi. Biofertilizers as the AMF has a demonstrated economic impact on agriculture and horticulture as they promote plant nutrient uptake and assimilation and confer pathogen protection (Mercy et al., 2017; Toscano et al., 2018) and on medicinal and aromatic plants AMF colonization have shown to influence bioactive compound biosynthesis such as ascorbic acid, flavonoids, polyphenols, carotenoids, and vitamins which are important in human health. Saffron (*Crocus sativus*) is an autumnal flowering geophyte whose dried stigmas, well known for their aromatic and coloring properties, first found in Mediterranean Asia being afterwards transported and distributed worldwide (Gresta et al., 2008). For long it has been neglected by farmers being considered a minor crop, however in the last few years it has gained the attention of farmers as alternative and low-input agricultural crop. In Italy its cultivation is mainly concentrated in Sardinia and Abruzzo but, being Italy the second saffron-importing country farmers from regions like the northern marginal Alps has been encouraged to cultivate it as a valid mean for increasing incomes of multifunctional farms, maintaining however the high standards of the saffron produced in the south or in the major producing countries. So, in the paper below we presented the results obtained by saffron root mycorrhization efficiency in potted conditions, and secondly to assess the AMF symbiosis in open field conditions and its effects on saffron plant growth, productivity, and bioactive compounds content in Alpine open field conditions, using both single and multispecies commercial inocula.

The objective of this research was to:

- evaluate saffron mycorrhization in potted conditions.
- evaluate, in two Alpine sites, how saffron plants inoculated with two types of inoculum: a single AMF species (*Rhizophagus intraradices*) or a mixture of *R. intraradices* and *Funneliformis mosseae* ones, are affected in terms of yield and bioactive compounds production if grown in field conditions.

We were able to confirm that commercial inocula can establish symbiotic relationships with saffron roots either in potted and open field conditions, and that both AMF inocula, and

particularly the mixture of *R. intraradices* and *F. mosseae*, positively affected saffron cultivation in the two Alpine sites.

Article

Saffron Cultivation in Marginal Alpine Environments: How AMF Inoculation Modulates Yield and Bioactive Compounds

Matteo Caser [1], Íris Marisa Maxaieie Victorino [2,3,4], Sonia Demasi [1], Andrea Berruti [2], Dario Donno [1], Erica Lumini [2], Valeria Bianciotto [2] and Valentina Scariot [1,*]

1 Department of Agricultural, Forest and Food Sciences, University of Torino, Largo Paolo Braccini 2, 10095 Grugliasco, TO, Italy; matteo.caser@unito.it (M.C.); sonia.demasi@unito.it (S.D.); dario.donno@unito.it (D.D.)

2 Institute for Sustainable Plant Protection, National Research Council (CNR), Viale Mattioli 25, 10125 Torino, Italy; irisvictorino@gmail.com (I.M.M.V.); berruti.andrea@gmail.com (A.B.); erica.lumini@unito.it (E.L.); valeria.bianciotto@ipsp.cnr.it (V.B.)

3 Department of Life Sciences and Systems Biology, University of Turin, Viale Mattioli 25, 10125 Torino, Italy

4 Biological Science Department, Science Faculty, Eduardo Mondlane University (UEM), Av. Julius Nyerere - Campus Universitário, Maputo 3453, Mozambique

* Correspondence: valentina.scariot@unito.it; Tel.: +39-011-670-8932

Received: 3 December 2018; Accepted: 30 December 2018; Published: 31 December 2018

Abstract: Arbuscular mycorrhizal fungi (AMF) establish mutualistic symbiotic associations with plant roots and act as biofertilizers by enhancing plant nutrient and water uptake. Moreover, AMF colonization may influence the biosynthesis of plant bioactive compounds in medicinal and aromatic plants. There is limited information on AMF associations with *Crocus sativus* L. (saffron) roots and their effect on crop performances and spice quality. In the present work we verified the efficiency of root mycorrhization in potted conditions, then we evaluated the yield and quality of the saffron produced in two Alpine sites during two cultivation cycles with the application of AMF. Two inocula were applied, either a single-species (*Rhizophagus intraradices*) or a multi-species mixture (*R. intraradices* and *Funneliformis mosseae*). The trial conducted in potted conditions confirmed that both AMF commercial inocula established symbiotic relationships with saffron roots. The multi-species inoculation yielded the highest content of arbuscules in colonized portions of the root (100%), while the single-species was slightly less (82.9%) and no AMF were recorded in untreated control corms. In open-field conditions, AMF colonization of the root systems, flower production, and saffron yields were monitored, and bioactive compounds contents and antioxidant activity in the dried spice were analysed using spectrophotometry and high performance liquid chromatography. Overall, the saffron produced was high quality (ISO category) and had high contents of bioactive compounds, with very high total polyphenol content and elevated antioxidant activity. The use of arbuscular mycorrhizal symbionts as biostimulants positively affected saffron cultivation, improving the crop performances and the content of important nutraceutical compounds. In particular, the inoculum composed by *R. intraradices* and *F. mosseae* increased flower production and the saffron yield. *R. intraradices* alone enhanced the spice antioxidant activity and the content of bioactive compounds such as picrocrocin, crocin II, and quercitrin. Since saffron is the world's highest priced spice, the increases in yield and quality obtained using AMF suggests that farms in marginal areas such as alpine sites can increase profitability by inoculating saffron fields with arbuscular mycorrhiza.

Keywords: *Crocus sativus* L.; biofertilization; arbuscular mycorrhizal fungi; antioxidant activity; crocin; picrocrocin; polyphenols; safranal

1. Introduction

Saffron (*Crocus sativus* L.) is a triploid herbaceous geophyte that is reproduced by means of replacement corms and is cultivated in environments with very different soil characteristics [1–3] for its red scarlet stigmas that are used worldwide as a spice and natural dye [4]. Origin, abiotic stresses, agronomical practices, and processing methods (stigma separation, drying, and storage) can influence both the plant and the saffron spice yield, composition, and quality [5,6]. The spice's organoleptic properties are ascribed to the relative percentage of peculiar secondary metabolites: crocin, picrocrocin, and safranal, which provide the unique color, bitter taste, and aroma, respectively. The concentration of these constituents combine to determine the saffron spice quality, as defined by the International Organization for Standardization [7]. Studies related to saffron quality are expanding mainly due to the antioxidant properties of this spice and their positive influence on human health [8]. Antitumor and cancer-preventive properties are mainly attributed to the high carotenoids content [9].

Reproductive, vegetative, and dormancy are the main phenological stages [10]. Saffron flower induction is a very complicated mechanism directly related to ecological conditions and field management [11,12]. As in most geophyte plants, both seasonal and daily thermoperiodism are involved as the main environmental factors [11]. Flower induction requires an incubation of the corms at high temperature (23–27 °C), followed by a period of exposure at moderately low temperature (17 °C) for flower emergence. In Mediterranean environments, flower induction occurs from early spring to mid summer, while flower emergence occurs from early-to late-autumn. Differences in the time required for flower initiation have mostly been attributed to the corm size [13]. In addition, Molina et al. [14] reported that air and soil temperatures might be responsible for differential flower induction and duration of up to two months. Flowering is followed by a vegetative stage throughout the winter and formation of replacement corms at the base of shoots. At the end of spring, the leaves reach the highest length, start to senesce, and wither, and the bulbs go into dormancy [14[.

Due to its unique biological, physiological, and agronomic traits, saffron is able to exploit marginal land and is included in low-input cropping systems, even if high amount of skilled labour is required [11]. In Italy, saffron cultivation is gaining increasing attention as an alternative crop for sustainable agriculture systems [11,15], where it could represent a valid mean for increasing incomes of multifunctional farms, with a positive impact on the recovery and economy of these areas [15,16]. Since saffron is the world's highest-priced spice due to the intensive hand labour required for daily flower picking and stigma separation [14], small increases in the yield and/or quality can connote a large increase in profitability. In this context, the adoption of sustainable cultivation techniques such as the use of biostimulants may represent further help in both the increase in spice yield and active ingredients accumulation [17].

Recent research has focused on the benefits of soil organisms to crops, especially to promote plant nutrient uptake and assimilation [18,19]. Indeed the soil is not only the location of plant life cycle stages, but also the main reservoir for a wide range of plant biostimulants (PBs), including arbuscular mycorrhizal fungi (AMF) [19–21]. Ubiquitous and abundant, AMF are obligate endosymbionts living inside most plant roots present in diverse environments, including productive agricultural systems [22–25]. When colonizing roots, hyphae extend root limits, improving water and inorganic nutrient acquisition from the soil, mainly phosphorus (P) and other minerals, in exchange for photosynthetic products. The use of AMF has a demonstrated economic impact on agriculture and horticulture and they may also confer pathogen protection by altering plant physiological parameters, and improving soil nutrition and aggregation under different growing conditions [26–28].

Mounting evidence indicates that AMF may induce changes in primary and secondary metabolism of host plants, increasing polyphenols, flavonoids, and phytohormone dynamics [29,30]. Such metabolic changes may be ascribed to a transient activation of host defence reactions in colonized roots [20,31]. The role of AMF symbiosis in flowering date and flower production is fragmented [32].

In medicinal and aromatic plants (MAPs), such as *Arnica montana* L., *Coriandrum sativum* L., and *Anethum graveolens* L., AMF colonization influenced bioactive compound biosynthesis such as ascorbic acid, flavonoids, polyphenols, carotenoids, and vitamins [33–36]. Inoculation with *Funneliformis mosseae* Gerd. & Trappe and *G. versiforme* P. Karst. improved plant growth and enhanced the glycyrrhizin concentration in *Glycyrrhiza uralensis* Fisch. plants [26]. Moreover, under low P availability, a mix of AMF increased the production of root biomass and of pseudohypericin and hypericin content in flowers of *Hypericum perforatum* L. [32]. Although widely applied, evidence for AMF symbiosis efficacy and persistence is scant, incomplete or lacking [37,38] and the use of AMF in crop production is facing some limitations due to product costs, producer awareness levels, and variability in mycorrhizal inoculum quality [21,27]. Many factors can affect the success of inoculation and AMF persistence, including environmental and cultivation conditions, species compatibility, degree of spatial competition with other soil organisms, and the time of inoculation. However, once AMF inoculation is restored and well established in soil, the AMF community will persist through time. If detrimental practices are minimized before and after cultivation, biodiverse mycorrhizal hyphal networks will remain unaltered and infective in the field [27]. Hence, it is important to assess the effects of AMF on crop traits both as early application and as residual persistence in the following crop cultivation seasons.

Incidence of AMF, alone or in combination with plant growth promoting bacteria (PGPB), was reported in corms of *C. sativus* [39–44]. Different authors report that well-established AMF colonization of saffron roots results in increased corm P content, chlorophyll, fresh and dry corm mass, and leaf matter, and greater soil P and nitrogen assimilation [43–45]. Shajari et al. [44] indicated a significant effect of AMF in corm growth and mineral assimilation during the second cultivation season, supporting their effective residual effects in saffron cultivation. However, little is known about the effects of AMF on spice yield, and phytochemical profiles in open field cultivation [46,47].

The possibility that AMF can enhance the economic value of saffron by increasing yield and quality is even more interesting if we consider the worldwide increase in use of biocompounds in the food and pharmaceutical industries. Thus, the aims of the present study were (1) to preliminarily verify the constitutive association of AMF with saffron roots in sterile pot conditions, and (2) to assess the AMF symbiosis in open field conditions and its effects on saffron plant growth, productivity, and bioactive compounds content in Alpine open field conditions.

2. Materials and Methods

2.1. AMF Inoculation in Pot

Saffron corms with horizontal diameters of 1.3–2.8 cm were sown in pots (4 L; 1 corm per pot) in the last ten days of August 2016. Pots were filled with sterile quartz sand (3 L per pot) on a layer of sterilized expanded clay (1 L per pot). Corms were treated with two inocula (MycAgro Lab, Breteniére, FR), one composed of a single fungus *Rhizophagus intraradices* (Ri) and one of *R. intraradices* and *Funneliformis mosseae* (Ri + Fm). Ten g of each inoculum were placed under each corm in order to guarantee the contact between the inoculum and the roots and therefore to favour the symbiosis between AMF and roots. Saffron corms used as controls were not inoculated (AMF-). Corms were not treated against fungal pathogens. A randomized block design was used with a total of 48 pots displayed in two experimental plot units (24 pots per unit) and three treatments (8 pots per treatment). Cultivation lasted for one cycle (August 2016–April 2017) in a heated glasshouse of the Department of Agricultural Forest and Food Sciences (DISAFA) of the University of Torino (Italy, 45°06′23.21″ N Lat, 7°57′82.83″ E Long; 293 m a.s.l.), with an average temperature of 22 °C during the day and 16 °C in the night. Irrigation water (pH 7.4, EC 505 μS cm) was added weekly (250 mL per pot) with a drip system. The corms were fertilized by fertigation (VIGORFLOR, AL.FE. srl, MN, Italy) every two weeks starting from the emergence of the spate, in quantities of 1.5 g L^{-1} of water. No flowering occurred because of the small size of the corms.

2.2. AMF Inoculation in Open Field

Saffron corms with horizontal diameters of 2.5–3.5 cm were planted in the last ten days of August 2016 in two Alpine experimental sites located in the municipality of Morgex (45°45′35.1″ N; 7°02′37.3″ E; 1000 m a.s.l.) and Saint Cristophe (45°45′06.9″ N; 7°20′37.0″ E; 700 m a.s.l.) in Italy and cultivation lasted for two cycles (2016–2017 and 2017–2018). Both sites were cultivated with saffron for at least the previous three years. Before starting the experiment both fields were milled. To assess the effects of AMF inocula on saffron cultivation and production, the same treatments used in the pot trial were applied (Ri, Ri + Fm or AMF-). A randomized block design was used, with three experimental plot units (blocks). Each plot unit consisted of 56 corms, planted in a 1.44 m^2 area (39 corms m^{-2}). Inter-row planting distance was of 7 cm, while between-row distance was of 25 cm. Plots were separated from each other with at least 4 m distance. Before planting, 10 g of inoculum was placed under the corms to ensure contact between plant and the treatment. Irrigation was provided when needed and hand weeding control was conducted during cultivation, while no pre-planting fertilization, tillage, or treatments against pathogens were applied. The two Alpine sites were characterized by semi-continental climate, with a long and cold winter (Supplementary Figure S1). In general, both sites had a sandy-loam texture according to the USDA classification and similar chemical characteristics (Supplementary Table S1).

2.3. AMF Evaluation

At the end of the vegetative phase in both pot (February 2017) and open field experiments (April 2017 and 2018), saffron roots were harvested, rid of topsoil, cleaned and stained with 0.1% (*w/v*) cotton blue in 80% lactic acid overnight, then de-stained 3 times with lactic acid for 18 h, cut into 1cm long segments and placed on microscope slides for further morphological analysis. Approximately 25 fragments were observed under light microscope for each replicate for a total of 300 root fragments. Fungal colonization was determined and calculated as described by Trouvelot et al. [48].

2.4. Plant Performance and Saffron Yield in Open Field

The daily number of picked flowers per corm (Supplementary Figure 2) and the yield of spice (i.e., stigmas dried at 40 °C for 8 h in an oven) were measured at flowering (November 2016 and 2017). When leaves were fully expanded (April 2017 and 2018), 50 mg of fresh leaves per treatment were used to determine chlorophyll and carotenoids content as described by Caser et al. [49]. Simultaneous with leaf sampling, the Chlorophyll Meter SPAD-502 (Konica Minolta Sensing Inc., Osaka, Japan) was used to determine the relative quantity of chlorophyll present in 27 randomly selected plants per treatment in the field.

At the end of full plant development (April 2017 and 2018), the leaves length of all corms was measured. Then, 27 plants per treatment were lifted, and corms rid of topsoil, cleaned and de-tunicated. The wilted rate as the ratio between the number of wilted corms and the total number of sown corms, the shoot caliber size, and the number, the size and the weight of replacement corms were determined.

2.5. Saffron Extract Preparation and Quality

The saffron aqueous extracts were prepared according to Cresta et al. [11]. Fifty mg of powdered saffron from each treatment and both cultivation years, were put into 5 mL of deionized water. After stirring for 1 h at room temperature (circa 21 °C) in the dark, the solution was filtered with polytetrafluoroethylene (PTFE, VWR international, Milano, Italy) filters of 25 mm diameter and 0.45 µm pore size. The saffron extract obtained was diluted 1:10 with deionized water (1 mg mL^{-1}). Saffron extracts were analysed with a spectrophotometer (Ultrospec 2100 Pro, GE Healthcare, UK Ltd., Little Chalfont, Buckinghamshire, UK) to determine the amount of picrocrocin, crocin, and safranal, according to ISO 3632 [7].

2.6. Total Phenols

The content of total phenols (TPC) was measured by using the Folin-Ciocalteau's phenolic method and determined as reported by Donno et al. [50]. Five hundred µl of saffron extract was added and mixed with 30 mL of deionized water, 2.5 mL of Folin-Ciocalteau's reagent (diluted 1:10), and, after eight minutes, 10 mL of 7.5% (*w/v*) saturated sodium carbonate solution. The solution was incubated at room temperature for 2 h in the dark and the absorbance was detected at 765 nm with a spectrophotometer (Ultrospec 2100 Pro, GE Healthcare, UK Ltd., Little Chalfont, Buckinghamshire, UK). The results were expressed as mg of gallic acid equivalents (GAE) per 100 g of fresh weight (FW).

2.7. Total Anthocyanins

The total anthocyanins content (TAC) was determined using the pH-differential method [50]. Saffron extracts were added to a pH 1 and pH 4.5 buffer solutions. Absorbance of samples was determined at 515 nm and 700 nm after a 15 min equilibration. The formula for calculating TAC is as follows:

$$\text{TAC (mg L}^{-1}) = (\text{A} \times \text{sample dilution factor} \times 1000)/(\text{molar absorptivity} \times 1) \quad (1)$$

where A is (Absorbance 515 nm—Absorbance 700 nm) at pH 1.0—(Absorbance 515 nm Absorbance 700 nm) at pH 4.5. The results were expressed as milligrams of cyanidin 3-O-glucoside (C3G) per 100 gof fresh weight (mg of C3G 100 g^{-1} FW).

2.8. Antioxidant Activity

The antioxidant activity (AOA) was determined using the ferric reducing antioxidant power (FRAP) method as reported by Caser et al. [51] and the 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) method as described by Urbani et al. [52].

For the FRAP method, a total of 30 µL of saffron extract was added and mixed with 90 µL of deionized water and 900 µL of the FRAP reagent. After incubation at 37 °C for 30 min, the absorbance of the solutions was measured at 595 nm using a spectrophotometer (Ultrospec 2100 Pro, GE Healthcare, UK Ltd., Little Chalfont, Buckinghamshire, UK). Results were expressed as millimoles of ferrous iron (Fe^{2+}) equivalents per kilogram of fresh weight.

The ABTS radical cation (ABTS +) was obtained by the reaction of 7.0 mM ABTS stock solution with 2.45 mM potassium persulfate solution. After the incubation for 12–16 h before use in the dark and at room temperature, the solution was diluted with distilled water to obtain an absorbance of 0.70 (+0.02) at 734 nm. After addition of 0.6 mL of diluted ABTS·+ solution to 180 µL of saffron extract, the reaction was left in the dark at room temperature for six min. The absorbance was then measured at 734 nm using a spectrophotometer (Ultrospec 2100 Pro, GE Healthcare, UK Ltd., Little Chalfont, Buckinghamshire, UK) . The antioxidant activity was expressed as µmol of Trolox equivalents per gram of dry weight (µmol TE g^{-1} DW).

All analyses were performed in three replicates.

2.9. Identification and Quantification of Bioactive Compounds

The chromatographic analysis of saffron extracts (Supplementary Table 2) was conducted with an Agilent 1200 high-performance liquid chromatograph coupled to a diode array detector (HPLC-DAD; Agilent Technologies, Santa Clara, CA, USA), according to established methods [53]. Different chromatographic methods were used for analysis: benzoic acids (ellagic and gallic acids), catechins ((−)catechin and (-)epicatechin), cinnamic acids (caffeic, chlorogenic, coumaric, and ferulic acids), flavonols (hyperoside, isoquercitrin, quercetin, quercitrin, and rutin), carotenoids (crocin I and II and safranal), and vitamin C (ascorbic + dehydroascorbic acids).

Four chromatographic methods were used to separate the bioactive molecules on a Kinetex C18 column (4.6 × 150 mm, 5 µm, Phenomenex, Torrance, CA, USA). Several mobile phases were used for bioactive compound identification and ultra violet (UV) spectra were recorded at different

wavelengths, based on HPLC methods, previously tested and validated [4], with some modifications. UV spectra were recorded at 330 nm (α); 280 nm (β); 310 and 441 nm (χ); 261 and 348 nm (δ).

All single compounds were identified in samples by comparison and combination of their retention times and UV spectra with those of authentic standards analysed with the same chromatographic conditions.

2.10. Chemicals and Reagents

All the chemicals and reagents used for the AMF evaluation, phenols, anthocyanins, FRAP and ABTS assays, and bioactive quantification were purchased from Sigma-Aldrich (Saint Louis, MO, USA).

2.11. Statistical Analysis

Arcsin transformation was performed on all percent incidence data before statistical analysis in order to improve homogeneity of variance (Levene test). All the analysed data were checked for normality of variance. For all indices analysed in the greenhouse assay, mean differences were computed using a one-way analysis of variance (ANOVA) with Tukey post hoc test ($p \leq 0.05$). Data from open field were analysed by means of a linear mixed effect models considering AMF treatments as a fixed factor, year as a repeated measure, and sites and blocks as random factors. The following interactions (year × AMF treatment) were included in the model. Pairwise comparisons according to a sequential Bonferroni post hoc tests were used to separate means when a treatment was significantly affecting the variable at a $p \leq 0.05$. All presented values are means of untransformed data. All computations were conducted with SPSS statistical package (version 25.0; SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Assessment of Saffron Mycorrhization at Pot and Open Field Scale

Values concerning intensity of colonization in the root system and abundance of arbuscules or coils in the saffron roots in potted conditions are shown in Table 1. Corms treated with Ri + Fm reached the highest level of mycorrhization (M%) (93.33%), however, high levels were also obtained with Ri inoculum (71.37%). The Ri + Fm treatment also had the highest occurrence of arbuscules (a%) in the mycorrhizal portions (100%), significantly higher than Ri (82.99%) and AMF-(0%).

Table 1. AMF colonization indices (intensity in the whole root system, M; intensity of the mycorrhizal portions, m; presence of arbuscules in the whole root system, A; presence of arbuscules in the mycorrhizal portions, a) of *Crocus sativus* L. roots treated with the inoculum composed by *Rhizophagus intraradices* and *Funneliformis mosseae* (Ri + Fm), *R. intraradices* alone (Ri), or the control (AMF-) in the saffron pot cultivation.

	Index (%)			
Treatment	**M**	**m**	**A**	**a**
Ri + Fm	93.33 a	93.33 a	93.33 a	100.00 a
Ri	71.37 b	80.28 b	58.98 b	82.99 b
AMF-	0.07 c	0.33 c	0.00 c	0.00 c
p	***	***	***	***

Mean values with the same letter are not statistically different at $p < 0.05$ according to Tukey post-hoc tests. The statistical relevance is provided (*** $p < 0.001$).

In open field conditions, the AMF root colonization measurements in *C. sativus* treated with Ri + Fm or with Ri alone during the two cultivation cycles are presented in Table 2. In general, both the presence of arbuscules in the mycorrhizal portions (a%) and in the whole root system (A%) indices

were affected by the inoculum composition only in the first cultivation year, while control plants (AMF-) were not colonized. In the second year, low root colonization was observed and no differences among the treated and untreated corms were detected.

Table 2. AMF colonization intensity in open field conditions after the first and second cultivation year of the whole root system (M) and of the mycorrhizal portions (m), and presence of arbuscules in the whole root system (A) and in the mycorrhizal portions (a) of *Crocus sativus* roots treated with inoculum composed of *Rhizophagus intraradices* and *Funneliformis mosseae* (Ri + Fm), *R. intraradices* alone (Ri), or the control (AMF-).

Effect	Index (%)			
Year 1	**M**	**m**	**A**	**a**
Ri + Fm	11.6 a	11.7	4.0 a	26.6 a
Ri	13.8 a	14.2	6.9 a	38.1 a
AMF-	1.7 b	3.4	0.0 b	0.0 b
p	*	ns	***	***
Year 2				
Ri + Fm	7.0	8.5	0.8	12.5
Ri	16.1	16.5	1.6	8.31
AMF-	4.73	6.1	2.5	18.8
p	ns	ns	ns	ns
Year × Treatment (*p*)	*	ns	*	*

Values with the same letter denote no significant differences. The statistical relevance is provided (ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

3.2. Impact of AMF Symbiosis on Saffron Productivity and Qualitative Traits in Open Field

Significant differences between the two cultivation years emerged for several studied parameters. In general, the wilting rate, all the main productivity traits (number of flowers m^{-2}, number of flowers per corm, mg of saffron m^{-2}, saffron per flower, and the number of replacement corms), and the content of leaf chlorophyll and carotenoids significantly increased after the second year of cultivation (Table 3). While, a reduction in leaf length, SPAD unit, and shoot size was observed.

Table 3. Effects of cultivation seasons (Year 1 and Year 2), AMF treatments (Ri + Fm was composed of *Rhizophagus intraradices* and *Funneliformis mosseae*, Ri of *R. intraradices* alone, and AMF-was the un-inoculated control), and their interaction (Year × AMF treatment) on saffron plant growth and productivity based on linear mixed-effects models considering AMF treatments as a fixed factor, year as a repeated measure, and sites and blocks as random factors.

	Growing Seasons			**AMF Treatments**				**Year × AMF**
Traits	**Year 1**	**Year 2**	***p***	**Ri + Fm**	**Ri**	**AMF-**	***p***	***p***
Wilting rate (%)	39.0	54.4	***	44.3	50.0	45.8	ns	ns
Flower (n m^{-2})	49.8	101.7	***	91.8 a	61.9 b	66.4 b	*	*
Flower/corm (n)	1.5	4.2	***	5.1 a	3.8 b	3.9 b	*	*
Saffron yield (mg m^{-2})	278.0	700.0	***	645.3 a	377.4 b	477.2 b	*	*
Saffron/flower (mg)	6.0	7.0	**	7.1 a	5.8 b	7.3 a	*	*
Leaf length (cm)	36.8	24.1	***	31.4	30.3	29.9	ns	ns
SPAD unit	74.8	45.7	***	60.0	61.1	59.7	ns	ns
Shoot size (mm)	5.3	4.1	**	5.5 a	3.3 b	4.2 ab	**	*
Corm size (mm)	21.1	20.2	ns	19.8	20.0	22.2	ns	ns
Replacement corm (n)	2.2	3.7	*	2.8	3.4	2.7	ns	ns
Corm weight (g)	7.7	6.5	ns	7.8	7.4	6.3	ns	ns
Chlorophyll (μg mg^{-1})	1.6	4.1	***	2.9	2.9	2.7	ns	ns

Carotenoids (μg mg^{-1})	0.6	2.2	***	1.4	1.5	1.4	ns	ns

In Table 3, the productivity and growth traits influenced by the AMF treatments are also reported. Particularly, the inoculum composed of the mix of *R. intraradices* and *F. mosseae* significantly increased the number of flowers m^{-2} (+138.2%), the number of flowers corm^{-1} (+130.8%), and the mg of saffron m^{-2} (+135.2%) in comparison to other treatments. In contrast, the mg of saffron flower^{-1} and the shoot size were significantly reduced (−20% and −40%, respectively) by the inoculum of *R. intraradices* alone in comparison to Ri + Fm and AMF-. Significant interaction between cultivation year and AMF treatments resulted for the number of flowers m^{-2}, the number of flowers corm^{-1}, saffron yield, saffron flower^{-1}, and shoot size.

Regarding the synthesis of bioactive molecules in the studied saffron spice, differences between the two cultivation seasons occurred (Table 4). Overall, the saffron produced at the two experimental sites belonged to the quality category I for the picrocrocin, safranal, and crocins analysis [7] with a significant increase after the second cultivation year. On the opposite, different bioactive compounds (isoquercitirin, quercitrin, ellagic acid, safranal and total vitamin C) were significantly reduced. Very few differences were observed among AMF treatments (Table 4). Both Ri + Fm and Ri positively affected the antioxidant activity (FRAP assay) of the saffron produced. While, the effect of the Ri inoculum significantly increased the absorbance value of picrocrocin (ISO 3632) and the content of quercitrin in comparison to Ri + Fm, and the content of crocin II compared to AMF-(Table 4). A significant interaction between cultivation seasons and AMF treatments resulted for picrocrocin (ISO), quercitrin, crocin II, and antioxidant activity (FRAP assay).

Table 4. Effects of cultivation seasons (Year 1 and Year 2), AMF treatments (Ri + Fm was composed of *Rhizophagus intraradices* and *Funneliformis mosseae*, Ri of *R. intraradices* alone, and AMF- was the un-inoculated control), and their interaction (year × AMF treatment) on bioactive compounds, total polyphenol content (TPC), anthocyanins, quality traits as defined by ISO 3632 [7], and antioxidant activity of the produced saffron based on liner mixed-effects models considering AMF treatments as a fixed factor, year as a repeated measure, and sites and blocks as random factors.

	Growing Seasons			AMF Treatments				Year × AMF
Traits	**Year 1**	**Year 2**	***p***	**Ri + Fm**	**Ri**	**AMF-**	***p***	***p***
ISO 3632 [7]								
Picrocrocin	131.4	135.0	*	130.2 b	138.7 a	136.1 a	*	*
Safranal	38.8	44.2	**	39.9	43.8	40.8	ns	ns
Crocins	207.1	368.5	***	275.9	303.5	284.1	ns	ns
Bioactive compounds (mg 100 g^{-1} dry weight)								
Coumaric acid	23.6	23.5	ns	23.6	23.5	23.7	ns	ns
Isoquercitrin	2.6	2.5	*	2.5	2.5	2.6	ns	ns
Quercitrin	22.8	16.0	***	17.0 b	22.3 a	18.9 ab	*	*
Gallic acid	5.0	4.9	ns	4.9	4.9	5.1	ns	ns
Ellagic acid	2.7	0.8	***	2.0	2.1	1.3	ns	ns
Catechin	3.4	3.1	ns	2.7	3.0	4.3	ns	ns
Epicatechin	6.1	8.3	ns	6.4	6.3	9.0	ns	ns
Safranal	4.4	4.0	***	4.2	4.3	4.2	ns	ns
Crocin I	32.5	67.9	**	49.2	37.7	63.8	ns	ns
Crocin II	31.1	36.6	*	35.0 ab	38.8 a	27.7 b	*	*
Total Vitamin C	76.5	67.0	**	71.4	70.1	73.2	ns	ns
TPC (mg$_{GAE}$ 100 g^{-1} DW)	1340.7	2355.5	ns	1906.1	1868.8	1819.5	ns	ns
Antocyanins (mg$_{C3G}$ 100 g^{-1} DW)	1866.5	1633.6	ns	964.1	2418.8	1867.5	ns	ns
Antioxidant activity								
FRAP (mmol Fe^{2+} kg^{-1})	408.9	1937.1	***	424.8 a	463.8 a	338.2 b	***	***
ABTS (μmole g^{-1})	4.2	4.6	ns	4.3	4.5	4.6	ns	ns

4. Discussion

4.1. AMF Colonization

In the literature, only some studies report AMF colonization of *C. sativus* roots. In the present study, their presence in potted cultivation was detected in *C. sativus* roots subjected to both AMF

treatments (Ri + Fm and Ri). Saffron root fragments showed extensive AM fungal colonization, characterized by a moderate to high intensity of colonization and arbuscule formation. Saffron root colonization in the present pot cultivation trial was markedly superior to the results obtained in the open field test. This could be due to the antagonistic action of the naturally occurring fungi in the soil that compete with the AMF and by the different cultivation substrate used. However, to the best of our knowledge, this is the first report clearly indicating and measuring successful symbiosis between *C. sativus* roots and AMF under pot cultivation conditions.

Our open field data are equal or lower than findings obtained in other open field trials as reported by Aimo et al. [40] and Lone et al. [43]. Applying the percent colonization method for root AMF evaluation, these authors reached a maximum of 30% and 60% mycorrhizal colonization in saffron roots in Italy and Kashmir cultivation fields when using a mix of AMF belonging to the genus *Glomus*., respectively. In a similar study conducted in Iranian fields, the percentage of root colonization of saffron was of 39% [42], while in a field in Kashmir, ranged between 15 and 90% on the basis of the season [43]. As reported in Supplementary Table 1, P Olsen values measured at the experimental sites are high (>69.2 mg kg^{-1}), indicating the potential for a detrimental effect of P on AMF colonization in our experiment. As the cost of the symbiosis to the plant outweighs the benefit of access to P via the fungal pathway, plants reduce fungal access to carbohydrate [54]. Similar data were reported also in other species such as *Zea mays* L. in which the AMF root colonization was reduced with a soil P content of 90 mg kg^{-1} [55]. In other geophyte plants, such as *Allium tricoccum* Aiton., a low level of AMF symbiosis was observed in the absence of leaves and photosynthetic activity. However, once leaves elongate in early spring, root colonization increases rapidly. This is similar to the pattern of *Maianthemum racemosum* L., where AMF colonization peaked during vegetative growth [56]. Here, AMF sampling was performed during maximum leaf elongation, and therefore, the detection of low colonization is likely more related to soil characteristics than to other physiological or biochemical parameters.

Taken together, all these findings indicated that under open field conditions in Alpine environments, AMF colonization was substantially lower than under pot conditions as already indicated in literature. This is in agreement with the meta-analysis of Berruti et al. [27], in which successful outcomes of AMF inoculation were more often found in controlled (greenhouse and growth chamber) conditions. In this condition, environmental extremes and variation are minimised or absent [38]. Moreover, one of the most important confounding factors in pot or field experiments is the effect of root temperature on the AMF growth [57]. The higher temperatures typical of greenhouse conditions favour greater growth and superior colonization by AMF [58].

4.2. AMF Modulate Crop Performance and Spice Quality

Flower yield is a difficult parameter to forecast in saffron since it is influenced by a combination of agronomic, biological, and environmental factors [11]. Generally, a saffron field may produce from 200 to 3000 mg m^{-2} of spice, depending on the cultivation factors [11] and obviously, by the planting density, which may vary considerably. By planting at a 55 corms m^{-2} density in southern Italy (Sicily), Gresta et al. [3] obtained more than 1200 mg m^{-2}. In the area of Navelli (central Italy) [59], with a similar corm density, the average yield ranged between 1000-1600 mg m^{-2}. In Iranian fields with a density of 150 and 100 corms m^{-2}, Mollafilabi et al. [60] and Koocheki et al. [61] obtained 740 and 370 mg m^{-2} of saffron, respectively. Recently, the path coefficient analysis conducted by Bayat et al. [62] highlighted that fresh stigma weight, flower number, dry stigma and flower weight, leaf size, and number and size of replacement corms have the highest positive correlation with saffron yield.

Arbuscular mycorrhizal fungi are known to be beneficial to several important plants, including some medicinal plants [30]. Unfortunately, very scarce reports of the effective role of AMF in saffron yield are available. Only, Aimo et al. [40] indicate an increase in flower production m^{-2} (equal to 68%, compared to control) using a mix of AMF species belonging to the genus *Glomus*. Our results are generally more supportive of the benefits of AMF inoculation with an increase of flower production

m^{-2} of circa 140%. Taken together, these findings suggest a beneficial effect of AMF inoculation with a mixture of *R. intraradices* and *F. mosseae* on saffron yield performance.

Few spices are able to provide the combination of color, taste, and aroma to the foods and possess several nutraceutical properties for human health as saffron. Most of the beneficial effects of saffron, recognized since ancient times, are due mainly to its total phenolic content (TPC) and antioxidant activity (FRAP and ABTS assays). *R. intraradices* alone was found to induce an increase in secondary metabolite contents, such as terpenes and phenolics, in *Salvia officinalis* L. [63] and *Echinacea purpurea* L. [64]. Overall, the saffron produced in the studied alpine areas had very high TPC (ranges between 1340.7–2355.5 mgGAE 100 g^{-1} DW), which was more than saffron cultivated in different areas of Lebanon (160 mgGAE 100g^{-1} DW) [65], and is much greater when compared with other common food additives and spices, such as *Eugenia caryophylate* (Thunb.), *Lavandula* spp., *Curcuma domestica* Val, and *Curcuma longa* L. (0.26, 0.22, 23m and 36 mgGAE 100 g^{-1} DW, respectively) (Table 4) [66,67]. Results of ABTS and FRAP assays also demonstrated elevated antioxidant activity (Table 4). ABTS assay values were comparable to what was found in Greek saffron by Ordoudi et al. [68]. FRAP assay values (ranges between 408.9 and 1937.1 mmol Fe2 + kg^{-1}) were generally higher in comparison to the Iranian samples (circa 570 mmol Fe2 + kg^{-1}) analysed by Karimi et al. [69]. The saffron produced in the west Italian Alps also had different bioactive compounds (Table 4) known for their health-promoting activity, that is, cinnamic acids, flavonols, benzoic acids, catechins, and carotenoids [50]. Other studies report that water-soluble carotenoids such as crocins have antioxidant effects superior to α-tocopherol [67]. It was recently observed in a clinical study that high crocin I and crocin II contents (4000 and 1000 mg, respectively) inhibit β-amyloid and tau aggregation [70]. Apart from crocins, Asdaq and Inamdar [71] suggest that flavonols are responsible for the synergistic antihyperlipidemic and antioxidant potential of saffron. Amin et al. [72] indicated that a concentration of 1 mg of safranal attenuated the behavioural symptoms of neuropathic pain. Our data indicate that the saffron produced presented high crocin II content (27.7–38.8 mg 100 g^{-1} DW), almost in line with the saffron produced in Sardinia (Italy, DOP Zafferano di Sardegna) [73], while also presenting a higher content of gallic acid compared to what was found in Iranian and Greek saffron (2 mg and 1.2 100 g^{-1} DW) by Karimi et al. [69] and Proestos et al. [74], respectively. Thus, the saffron obtained could be of particular interest for its elevated antioxidant properties.

5. Conclusions

Saffron quality may vary greatly by site on the basis of several factors, among which are climatic conditions and cultivation techniques. We hereby provide data indicating the production of high quality saffron in marginal Alpine areas, thus confirming that this crop is a strategic resource and good alternative for mountainous areas building multifunctional economies. Besides the phytochemical profile highlighted, the crop had many bioactive compounds. The use of arbuscular mycorrhizal symbionts as biostimulants positively affected saffron cultivation, mainly by increasing the crop productivity, and partially also the content of important nutraceutical compounds. Specifically, the inoculum composed by *R. intraradices* and *F. mosseae* was particularly effective in increasing flower production and saffron yield, while *R. intraradices* alone increased the content of some bioactive compounds such as picrocrocin, quercitrin, crocin II, and antioxidant activity. Since saffron is the world's highest priced spice, the increases in yield and quality obtained using AMF should allow for an increase in profitability.

Furthermore, a new perspective can be envisaged. Since AMF symbiosis was more effective under soilless pot cultivation, this system may be a valuable alternative for saffron production and further work is underway to assess the potential of AMF inocula in saffron soilless cultivation.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: title, Figure S2: , Table S1: title, Table S2: title.

Author Contributions: M.C., A.B., E.L., V.B., and V.S. contributed to the experimental design. M.C., I.M.M.V., S.D., D.D., A.B., and V.S. acquired and interpreted data. M.C. drafted the manuscript. V.B. and V.S. conceived, coordinated the work, and critically revised the manuscript.

Funding: This research was funded by the project titled 'SaffronALP—Lo zafferano di montagna: tecniche sostenibili per una produzione di qualità'—Fondazione Cassa di Risparmio di Torino (RF = 2017.1966) and by the program Interreg V-A Francia Italia Alcotra "Attività innovative per lo sviluppo della filiera transfrontaliera del fiore edule—Antea' n. 1139.

Acknowledgments: Authors acknowledge Dario Sacco for statistical assistance, Alessandro Putzolu for technical assistance, and Azienda Agricola La Branche di Diego Bovard and Azienda Agricola Rosset for providing plant material and hosting cultivation.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Molina, R.; García-Luis, A.; Coll, V.; Ferrer, C.; Valero, M.; Navarro, Y.; Guardiola, J. Flower formation in the saffron crocus (*Crocus sativus* L.). The role of temperature. *Acta Hortic.* **2004**, *650*, 39–47.
2. Mollafilabi, A. *Production and Modern Agronomic of Saffron*; Scientific and Industrial Research Organization of Khorasan: Mashhad, Iran, 2004; pp. 22–35.
3. Gresta, F.; Santonoceto, C.; Avola, G. Crop rotation as an effective strategy for saffron (*Crocus sativus* L.) cultivation. *Sci. Hortic.* **2016**, *211*, 34–39.
4. D'Archivio, A.; Giannitto, A.; Maggi, M.; Ruggieri, F. Geographical classification of Italian saffron (*Crocus sativus* L.) based on chemical constituents determined by high-performance liquid-chromatography and by using linear discriminant analysis. *Food Chem.* **2016**, *212*, 110–116.
5. Carmona, M.; Zalacain, A.; Pardo, J.; López, E.; Alvarruiz, A.; Alonso, G. Influence of different drying and aging conditions on saffron constituents. *J. Agric. Food Chem.* **2005**, *53*, 3974–3979.
6. Tong, Y.; Yan, Y.; Zhu, X.; Liu, R.; Gong, F.; Zhang, L.; Wang, P. Simultaneous quantification of crocetin esters and picrocrocin changes in Chinese saffron by high-performance liquid chromatography-diode array detector during 15 years of storage. *Pharmacogn. Mag.* **2015**, *11*, 540–545.
7. ISO 3632-1:2011. *Spices—Saffron (Crocus sativus L.). Food Products SC 7, Spices, Culinary Herbs and Condiments*; International Organization for Standardization: Geneva, Switzerland, 2011.
8. Shahi, T.; Assadpour, E.; Jafari, S. Main chemical compounds and pharmacological activities of stigmas and tepals of 'red gold'; saffron. *Trends Food Sci. Technol.* **2016**, *58*, 69–78.
9. Bagur, M.; Salinas, G.; Jiménez-Monreal, A.; Chaouqi, S.; Llorens, S.; Martínez-Tomé, M.; Alonso, G. Saffron: An Old Medicinal Plant and a Potential Novel Functional Food. *Molecules* **2018**, *23*, 30.
10. De Juan, J.A.; Córcoles, H.L.; Muñoz, R.M.; Picornell, M.R. Yield and yield components of saffron under different cropping systems. *Ind. Crops Prod.* **2009**, *30*, 212–219.
11. Gresta, F.; Lombardo, G.; Siracusa, L.; Ruberto, G. Saffron an alternative crop for sustainable agricultural systems. A review. *Agron. Sustain. Dev.* **2008**, *28*, 95–112.
12. Yarami, N.; Kamgar-Haghighi, A.A.; Sepaskhah, A.R.; Zand-Parsa, S. Determination of the potential evapotranspiration and crop coefficient for saffron using a water-balance lysimeter. *Arch. Agron. Soil Sci.* **2011**, *57*, 727–740.
13. Maggio, A.; Raimondi, G.; Martino, A.; De Pascale, S. Soilless cultivation of saffron in mediterranean environment. *Acta Hortic.* **2006**, *718*, 515–522.
14. Molina, R.; Valero, M.; Navarro, Y.; Guardiola, J.; Garcia-Luis, A. Temperature effects on flower formation in saffron (*Crocus sativus* L.). *Sci. Hortic.* **2005**, *103*, 361–379.
15. Giorgi, A.; Pentimalli, D.; Giupponi, L.; Panseri, S. Quality traits of saffron (*Crocus sativus* L.) produced in the Italian Alps. *Open Agric.* **2017**, *2*, 52–57.
16. Manzo, A.; Panseri, S.; Bertoni, D.; Giorgi, A. Economic and qualitative traits of Italian Alps saffron. *J. Mt. Sci.* **2015**, *12*, 1542–1550.
17. De Pascale, S.; Rouphael, Y.; Colla, G. Plant biostimulants: Innovative tool for enhancing plant nutrition in organic farming. *Eur. J. Hortic. Sci.* **2017**, *82*, 277–285.
18. De Ponti, T.; Rijk, B.; Van Ittersum, M. The crop yield gap between organic and conventional agriculture. *Agric. Syst.* **2012**, *108*, 1–9.

19. Colla, G.; Nardi, S.; Cardarelli, M.; Ertani, A.; Lucini, L.; Canaguier, R.; Rouphael, Y. Protein hydrolysates as biostimulants in horticulture. *Sci. Hortic.* **2015**, *196*, 28–38.
20. Rouphael, Y.; Cardarelli, M.; Colla, G. Role of arbuscular mycorrhizal fungi in alleviating the adverse effects of acidity and aluminium toxicity in zucchini squash. *Sci. Hortic.* **2015**, *188*, 97–105.
21. Mercy, L.; Lucic-Mercy, E.; Nogales, A.; Poghosyan, A.; Schneider, C.; Arnholdt-Schmitt, B. A Functional Approach towards Understanding the Role of the Mitochondrial Respiratory Chain in an Endomycorrhizal Symbiosis. *Front. Plant Sci.* **2017**, *8*, 417.
22. Błaszkowski, J.; Kovács, G.; Balázs, T.; Orłowska, E.; Sadravi, M.; Wubet, T.; Buscot, F. *Glomus africanum* and *G. iranicum*, two new species of arbuscular mycorrhizal fungi (Glomeromycota). *Mycologia* **2010**, *102*, 1450–1462.
23. Johnson, N.; Gehring, C. Mycorrhizas: Symbiotic mediators of rhizosphere and ecosystem processes. *Rhizosphere* **2007**, 73–100, doi:10.1016/B978-012088775-0/50006-9.
24. Berruti, A.; Borriello, R.; Lumini, E.; Scariot, V.; Bianciotto, V.; Balestrini, R. Application of laser microdissection to identify the mycorrhizal fungi that establish arbuscules inside root cells. *Front. Plant Sci.* **2013**, *4*, 135.
25. Borriello, R.; Berruti, A.; Lumini, E.; Della Beffa, M.T.; Scariot, V.; Bianciotto, V. Edaphic factors trigger diverse AM fungal communities associated to exotic camellias in closely located Lake Maggiore (Italy) sites. *Mycorrhiza* **2015**, *25*, 253–265.
26. Liu, J.; Wu, L.; Wei, S.; Xiao, X.; Su, C.; Jiang, P.; Song, Z.; Wang, T.; Yu, Z. Effects of arbuscular mycorrhizal fungi on the growth, nutrient uptake and glycyrrhizin production of licorice (*Glycyrrhiza uralensis* Fisch). *Plant Growth Regul.* **2007**, *52*, 29–39.
27. Berruti, A.; Lumini, E.; Balestrini, R.; Bianciotto, V. Arbuscular mycorrhizal fungi as natural biofertilizers: Let's benefit from past successes. *Front. Microbiol.* **2016**, *6*, 1559.
28. Bianciotto, V.; Victorino, I.; Scariot, V.; Berruti, A. Arbuscular mycorrhizal fungi as natural biofertilizers: Current role and potential for the horticulture industry. *Acta Hortic.* **2018**, *1191*, 207–215.
29. López-Ráez, J.; Verhage, A.; Fenández, I.; García, J.; Azcón-Aguilar, C.; Flors, V.; Pozo, M. Hormonal and transcriptional profiles highlight common and differential host responses to arbuscular mycorrhizal fungi and the regulation of the oxylipin pathway. *J. Exp. Bot.* **2010**, *61*, 2589–2601.
30. De Sousa Oliveira, M.; da Silva Campos, M.; de Albuquerque, U.P.; da Silva, F.S.B. Arbuscular mycorrhizal fungi (AMF) affects biomolecules content in *Myracrodruon urundeuva* seedlings. *Ind. Crops Prod.* **2013**, *50*, 244–247.
31. Bona, E.; Lingua, G.; Manassero, P.; Cantamessa, S.; Marsano, F.; Todeschini, V.; Copetta, A.; D'Agostino, G.; Massa, N.; Avidano, L.; et al. AM fungi and PGP pseudomonads increase flowering, fruit production, and vitamin content in strawberry grown at low nitrogen and phosphorus levels. *Mycorrhiza* **2015**, *25*, 181–193.
32. Lazzara, S.; Militello, M.; Carrubba, A.; Napoli, E.; Saia, S. Arbuscualr mycorrhizal fungi altered the hypericin, pseudohypericin, and hyperforin conten in flowers of Hypericum perforatum grown under contrasting P availability in a highly organic substrate. *Mycorrhiza* **2017**, *27*, 345–354.
33. Jurkiewicz, A.; Ryszka, P.; Anielska, T.; Waligórski, P.; Białońska, D.; Góralska, K.; Tsimilli-Michael, M.; Turnau, K. Optimization of culture conditions of *Arnica montana* L.: Effects of mycorrhizal fungi and competing plants. *Mycorrhiza* **2010**, *20*, 293–306.
34. Zeng, Y.; Guo, L.; Chen, B.; Hao, Z.; Wang, J.; Huang, L.; Yang, G.; Cui, X.; Yang, L.; Wu, Z.; et al. Arbuscular mycorrhizal symbiosis and active ingredients of medicinal plants: Current research status and prospectives. *Mycorrhiza* **2013**, *23*, 253–265.
35. Giovannetti, M.; Avio, L.; Sbrana, C. Improvement of nutraceutical value of food by plant symbionts. In *Natural Products, Phytochemistry, Botany and Metabolism of Alkaloids, Phenolics and Terpenes*; Ramawat, K.G., Merillon, J.M., Eds.; Springer: Berlin, Germany, 2013; pp. 2641–2662.
36. Rydlová, J.; Jelínkova, M.; Dušek, K.; Dušková, E.; Vosátka, M.; Püschel, D. Arbuscular mycorrhiza differentially affects synthesis of essential oils in coriander and dill. *Mycorrhiza* **2016**, *26*, 123–131.
37. Hart, M.M.; Antunes, P.M.; Chaudhary, V.B.; Abbott, L.K. Fungal inoculants in the field: Is the reward greater than the risk? *Funct. Ecol.* **2017**, *32*, 126–135.
38. Ryan, M.H.; Graham, J.H. Little evidence that farmers should consider abundance or diversity of arbuscular mycorrhizal fungi when managing crops. *New Phytol.* **2018**, *220*, 1092–1107.

39. Berg, G. Plant-microbe interactions promoting plant growth and health: Perspectives for controlled use of microorganisms in agriculture. *Appl. Microbiol. Biotechnol.* **2009**, *84*, 11–18.
40. Aimo, S.; Gosetti, F.; D'Agostino, G.; Gamalero, E.; Gianotti, V.; Bottaro, M.; Gennaro, M.; Berta, G. Use of arbuscular mycorrhizal fungi and beneficial soil bacteria to improve yield and quality of saffron (*Crocus sativus* L.). *Acta Hortic.* **2010**, *850*, 159–162.
41. Venkateshwaran, M.; Volkening, J.; Sussman, M.; Ané, J. Symbiosis and the social network of higher plants. *Curr. Opin. Plant Biol.* **2013**, *16*, 118–127.
42. Mohebi-Anabat, M.; Riahi, H.; Zanganeh, S.; Sadeghnezhad, E. Effects of arbuscular mycorrhizal inoculation on the growth, photosynthetic pigments and soluble sugar of *Crocus sativus* L. (saffron) in autoclaved soil. *Int. J. Agron. Agric. Res.* **2015**, *6*, 296–304.
43. Lone, R.; Shuab, R.; Koul, K. AMF association and their effect on metabolite mobilization, mineral nutrition and nitrogen assimilating enzymes in saffron (*Crocus sativus*) plant. *J. Plant Nutr.* **2016**, *39*, 1852–1862.
44. Shajari, M.A.; Moghaddam, P.R.; Ghorbani, R.; Koocheki, A. Increasing saffron (*Crocus sativus* L.) corm size through the mycorrhizal inoculation, humic acid application and irrigation managements. *J. Plant Nutr.* **2018**, *41*, 1047–1064.
45. Moghaddam, P.R.; Koocheki, A.; Molafilabi, A.; Seyyedi, M. Effect of biological and chemical fertilizers on replacement corm and flower yield of saffron (*Crocus sativus* L.). *Iran. J. Crop Sci.* **2013**, *15*, 234–246.
46. Cusano, E.; Consonni, R.; Petrakis, E.A.; Astraka, K.; Cagliani, L.R.; Polissiou, M.G. Integrated analytical methodology to investigate bioactive compounds in *Crocus sativus* L. flowers. *Phytochem. Anal.* **2018**, *29*, 476–486.
47. Vahedi, M.; Kabiri, M.; Salami, S.A.; Rezadoost, H.; Mirzaie, M.; Kanani, M.R. Quantitative HPLC-based metabolomics of some Iranian saffron (*Crocus sativus* L.) accessions. *Ind. Crops Prod.* **2018**, *118*, 26–29.
48. Trouvelot, A.; Kough, J.; Gianinazzi-Pearson, V. Mesure du taux de mycorhization VA d'un système radiculaire. Recherche de méthodes d'estimation ayant une signification fonctionnelle. In *Mycorrhizae: Physiology and Genetics*; Gianinazzi-Pearson, V., Gianinazzi, S., Eds.; INRA Press: Paris, France, 1986; pp. 217–221.
49. Caser, M.; D'Angiolillo, F.; Chitarra, W.; Lovisolo, C.; Ruffoni, B.; Pistelli, L.; Pistelli, L.; Scariot, V. Ecophysiological and phytochemical responses of *Salvia sinaloensis Fern.* to drought stress. *Plant Growth Regul.* **2018**, *84*, 383–394.
50. Donno, D.; Beccaro, G.; Mellano, M.; Cerutti, A.; Bounous, G. Goji berry fruit (*Lycium* spp.): Antioxidant compound fingerprint and bioactivity evaluation. *J. Funct. Foods* **2015**, *18*, 1070–1085.
51. Caser, M.; D'Angiolillo, F.; Chitarra, W.; Lovisolo, C.; Ruffoni, B.; Pistelli, L.; Pistelli, L.; Scariot, V. Water deficit regimes trigger changes in valuable physiological and phytochemical parameters in *Helichrysum petiolare* Hilliard & B.L. Burtt. *Ind. Crops Prod.* **2016**, *83*, 680–669.
52. Urbani, E.; Blasi, F.; Simonetti, M.; Chiesi, C.; Cossignani, L. Investigation on secondary metabolite content and antioxidant activity of commercial saffron powder. *Eur. Food Res. Technol.* **2016**, *242*, 987–993.
53. Donno, D.; Mellano, M.G.; Prgomet, Z.; Beccaro, G.L. Advances in *Ribes x nidigrolaria* Rud. Bauer & A. Bauer fruits as potential source of natural molecules: A preliminary study on physico-chemical traits of an underutilized berry. *Sci. Hortic.* **2018**, *237*, 20–27.
54. Gosling, P.; Mead, A.; Proctor, M.; Hammond, J.P.; Bending, G.D. Contrasting arbuscular mycorrhizal communities colonizing different host plants show a similar response to a soil phosphorus concentration gradient. *New Phytol.* **2013**, *198*, 546–556.
55. Watt-Williams, S.J.; Smith, F.A.; Jakobsen, I. Soil phosphorus availability is a driver of the responses of maize (*Zea mays*) to elevated CO_2 concentration and arbuscular mycorrhizal colonization. *Symbiosis* **2018**, 1–10, doi:10.1007/s13199-018-0573-0.
56. Brundrett, M.; Kendrick, B. The roots and mycorrhizas of herbaceous woodland plants. II. Structural aspects of morphology. *New Phytol.* **1990**, *114*, 469–479.
57. Gavito, M.E.; Olsson, P.A.; Rouhier, H.; Medina-Peñafiel, A.; Jakobsen, I.; Bago, A.; Azcón-Aguilar, C. Temperature constraints on the growth and functioning of root organ cultures with arbuscular mycorrhizal fungi. *New Phytol.* **2005**, *168*, 179–188.
58. Gavito, M.E.; Azcón-Aguilar, C. Temperature stress in arbuscular mycorrhizal fungi: A test for adaptation to soil temperature in three isolates of *Funneliformis mosseae* from different climates. *Agric. Food Sci.* **2012**, *21*: 2–11.

59. Tammaro, F. Saffron (*Crocus sativus* L.) in Italy. In *Saffron: Crocus sativus L.*; Negbi, M., Ed.; Harwood Academic Publishers: Amsterdam, NL, 1999; pp. 53–62.
60. Mollafilabi, A.; Koocheki, A.; Moeinerad, H.; Kooshki, L. Effect of plant density and weight of corm on yield and yield components of saffron (*Crocus sativus* L.) under soil, hydroponic and plastic tunnel cultivation. *Acta Hortic.* **2013**, *997*, 51–58.
61. Koocheki, A.; Seyyedi, S. How irrigation rounds and mother corm size control saffron yield, quality, daughter corms behavior and phosphorus uptake. *Sci. Hortic.* **2016**, *213*, 132–143.
62. Bayat, M.; Amirnia, R.; Rahimi, M. Phenotypic and genotypic relationships between traits in saffron (*Crocus sativus* L.) as revealed by path analysis. *J. Appl. Res. Med. Aromat. Plants* **2017**, *5*, 33–40.
63. Geneva, M.; Stancheva, I.; Boychinova, M.; Mincheva, N.; Yonova, P. Effects of foliar fertilization and arbuscular mycorrhizal colonization on *Salvia officinalis* L. growth, antioxidant capacity, and essential oil composition. *J. Sci. Food Agric.* **2010**, *90*, 696–702.
64. Araim, G.; Saleem, A.; Arnason, J.; Charest, C. Root colonization by an arbuscular mycorrhizal (AM) fungus increases growth and secondary metabolism of purple coneflower, *Echinacea purpurea* (L.) Moench. *J. Agric. Food Chem.* **2009**, *57*, 2255–2258.
65. Makhlouf, H.; Saksouk, M.; Habib, J.; Chahine, R. Determination of antioxidant activity of saffron taken from the flower of *Crocus sativus* grown in Lebanon. *Afr. J. Biotechnol.* **2011**, *10*, 8093–8100.
66. Gülçin, I.; Güngör, Ş.; Beydemir, Ş.; Elmastas, M.; İrfan, K. Comparison of antioxidant activity of clove (*Eugenia caryophylata* Thunb) buds and lavender (*Lavandula stoechas* L.). *Food Chem.* **2004**, *87*, 393–400.
67. Chen, Y.; Zhang, H.; Tian, X.; Zhao, C.; Liu, Y.; Jia, L.; Yin, H.X.; Chen, C. Antioxidant potential of crocins and ethanol extracts of *Gardenia jasminoides* ELLIS and *Crocus sativus* L. A relationship investigation between antioxidant activity and crocin contents. *Food Chem.* **2008**, *109*, 484–492.
68. Ordoudi, S.; Befani, C.; Nenadis, N.; Koliakos, G.; Tsimidou, M. Further Examination of Antiradical Properties of *Crocus sativus* Stigmas Extract Rich in Crocins. *J. Agric. Food Chem.* **2009**, *57*, 3080–3086.
69. Karimi, E.; Oskoueian, E.; Hendra, R.; Jaafar, H. Evaluation of *Crocus sativus* L. stigma phenolic and flavonoid compounds and its antioxidant activity. *Molecules* **2010**, *15*, 6244–6256.
70. Inoue, E.; Shimizu, Y.; Masui, R.; Hayakawa, T.; Tsubonoya, T.; Hori, S.; Sudoh, K. Effects of saffron and its constituents, crocin-1, crocin-2, and crocetin on α-synuclein fibrils. *J. Nat. Med.* **2018**, *72*, 274–279.
71. Asdaq, S.; Inamdar, M. Potential of garlic and its active constituent, S-allyl cysteine, as antihypertensive and cardioprotective in presence of captopril. *Phytomedicine* **2010**, *17*, 1016–1026.
72. Amin, S.; Parker, M.; Armbrust, E. Interactions between diatoms and bacteria. *Microbiol. Mol. Boil. Rev.* **2012**, *76*, 667–684.
73. Del Campo, P.; Carmona, M.; Maggi, L.; Kanakis, C.; Anastasaki, E.; Tarantilis, P.; Polissiou, M.; Alonso, G. Picrocrocin Content and Quality Categories in Different (345) Worldwide Samples of Saffron (*Crocus sativus* L.). *J. Agric. Food Chem.* **2010**, *58*, 1305–1312.
74. Proestos, C.; Chorianopoulos, N.; Nychas, G.; Komaitis, M. RP-HPLC Analysis of the Phenolic Compounds of Plant Extracts. Investigation of Their Antioxidant Capacity and Antimicrobial Activity. *J. Agric. Food Chem.* **2005**, *53*, 1190–1195.

© 2018 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

Supplementary

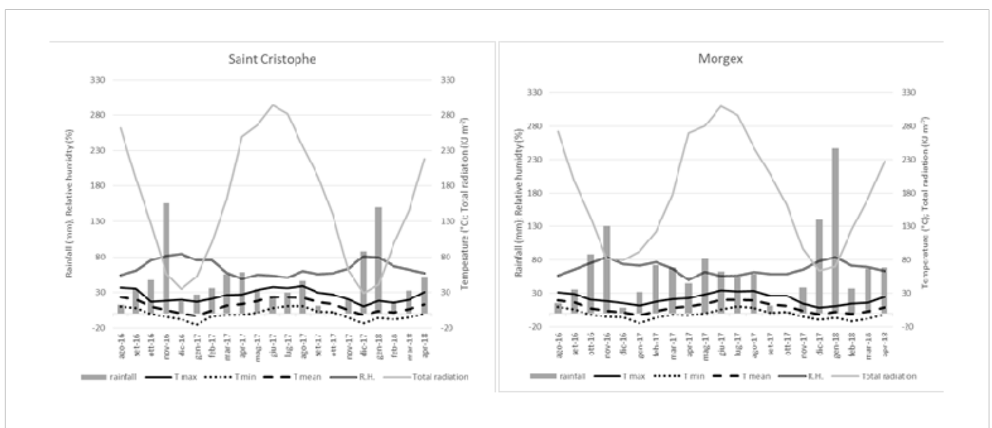


Figure S1. Climatic conditions of the Alpine experimental sites.

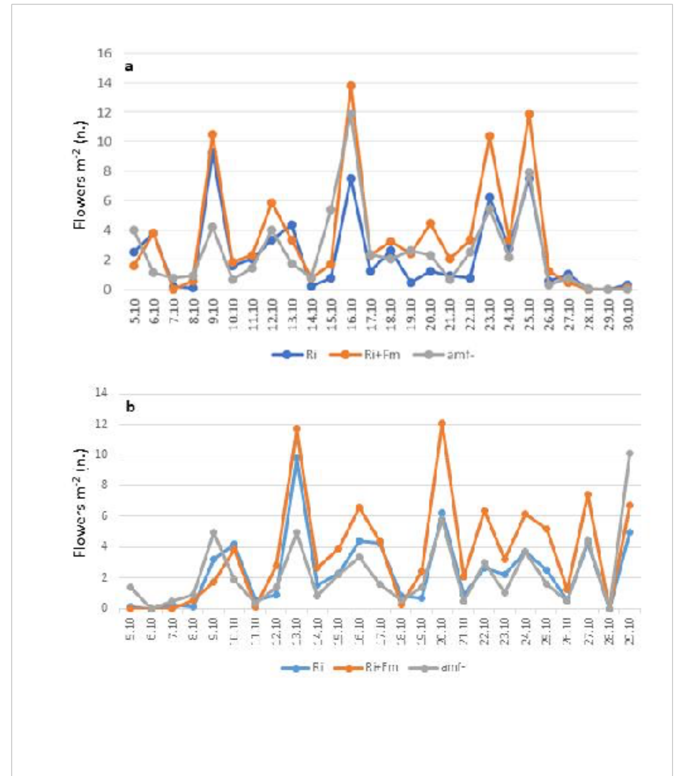


Figure S2. Effects of AMF inoculum composed by *Rhizophagus intraradices* and *Funneliformis mosseae* (Ri + Fm), *R. intraradices* alone (Ri), or control (AMF-) on flower production m^{-2} during the first (**a**) and second (**b**) cultivation cycle.

Table S1. Physical and chemical properties of the soils collected in the three saffron experimental fields located in the municipality of Saint Cristophe and Morgex (north west Italy).

		Saint Cristophe	Morgex
Texture	Clay (%)	3.7	3.8
	Fine Silt (%)	21.8	21.0
	Coarse Silt (%)	20.2	14.4
	Fine Sand (%)	24.3	25.0
	Coarse Sand (%)	29.9	35.8
Bulk density (g L^{-1})		1123.7	1075.6
Moisture (%)		17.3	20.2
P Olsen (mg Kg^{-1})		69.2	113.0
pH		6.9	7.4
Electrical conductivity (μS/cm^{-1})		316	243
N tot (%)		0.31	0.23
C tot (%)		3.50	3.79
Cation-exchange capacity (meq $100g^{-1}$)		19.2	15.7
Exchangeable Ca (meq $100g^{-1}$)		17.31	16.61
Exchangeable K (meq $100g^{-1}$)		1.47	0.53
Exchangeable Mg (meq $100g^{-1}$)		1.90	0.76

Table S2. Characteristics of the HPLC methods applied to analyse the bioactive compounds present in the studied saffron samples.

HPLC Method	Class	Standard	Stationary Phase	Mobile Phase	Flow (mL min^{-1})	Time (min)	Gradient	Wavelenght (nm)
α	Cinnamic acids Flavonols	caffeic acid chlorogenic acid coumaric acid ferulic acid hyperoside isoquercitrin quercetin quercitrin rutin	KINETEX – C18 column (4.6 × 150mm, 5 μm)	A: 10mM KH_2PO_4/H_3PO_4, pH=2.8 B: CH_3CN	1.5	20 + 2 (CT)	yes	330
β	Benzoic acids Catechins Tannins	ellagic acid gallic acid catechin epicatechin castalagin vescalagin	KINETEX – C18 column (4.6 × 150mm, 5 μm)	A: $H_2O/CH_3OH/HCOOH$ (5:95:0.1 v/v/v), pH=2.5 B: $CH_3OH/HCOOH$ (100:0.1 v/v)	0.6	23 + 2 (CT)	yes	280
γ	Carotenoids	crocin I crocin II safranal	KINETEX – C18 column (4.6 × 150mm, 5 μm)	A: H_2O B: CH_3CN	0.6	35 – 10 (CT)	yes	310, 441
δ	Vitamin C	ascorbic acid dehydroascorbic acid	KINETEX – C18 column (4.6 × 150mm, 5 μm)	A: 5 mM $C_{16}H_{33}N(CH_3)_3Br$/50 mM KH_2PO_4, pH=2.5 B: CH_3OH	0.9	10 + 5 (CT)	no	261, 348

* CT = conditioning time. Method α—gradient analysis: 5% B to 21% B in 17 min + 21% B in 3 min + 2 min of conditioning time. Method β—gradient analysis: 3% B to 85% B in 22 min + 85% B in 1 min + 2 min of conditioning time. Method χ - gradient analysis: 5%B to 95%B in 30 min – 95%B to 5%B in 5 min + 10 min of conditioning time. Method δ - isocratic analysis: 10 min + 5 min of conditioning time.

Chapter IV

MiSeq Illumina analysis as an additional tool in the labyrinth of studying Italian Alps microbiome

Preamble

Microbiome is an essential component of diverse habitats (air, soil, water and the gut of simple and complex organisms), playing a crucial role in metabolic processes of both abiotic and biotic systems, mineral recycling and breakdown, nitrogen fixation, modulation of host immune responses and production of vitamins and secondary metabolites (Reese and Dunn, 2018; Turnbaugh et al., 2007; Kamada et al., 2013; Philippot et al., 2013 and Zilber et al, 2008). To unfold ´the microbiome´ researchers have relied on the advances in high-throughput sequencing as the Illumina MiSeq system, which dominates more than 70% of the sequencing market, providing sequencing results in 1 to 2 days at a cost of $0.18 for each run. Although Illumina MiSeq technologies faces many experimental and computational challenges, it also promises to, in the near future, bring resourceful inputs to microbiome analysis (arbuscular mycorrhizal fungal and also other microorganism's communities) (Barthi and Grimm, 2021). Therefore, best-practices and standardized protocols on experimental design, sample collection, sequencing, assembly, binning, annotation and visualization are crucial to obtain comparable and reproducible results.

Italian Alpine area has become a valid means for increasing incomes of multifunctional farms due to medicinal and aromatic plants production however the microbiome present there is far from being known, so that the following paper presents the results of a study to investigate the fungal communities of two Alpine experimental sites cultivated with saffron, and to rank the relative impact of two AMF inocula, applied to soil as single species (R = *Rhizophagus intraradices, C. Walker & A. Schüßler*) or a mixture of two species (M = *R. intraradices and Funneliformis mosseae, C. Walker & A. Schüßler)*, on the resident fungal communities.

The main objective was to:

- characterize the diversity and composition of fungal communities associated to *Crocus sativus* cultivation by using Illumina MiSeq metabarcoding on nuclear ribosomal ITS2 region which might be influenced in their two fields, located in the municipalities of Saint Christophe (SC) and Morgex (MG), (Aosta Valley, Italy), treated or not with AMF inocula and sampled for two consecutive years (Y1; Y2);

As a result, data analyses consistently indicated that *Basidiomycota* were particularly abundant in both sites and sampling years. Also, significant differences in the distribution of fungal taxa assemblages at phylum and class levels between the two sites were found whether no significant difference was seen through inoculation. Further differences concerned OTUs, of other classes, significantly represented only in the first or second year of sampling. These findings altogether highlighted the fact that neither sites nor inoculation significantly impacted

Alpine saffron-field fungal communities; instead, the year of sampling had the most appreciable influence on the resident communities.

Journal of *Fungi*

Article

Metabarcoding of Soil Fungal Communities Associated with Alpine Field-Grown Saffron (*Crocus sativus* L.) Inoculated with AM Fungi

Íris Marisa Maxaieie Victorino [1,2,3,†], Samuele Voyron [1,2,†], Matteo Caser [4], Alberto Orgiazzi [5], Sonia Demasi [4], Andrea Berruti [1], Valentina Scariot [1,4], Valeria Bianciotto [1,‡] and Erica Lumini [1,*,‡]

1 Institute for Sustainable Plant Protection, National Research Council (CNR), Viale Mattioli 25, 10125 Torino, Italy; irisvictorino@gmail.com (Í.M.M.V.); samuele.voyron@unito.it (S.V.); andrea.berruti@gmail.com (A.B.); valentina.scariot@unito.it (V.S.); valeria.bianciotto@ipsp.cnr.it (V.B.)
2 Department of Life Sciences and Systems Biology, University of Turin, Viale Mattioli 25, 10125 Torino, Italy
3 Biological Science Department, Science Faculty, Eduardo Mondlane University (UEM), Av. Julius Nyerere nr 3453 Campus Principal, Maputo, Mozambique
4 Department of Agricultural, Forest and Food Sciences, University of Torino, Largo Paolo Braccini 2, 10095 Grugliasco, Italy; matteo.caser@unito.it (M.C.); sonia.demasi@unito.it (S.D.)
5 European Commission, Joint Research Centre, Via E. Fermi, 2749, 21027 Ispra, VA, Italy; alberto.orgiazzi@ec.europa.eu
* Correspondence: erica.lumini@ipsp.cnr.it; Tel.: +39 011 650 2927
† These authors equally contributed to this work.
‡ Both senior authors.

Abstract: Soil fungi strongly influence ecosystem structure and functioning, playing a key role in many ecological services as decomposers, plant mutualists and pathogens. Arbuscular mycorrhizal fungi (AMF) establish mutualistic symbiotic associations with plant roots and act as biofertilizers by enhancing plant nutrients and water uptake. Information about the AMF association with *Crocus sativus* L. (saffron) and their impact on crop performances and spice quality has been increasing in recent years. Instead, there is still little data on the biodiversity of soil microbial communities associated with this crop in the Alpine environments. The aims of this study were to investigate the fungal communities of two Alpine experimental sites cultivated with saffron, and to rank the relative impact of two AMF inocula, applied to soil as single species (R = *Rhizophagus intraradices*, C. Walker & A. Schüßler) or a mixture of two species (M = *R. intraradices* and *Funneliformis mosseae*, C. Walker & A. Schüßler), on the resident fungal communities which might be influenced in their diversity and composition. We used Illumina MiSeq metabarcoding on nuclear ribosomal ITS2 region to characterize the fungal communities associated to *Crocus sativus* cultivation in two fields, located in the municipalities of Saint Christophe (SC) and Morgex (MG), (Aosta Valley, Italy), treated or not with AMF inocula and sampled for two consecutive years (Y1; Y2). Data analyses consistently indicated that Basidiomycota were particularly abundant in both sites and sampling years (Y1 and Y2). Significant differences in the distribution of fungal taxa assemblages at phylum and class levels between the two sites were also found. The main compositional differences consisted in significant abundance changes of OTUs belonging to Dothideomycetes and Leotiomycetes (Ascomycota), Agaricomycetes and Tremellomycetes (Basidiomycota), Mortierellomycetes and Mucoromycetes. Further differences concerned OTUs, of other classes, significantly represented only in the first or second year of sampling. Concerning Glomeromycota, the most represented genus was *Claroideoglomus* always detected in both sites and years. Other AMF genera such as *Funneliformis*, *Septoglomus* and *Microdominikia*, were retrieved only in MG site. Results highlighted that neither sites nor inoculation significantly impacted Alpine saffron-field fungal communities; instead, the year of sampling had the most appreciable influence on the resident communities.

Keywords: soil fungal metabarcoding; saffron cultivation; arbuscular mycorrhizal fungi; AMF inocula; alpine field environments

Citation: Victorino, Í.M.M.; Voyron, S.; Caser, M.; Orgiazzi, A.; Demasi, S.; Berruti, A.; Scariot, V.; Bianciotto, V.; Lumini, E. Metabarcoding of Soil Fungal Communities Associated with Alpine Field-Grown Saffron (*Crocus sativus* L.) Inoculated with AM Fungi. *J. Fungi* 2021, *7*, 45. https://doi.org/10.3390/jof7010045

Received: 27 November 2020
Accepted: 8 January 2021
Published: 12 January 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Soil has always been known to be a source of microorganisms and although studied for many years its microorganism diversity is far from being fully known. Microbial communities present in soil are responsible for carrying out key ecosystem services for life on our planet but unfortunately, many beneficial functions are threatened due to climate change, soil degradation and agricultural exploitation [1,2]. The availability of soil nutrients to plants is key to obtaining healthier and better fitted crops, as they are agricultural productivity dependent on a wide range of ecosystem services provided by the soil microbiota [3]. However, most crops of global and local interest are still heavily dependent on the use of fertilizers and other chemicals which are hazardous to human and animal health and to the soil itself [4,5]. As concerns have been raised about the impact on soil microbiome of herbicides, pesticides and inorganic fertilizers, and more generally on the soil nutrient availability and plant phytotoxicity, the interest in alternative strategies for ecosystem management has greatly increased [6]. Plant growth promoting microorganisms (PGPM), i.e., soil and rhizosphere-inhabiting microorganisms, in minute quantities, promote plant growth [7], and have become one of the most important components of bio-fertilizers for sustainable agriculture. They are applied due to their role in plant growth promotion by regulating the dynamics of various processes (e.g., decomposition of the organic matter), the accessibility of various nutrients to plants (iron, magnesium, nitrogen, potassium and phosphorus), as well as acting against pathogens [8,9].

Within the categories of PGPM are the plant growth-promoting rhizobacteria (PGPR) and among the beneficial fungi, the arbuscular mycorrhizal fungi (AMF) [10,11]. It is well known that fungi can interact with plant roots in different ways, from mutualistic mycorrhizal symbioses (i.e., when both organisms live in direct contact with each other and establish mutually beneficial relationships [12], to parasitism [13]). Among mycorrhizal symbioses, arbuscular mycorrhizal (AM) fungi represent a fungal mutualistic endophytic group which establishes symbioses with over 90% of all plant species since the origin of terrestrial plants [12]. There is an increasing interest for the use of AM fungi to promote sustainable agriculture, considering the widely accepted benefits of the association with plants to nutrition efficiency (for both macronutrients, especially phosphorus, and micronutrients), water balance, and biotic and abiotic stress protection [14]. Successful progresses in AMF inoculation have been achieved and reported worldwide, but studies regarding its application from laboratory and greenhouse to field trials are still encouraged.

Agricultural practices strongly affect soil physical and chemical properties, and impact on the microbial communities affecting their abundance, diversity, and activity [15]. The effects of management practices could be positive or negative [16,17]. On the negative side, they may affect the interaction between different microbial communities, including bacteria and fungi [18], which are known to be key drivers for a more sustainable soil management [19].

High-throughput DNA sequencing techniques have greatly expanded our capability to characterize soil microbiome and identify the factors, including land management, that shape soil microbial communities across space and time [20]. In light of these studies, although most soil microorganisms still remain undescribed, some of them have recently been characterized based on their ecological strategies [21]. This aspect is of importance to identify and predict functional attributes of individual taxa that could be manipulated and managed to maintain or increase soil fertility and crop production under severe threats, including intensive exploitation [22].

While studying a soil sample it is important to consider that there is no "typical" soil microbiome, but the relative abundances of major prokaryotic and eukaryotic taxa found in the soil microbiome can vary considerably depending on the soil in question [23]. It has been widely reported that soil samples, collected from the same sampling sites just a few centimeters apart from each other, may retain very different microbiomes [20,24]. The microbiome variation can be attributed to spatial variability in the soil environment and to specific characteristics of the sampling site, sampling time and crop species and

management [3]. For this reason, over the last years, some protocols have been endorsed by international projects, such as the Earth Microbiome Project (http://earthmicrobiome.org/), to analyze and compare soil microbial diversity at a large scale [25].

Fungi are widely distributed among all terrestrial ecosystems with a huge biodiversity and ecological importance by their principal role in ecosystems processes such as carbon cycling, plant nutrition, and phytopathology [26]. However, the distribution of fungal species, phyla, and functional groups as well as the determinants of fungal diversity and biogeographic patterns are still poorly understood despite recent large-scale sampling campaigns [27]. So far, information on soil fungal biodiversity in ecosystems such as Italian Alpine cultivated areas is still scarce, with the exception of some studies on vineyards [28,29] and apple orchards [30], compared to those concerning European alpine meadows, pastures, woods or specific alpine endemic plants and environments [31–34]. In the Aosta Valley region (north west Italy), smallholder farming systems, such as saffron cultivation, lead to interesting and unique Alpine agricultural ecosystems. Indeed, they are characterized by a high level of agricultural diversity, being mainly focused on meeting farmers' needs. In this regard, they could represent a valid means for increasing incomes of multifunctional farms, with a positive impact on the recovery and economy of these often remote areas [35,36]. In particular, saffron is gaining increasing attention as an alternative crop in sustainable agricultural systems due to its unique biological, physiological, and agronomic traits, such as the capability to exploit marginal land. The application of AMF or PGPR inocula has also found interest due to the possibility to increase the overall cultivation sustainability and quality [37–39] In this context, we have already gained insight into the impact of AMF inoculants on growth and secondary metabolites production in saffron plantations [40].

Specifically, we record an increasing interest in applying low-input cropping systems (e.g., saffron cultivation) in mountain regions. Nonetheless, this is accompanied by a scant data collection on soil microbial diversity in saffron productive areas along with limited information on the effect of AMF inoculation on field-grown *Crocus sativus* L. (saffron). To fill this knowledge gap, the aims of this study were to investigate the fungal communities of two Alpine experimental sites cultivated with saffron, and to rank the relative impact of two AMF inocula, applied to soil as single species (R = *Rhizophagus intraradices*, C. Walker & A. Schüßler) or a mixture of two species (M *R. intraradices* and *Funneliformis mosseae*, C. Walker & A. Schüßler), on the resident fungal communities which might be influenced in their diversity and composition. We used Illumina MiSeq metabarcoding on nuclear ribosomal ITS2 region to characterize the fungal communities associated to Crocus sativus cultivation in two fields, located in the municipalities of Saint Christophe (SC) and Morgex (MG), (Aosta Valley, Italy), treated or not with AMF inocula and sampled for two consecutive years (Y1; Y2). In the frame of an increasing demand to reduce chemical inputs in agriculture, the results of this study could reveal useful information on the real impact of AMF inoculation on the resident fungal communities opening new perspectives on the possible roles of AMF and/or other most competitive beneficial microbes to be further exploited in a sustainable agriculture perspective.

2. Materials and Methods

2.1. Sampling Sites

Two Italian western alpine experimental sites, located in the municipality of Morgex (45°45′35.1″ N; 7°02′37.3″ E; 1000 m a.s.l.) and Saint Christophe (45°45′06.9″ N; 7°20′37.0″ E; 700 m a.s.l.) (Aosta Valley, AO) and cultivated with saffron for at least the previous three years, were selected for our analyses (Figure 1). The experiment covered two successive cultivation cycles: year 1 (Y1) (2016–2017) and year 2 (Y2) (2017–2018) as reported by Caser et al. [40]. Before starting the experiment, both fields were milled and, at time of plantation (mid-August), *Crocus sativus* corms (caliber size of 6–7 cm, without antifungal application), kindly provided by the Azienda Agricola "La Branche di Diego Bovard" (Morgex, AO, Italy), were treated with two AMF inocula (MycAgro Lab, Breteniére, France).

One was composed by the single species *Rhizophagus intraradices* (R), the other by the mix of *R. intraradices* and *Funneliformis mosseae* (M). As Control (C), corms were not inoculated with AMF but with their sterile carrier. Just before the first year of planting (August 2016), 10 g of inoculum/carrier was placed under the corms to ensure contact between plant and the treatment [40], while in the second year (August 2017) corms were planted without any further inoculum supply.

A randomized block design was used, with three experimental plot units (blocks) (Figure 1). Each plot unit consisted of 56 corms, planted in a 1.44 m^2 area (39 corms m^2). Inter-row planting distance was of 7 cm, while between-row distance was of 25 cm. Plots were separated from each other with at least 4 m distance. Irrigation was provided when needed and hand weeding control was conducted during cultivation, while no pre-planting fertilization, tillage, or treatments against pathogens were applied. Sites were characterized by semi-continental climate, with a long and cold winter, and both of them had a sandy-loam texture, according to the USDA classification, and similar chemical characteristics [41]. At each experimental site, samples were collected for 2 following cultivation cycles (Y1 and Y2) at flowering time (November 2016 and 2017). Five replicates of bulk soil were collected in each of the three experimental plot unit (C; M; R) with an earth drill from the first 30 cm of soil following a V-shaped sampling pattern, pooled to generate a total of three biological replicates and kept in zip lock bags at 4 °C before being processed in the laboratory. Soil samples were sieved using 2 mm stainless steel sieve mesh and then put in tubes at −20 °C for further molecular analysis. A total of 9 soil samples for Y1 and 9 samples for Y2 were collected respectively in Morgex and Saint Christophe for a grand total of 36 soil samples.

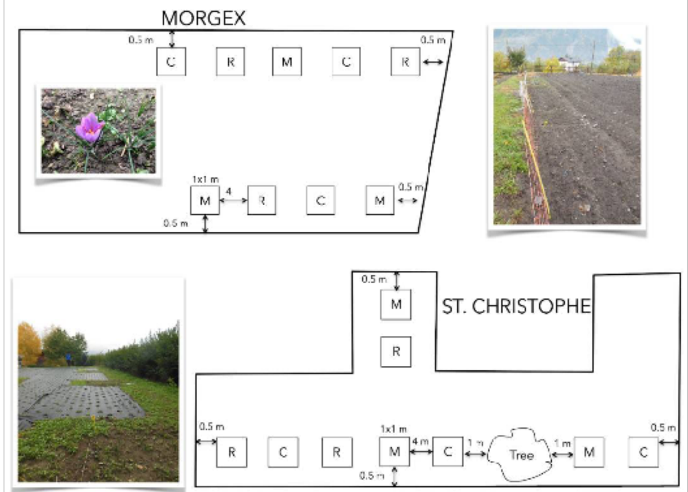


Figure 1. Scheme of the randomized block design in the experimental sites located in the municipality of Morgex (45°45′35.1″ N; 7°02′37.3″ E, 1000 m a.s.l.) and Saint Christophe (45°45′06.9″ N; 7°02′37.0″ E, 700 m a.s.l.), Aosta Valley (AO), Italy. (C = Carrier (Control); R = Single-species inoculum (*Rhizophagus intraradices*); M = Multi-species inoculum (*R. intraradices* and *Funneliformis mosseae*).

2.2. Site Environmental Conditions

In the first cultivation season (2016–2017), average temperatures ranged from −3.5 °C to 23.4 °C in Saint Christophe, and from −2.7 °C to 20.0 °C in Morgex. In particular, Morgex showed greater precipitation rate (57.5 mm/month) and higher relative humidity (R.H.), with the peak in November (89.3%) compared to Saint Cristophe. Conversely, the total radiation was generally higher in Saint Cristophe, from −2.3 °C to 22.6 °C than in Morgex, from −1.2 °C to 19.5 °C. In fact, Morgex showed, in general, more wet conditions and more

rainfall weather conditions, with the highest precipitation rate and R.H. (74.5 mm/month and 84.0%, respectively).

2.3. Soil DNA Extraction, PCR Amplification and Sequencing

In order to achieve our main objective, DNeasy PowerSoil Kit, (formerly sold by MO BIO as PowerSoil DNA Isolation Kit) (Qiagen, Hilden, Germany) endorsed by the Earth Microbiome Project (http://earthmicrobiome.org/) was used. Extractions were carried out from 250 mg of soil samples, following the manufacturer's protocol.

To investigate the total fungal community, the nuclear ribosomal ITS2 region was amplified using Invitrogen Platinum HotStart PCR Master MIx (Thermo Fisher Scientific, Waltham, Massachusetts, USA) from all DNA extracts by means of a semi-nested PCR approach. In the first PCR, the entire ITS (ITS1-5.8S-ITS2) region was amplified with the generic fungal primer pair ITS1F-ITS4 [42,43]. The cycling conditions were an initial step at 95 °C for 15 min, 35 cycles at 95 °C for 35 s, 57 °C for 35 s, 72 °C for 45 s, and a final extension step of 72 °C for 7 min. Each PCR product was checked on agarose gel, diluted at 1:20 and used as a template in the semi-nested PCR targeting the ITS2 region, with primer fITS9 (5′–GAACGCAGCRAAIIGYGA–3′), which has been previously reported to match only 20% of 5.8S AMF sequences at NCBI [44], and ITS4 (5′–TCCTCCGCTTATTGATATGC–3′). This second couple of primers was added to Illumina overhang adapter sequences: forward overhang: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG- [locus specific target primer], reverse overhang: 5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG- [locus specific target primer]. The semi-nested PCR cycling conditions were an initial step at 95 °C for 15 min, 27 cycles at 95 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s, and a final extension step of 72 °C for 7 min.

For the 36 samples (18 in Y1 and 18 in Y2) DNA extracted was amplified in triplicate and pooled prior the purification using the Wizard® SV Gel and PCR Clean-Up System (Promega, Milano, Italy). A final number of 36 PCR purified products were quantified with Qubit 2.0 (Invitrogen, Carlsbad, California, USA) following manufacturer's protocol and sent for Illumina MiSeq sequencing (2 × 250 bp) to IGA technologies (Udine, Italy).

2.4. Bioinformatic and Statistical Analyses on Soil Fungal Communities

DNA reads from each sampling point included forward and reverse sequences in separate files. Sequencing adapters and primers were removed, and the sequences were then analyzed by means of the microbiome bioinformatics platform QIIME2 (Quantitative Insights Into Microbial Ecology 2, version 2019.7 [45]. Denoising and quality control, including removal of chimeras, were achieved by means of the DADA2 [46] plugin (qiime dada2 denoise-paired). To avoid low quality sequences, reads were truncated (300 bp for forward, 290 bp for reverse reads). The classifier adopted for the taxonomic assignment of the total fungal community was generated using the UNITE Community (2019): UNITE QIIME release for Fungi version 02.02.2019.

The generated dataset, including OTU table, taxonomy table and metadata, was then imported in Rstudio (RStudio Team 2016) and was used to create a phyloseq object with the R package phyloseq [47] that was employed for all the following analyses. To allow statistical comparisons, the OTU table was rarefied at 8185 sequences per sample, by means of the rarefy_even_depth function of the R package phyloseq. Rarefaction curves of the non-rarefied and rarefied OTU table were obtained by means of the function rarecurve of the R package vegan v. 2.5-4 [48]. Biodiversity analyses were carried out by comparing the richness (number of species) and evenness (richness taking into account relative abundances) of fungal communities. Within-sample (alpha) diversity was assessed by three estimators: "observed fungal species," "Chao1," and "Shannon". The alpha diversity indices were calculated and plotted by means of the functions estimate_richness and plot_richness implemented in the R package phyloseq [47]. Bar plots were then generated with the R package ggplot2 version 3.1.0 [49]. Ordination plots (NMDS) were generated by means of the R packages phyloseq, ggplot2 and plyr version 1.8.4 [50]. The

trophism of retrieved OTUs was defined by means of the FUNGuild package [51]. In order to evaluate significant differences ($p < 0.05$) between alpha diversity indexes or influences of the site, inoculum and time of sampling over the fungal populations' distributions, the adonis function from R package vegan was used to perform PERMANOVA tests. PERMANOVA tests were run for every metadata, or combinations of metadata using dissimilarity index of Bray-Curtis. Permutest was also run in order to check the validity of the previous tests by means of the function betadisper of the R package vegan. Significant differences between taxonomic distribution at the phylum level of fungal communities retrieved from the two sites was evaluated by means of the software Past 4 (https://past.en.lo4d.com/windows), and the test Anova, Mann-Whitney pairwise comparison; Bonferroni corrected ($p < 0.05$). ITS2 representative sequences were deposited in GenBank under the accession numbers (MW162630-164623).

3. Results

3.1. Composition and Structure of Fungal Soil Communities

After the bioinformatic analysis, 487,815 high-quality ITS2 sequences (out of a total of 2,572,327 raw sequences) were retained and clustered in 1391 operational taxonomic units (OTUs). In order to perform statistical analysis, the resulting OTU tables were then rarefied at 8185 sequences per sample for a total number of 1100 OTUs. The fungal taxonomic diversity retrieved in the two sites (MG and SC) for the two years of sampling (Y1 and Y2) is reported at phylum level in Table 1.

Table 1. Taxonomic distribution, at phylum level, of operational taxonomic units (OTUs) retrieved from the two sampled sites (Morgex: MG; Saint Christophe: SC) at Y1 (year 1) and Y2 (year 2). Data are expressed as percentage (mean ± standard deviation). Different letters indicate significant differences (Anova; Mann-Whitney pairwise comparison; Bonferroni corrected p values; $p < 0.05$).

	Y1		Y2	
Phylum	**MG**	**SC**	**MG**	**SC**
Ascomycota	31.06 ± 5.78 ^{a}	18.39 ± 1.96 ^{b}	28.80 ± 7.41 ^{a}	16.12 ± 4.36 ^{b}
Basidiomycota	45.14 ± 7.33 ^{a}	59.83 ± 6.59 ^{b}	46.86 ± 11.36 ^{a}	66.02 ± 6.21 ^{b}
Chytridiomycota	1.46 ± 0.86 ^{a}	1.14 ± 0.58 ^{a}	2.01 ± 1.18 ^{a}	0.66 ± 0.54 ^{b}
Glomeromycota	0.50 ± 0.57 ^{a}	0.22 ± 0.19 ^{a}	0.68 ± 1.04 ^{a}	0.29 ± 0.31 ^{a}
Kickxellomycota	7.42 ± 1.89 ^{a}	7.08 ± 0.75 ^{a}	7.35 ± 2.08 ^{a}	9.35 ± 1.52 ^{a}
Mortierellomycota	2.92 ± 1.30 ^{a}	0.93 ± 0.63 ^{b}	5.63 ± 3.41 ^{a}	0.79 ± 0.65 ^{b}
Mucoromycota	5.64 ± 1.38 ^{a}	3.43 ± 1.40 ^{a}	4.87 ± 1.78 ^{a}	1.32 ± 1.08 ^{b}
unidentified	5.28 ± 2.77 ^{a}	8.94 ± 4.82 ^{a}	3.71 ± 1.43 ^{a}	5.43 ± 5.78 ^{a}

The percentage of the different phyla was variable among the two sampled sites. As a matter of fact (Table 1), Basidiomycota were particularly abundant in the two sites sampled and for both years (Y1 and Y2). More in detail, Basidiomycota, over the two sampling times, are significantly more present in SC (approx. 59–66%; Y1 and Y2 respectively) than in MG (approx. 45–46%; Y1 and Y2 respectively). Conversely, Ascomycota were significantly more present in MG (approx. 31–28%), both for Y1 and Y2, than in SC (approx. 18–16%). A similar trend is shown by Mortierellomycota and only for Y2 by Mucoromycota. Our data showed a significant decrease of Chytridiomycota in SC at Y2, while for Kickxellomycota, Glomeromycota, and for the unidentified fungi, we did not report any significant differences.

In particular, in MG, the Basidiomycota most represented classes were Agaricomycetes (25–31%) followed by Tremellomycetes (16–13%), Pucciniomycetes (2–0.67%) and Microbotriomycetes (1.4–0.90%), in Y1 and Y2 respectively, while in SC Agaricomycetes represented up to 49–54% of the total followed by a smaller percentage of Tremellomycetes (5.6–8.5%) and a percentage less than 1–2% of both Pucciniomycetes and Microbotriomycetes. Considering Ascomycota, the most represented classes were for MG Dothideomycetes (12–7.8%) followed by Sordariomycetes (8–9%) and Leotiomycetes (5.5–7%)

for Y1 and Y2 respectively. Concerning SC, the most represented class is Sordariomycetes (6–7%) followed by Dothideomycetes (6–3%) and Leotiomycetes (3.3–3%) for Y1 and Y2 respectively. The complete taxonomic association at class level is reported in Table S1.

Regarding Glomeromycota, our results underline that, when considering site and time of sampling, there were not significant differences in terms of taxa abundance while AM fungal community composition was variable in terms of retrieved genera (Figure 2A,B). As a matter of fact, SC was mostly dominated by the genus *Claroideoglomus* both for Y1 and Y2, whereas MG showed higher diversity: *Claroideoglomus* was again the most represented genus, but in MG we retrieved also *Funneliformis*, *Septoglomus*, *Glomus* and *Microdominikia* genera.

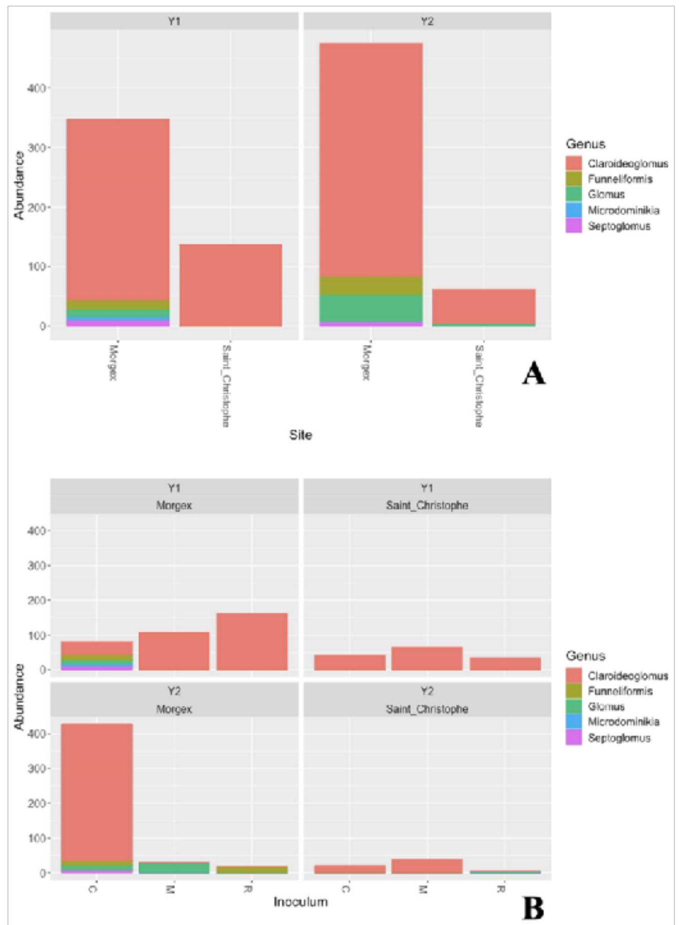


Figure 2. (**A**) Bar chart showing the taxonomic distribution (based on fungal ITS2), at genus level, of OTUs belonging to the phylum Glomeromycota, retrieved from Morgex, and Saint Christophe; (**B**) Bar chart showing the effect of the inoculum on the taxonomic distribution (based on fungal ITS2), at genus level, of OTUs belonging to the phylum Glomeromycota, retrieved from Morgex, and Saint Christophe. (Y1: year 1; Y2: year 2; C – Carrier (Control); R – Single-species inoculum (*Rhizophagus intraradices*); M – Multi-species inoculum (*R. intraradices* and *Funneliformis mosseae*).

Community shifts from Y1 and Y2, due to inoculation with AMF, are shown in Figure 2B. In SC all the treatments displayed lower relative abundance levels of the most represented genus (i.e., *Claroideoglomus*) which was nearly to disappear only in the single species inoculated plots (R). In MG, the abundance of *Claroidoglomus* OTUs sharply decreased in both the inoculated plots (M and R) giving way to OTUs belonging to *Glomus* and *Funneliformis* genera, which were not retrieved in the Y1. On the other hand, in the MG control plot (C), the abundance of *Funneliformis*, *Glomus* and *Septoglomus* remained almost unchanged, *Microdominikia* instead was no longer detected and *Claroidoglomus* represented the most retrieved genus ever found.

The ITS region sequence data were also used to infer the putative ecological roles of the total fungal communities at each sampled site (Figure 3). The assignment to a strategy was possible for 546 (49, 63%) out of 1100 ITS2 OTUs. In terms of trophic diversity, the abundance of saprotroph, pathotroph and saprotroph/symbiotroph fungi was significantly higher in MG than in SC, both at Y1 and Y2, and only for pathotroph/saprotroph guilds at Y2. Symbiotrophs are instead significantly more represented at SC, both at Y1 and Y2.

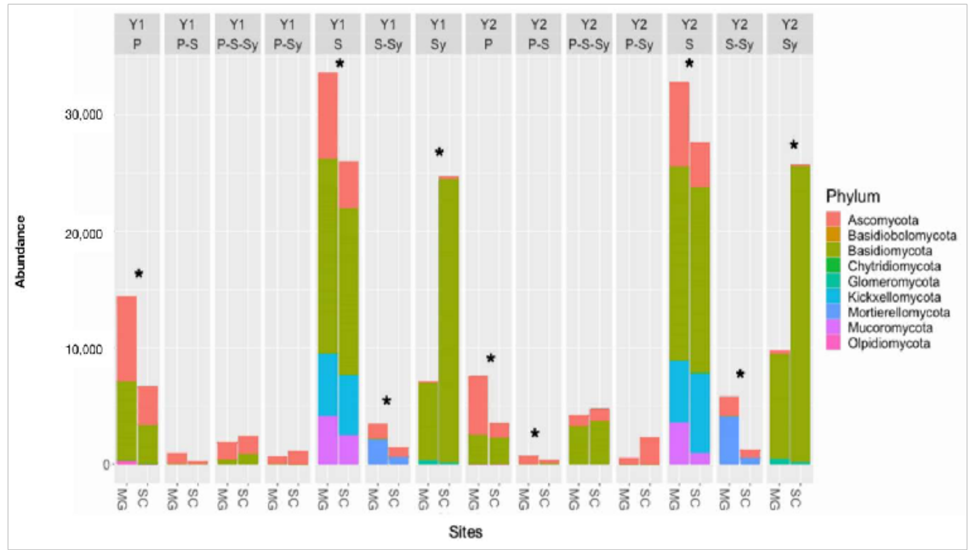


Figure 3. Trophic distribution and phylum composition of OTUs retrieved from Morgex (MG) and Saint Christophe (SC) at sampling time Y1 (first year) and Y2 (second year). S, saprotrophs; Sy symbiotrophs; P, pathotrophs (fungi showing different trophic behaviors are separately annotated). Data are expressed as percentage (mean ± standard deviation). *: indicate significant differences (Anova; Mann-Whitney pairwise comparison; Bonferroni corrected p values; $p < 0.05$).

3.2. Soil Fungal Microbiota Assembly and AMF Inocula Impact

The evaluation of alpha diversity highlighted that the variable most significantly affecting the fungal community was the year of sampling (Y1 and Y2), while AM fungal inocula did not exert significant effect (Figure S1). The homogeneity of dispersion among groups was supported by a non-significant result in permutest. PERMANOVA analyses also revealed that the fungal communities thriving in the soil of Morgex and Saint Christophe were not significantly affected by the AMF inocula but only by the sampling time (Y1; Y2) ($p \leq 0.05$, Bray–Curtis) (Figure 4).

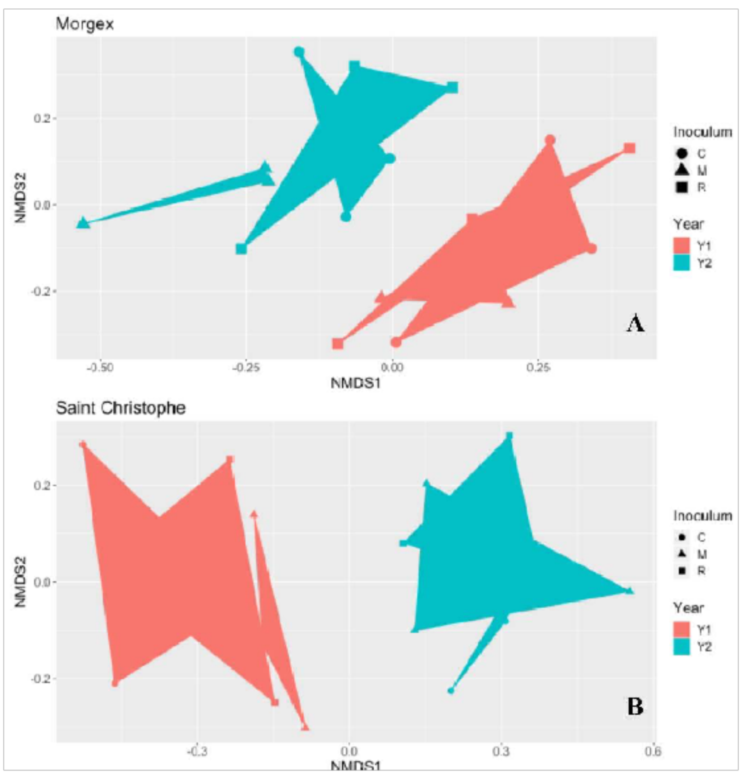


Figure 4. Ordination plots (NMDS) showing beta diversity of fungal communities of Morgex (**A**) and Saint Christophe (**B**). The two fungal communities are significantly affected by the sampling time (Y1; Y2) ($p \leq 0.05$, Permanova; Bray–Curtis) but not by the inoculum (C − Carrier Control; R − Single-species inoculum (Rhizophagus intraradices); M − Multi-species inoculum (R. intraradices and Funneliformis mosseae).

4. Discussion

Despite growing interest, the variability of soil microbial fungal communities (fungi and bacteria) and the biotic and abiotic factors that drive their differentiation are still poorly understood in some remote, but still ecologically important, environments such as Alpine marginal cultivated fields. Soil microbiome could be of particular relevance in saffron (*Crocus sativus* L.) cultivation, since soil has been shown to serve as a reservoir of microorganisms which, once colonizing roots, might contribute to saffron plant growth, nutrient availability and pathogen defense [38,39,52].

Besides bacteria, the major component of soil microbiota is represented by fungi which play crucial roles as saprotrophs, plant mutualists, symbionts and pathogens [53]. Moreover, fungi are key in controlling the soil structure and its water content and in regulating the aboveground biodiversity and productivity [54]. Due to their large number of species, specialization, and important ecological functions, fungi are also considered excellent bioindicators of soil quality [55]. This aspect is especially relevant in the case of marginal alpine areas where saffron yield and quality may vary greatly by site on the basis of several factors such as soil types, climatic conditions and cultivation techniques [35,36]. However, one of the most important factors, namely the soil microbiota associated with cultivation sites, has been poorly explored in *C. sativus* worldwide, in spite of its economic value both at national and international level. Here, for the first time, we reported results

obtained by profiling the fungal components of soils cultivated with saffron through Illumina MiSeq metabarcoding on fungal ITS2 from the Valle d'Aosta agriculture sites of Saint Christophe (SC) and Morgex (MG), and covering two years of sampling (Y1, Y2).

In general, the two sites were characterized by slightly different fungal phyla assemblages: in both sites and years (Y1 and Y2), Basidiomycota were particularly abundant in soil. This findings is not in line with previous reports identifying dominant fungal phylotypes as belonging to generalist Ascomycota that dominate soils globally [56]. Our results are also different from those obtained by Coller et al. [29] in Alpine vineyards soils showing Ascomycota (51.8%) and Zygomycota (20.1%) as dominant Phyla, while Basidiomycota representing only a small fraction (11.2%). The surprising percentage of Basidiomycota in saffron cultivated fields, where some of the OTUs were affiliated to ectomycorrhizal species (e.g., Inocybe vulpinella Bruylants; Suillus granulatus (L.) Roussel, S. viscidus (L.) Roussel), could be explained by the fact that most major genera of fungi, such as the ectomycorrhizal genera *Russula*, *Boletus*, *Inocybe*, *Cortinarius* and *Amanita*, seem to be present on all habitable continents [56]. It is worth noting that the site of SC is also characterized by: a shrubby fence, the presence of a tree inside the plot, several plants of birch just around the plot fence and very few grass (Figure 1). We can speculate that our data on the belowground fungal community may provide useful elements on the aboveground features such as previous and actual vegetation coverage and/or agronomic procedures, allowing to assess the impact of anthropogenic land use to hidden diversity in soil [31]. Through the analysis of fungal ecological guilds, we indeed highlighted that fungal symbiotrophs were significant more in SC than in MG. On the other hand, the higher abundance of saprotroph, pathotroph and saprotroph/symbiotroph fungi in MG could be explained by some other site specific edaphic characteristics.

Our results are in line with those obtained in the same experimental plots by Caser and colleagues [41]. Indeed, they clearly demonstrated that the largest difference in physiological and biochemicals flower-related traits and corm properties of saffron plants cultivated in the same sites (i.e., Morgex and Saint Christophe) were between the growing seasons (Y1 to Y2). In particular, they found that many more moldy corms (wilted) occurred in the first cultivation season (36.8%) than in the second (16.8%), and more in Morgex (52.4%) than Saint Christophe (39.1%). They argued that elevated percentage of wilted corms was probably due to the high relative humidity and precipitation rate (more than 550 mm/year), mainly occurring in MG than in SC and, to the absence of corm antifungal treatments. In accordance with those findings, our results showed that Morgex's soil seems to thrive not only significantly higher saprotroph but more important, pathotroph and saprotroph/symbiotroph fungi.

Even if a specific characterization of plant pathogens was not conducted, the high wilting rate found in MG could be related and favored by the presence, in this site, of several fungal species belonging to *Ascomycetes* such as *Blumeria*, *Colletotrichum*, *Curvularia*, *Gibberella*, *Leptosphaeria*, *Plectosphaerella*, *Ramularia*, *Stigmina*. Indeed, all these have been reported to be associated with saffron diseases [57,58]. We also detected some taxa, previously reported as endophytic fungal isolates, belonging to two Ascomycota lineages representing two orders (Helotiales and Pleosporales) and one order each in Basidiomycota and Mortierellomycota, namely Agaricales and Mortierellales, respectively. The presence in soil of microbial endophytes is very important. In fact, some endophytes (e.g., *M. alpina*) showed positive effects on many growth parameters (i.e., total biomass, size of corms, number of apical sprouting buds, number of adventitious roots) and plant secondary metabolite production [59]. Furthermore, the endophyte can enhance biotic stress tolerance to corm rot fungus by releasing arachidonic acid [60].

Despite being also designed to rank the relative impact of two AMF inocula on the resident soil fungal communities, this study highlighted that the variable significantly affecting the fungal communities was instead only the year of sampling (Y1 and Y2). Neither the sites nor inoculum application significantly influenced soil fungal diversity and composition. In particular, our results also showed that apparently, the resident AM fungal

communities found in the treated or control plots of Morgex and Saint Christophe were not significantly affected by the introduction of commercial AMF-based inoculum and that the AMF sequences retrieved from the soil metagenome very partially reflected the species inoculum composition. In addition, unlike what has been previously reported [61] the inoculation process seems to increase the dominance of a single species (i.e., *Claroidoglomus*) and decrease diversity of the preexisting AMF communities. Unlike the results obtained in other Alpine environments of Northern Italy by Berruti et al. [28], on vineyards of Aosta Valley, and Turrini et al. [30], on apple orchards of South Tyrol, the most abundant genus retrieved in saffron alpine agriculture sites, was not *Glomus* but *Claroideoglomus*, representing up to 80% of the *Glomeromycota* sequences. Similar to previous reports, AMF taxa belonging to *Septoglomus* and *Funneliformis* corresponded to less than 4% of total sequences in these agriculture sites. However, we must point out that the differences found may be due to the different methods, which were used in the studies cited above, to investigate AM soil fungal communities and only partially overlapping with ours. Regarding the higher AMF abundance and biodiversity found in Morgex we could speculate that this site is characterized by some patchy areas dominated by grasses; an environment more favorable to AM Fungi such as surrounding herbaceous plants, from which AMF propagules and/or healthy AMF mycelial networks could gradually have colonized saffron cultivated fields. Another hypothesis could be that in MG the vegetation-mediated legacy effects on soil microbial communities is still maintained.

Furthermore, no sequences of *Rhizophagus/Rhizoglomus* spp. that represented the main taxon of one of the applied inoculum (i.e., R) were found. Many factors can affect the success of inoculation and AMF persistence, including environmental and cultivation conditions, species compatibility, degree of spatial competition with other soil organisms, and the time of inoculation. Hence, it is important to assess the effects of AMF on crop traits both as early application and as residual persistence in the following crop cultivation seasons [61]. This aspect of particular importance is in accordance with previous results showing that AMF root colonization of *C. sativus*, treated with mixed or with single inoculum, during the two successive cultivation cycles (year Y1 and Y2), was very low in terms of both intensity of colonization (0.0–9.0%) and percentage of arbuscules (0.0–4.4%) [40].

These results are, however, in line with those reported by Ceballos et al. [62] and Berruti et al. [61] on cassava and maize plants inoculated by AMF, respectively. In fact, even if root mycorrhization was very low for both plants (which are usually very well mycorrhized in the field) they eventually produced higher yields anyway. Beside the results regarding the belowground aspect of our experiment, it is important instead to underline that in many experiments AM fungal applications exerted an important impact on the above-grown features [37,39]. In particular, the inoculation of saffron with single or multiple AM fungal isolates was demonstrated to increase either flower production and, saffron yield as well as spice antioxidant activity and the content of some important bioactive compounds (i.e., picrocrocin, crocin I, and quercitrin) [39,40].

We can assume that the AM fungal inoculum may have had a side stimulating effect on other resident soil microbial components (i.e., PGPR) that affect plant growth and physiology. Another possible explanation could be the presence of PGPR strictly associated with spores of some AM fungal species commonly used as inoculum [63].

5. Conclusions

For the first time, we have characterized the fungal communities of an Italian alpine agroecosystem cultivated with saffron and treated with arbuscular mycorrhizal fungal inocula. Since saffron is the world's highest priced spice, the increases in yield and quality obtained using AMF inoculum along with having no evident impact on resident microbial populations suggests that farms in marginal areas such as alpine sites can increase profitability by inoculating saffron bulbs with arbuscular mycorrhizal fungi.

In addition to AMF, it would be advisable to investigate other endophytes to be co-inoculated with saffron bulbs, or directly recruited from soils, that could offer further advantages, for example, an increasing tolerance to biotic and/or abiotic stresses. This last aspect is of particular importance because, after a few years of continuous cultivation, saffron could exert allelopathic activity against many soil components [64,65], thus compromising both its replanting and the cultivation of alternative crops (i.e., lettuce) [66].

Lastly, the provided datasets may contribute to future searches on fungal bio-indicators to be applied as biodiversity markers of a specific site and/or agriculture cultivation.

Supplementary Materials: The following are available online at https://www.mdpi.com/2309-608X/7/1/45/s1, Table S1. Taxonomic distribution, at Class level, of OTUs retrieved from the two sampled sites (Morgex: MG; Saint Christophe: SC) at Y1 (year 1) and Y2 (year 2); Figure S1: Alpha diversity measures of saffron fields: Observed OTUs, Chao1 and Shannon.

Author Contributions: Conceptualization, V.B., E.L., M.C., and V.S.; methodology, E.L., A.B., Í.M.M.V., V.B. and A.O.; field sampling, Í.M.M.V., M.C., S.D., E.L., V.B.; wet lab research and data Í.M.M.V. and E.L.; bioinformatics S.V., and A.B.; data analyses Í.M.M.V., E.L., S.V. and A.B. Original draft manuscript preparation E.L., Í.M.M.V., S.V. A.O. partially contributed to funding acquisition. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the project titled "SaffronALP—Lo zafferano di montagna: tecniche sostenibili per una produzione di qualità"—Fondazione Cassa di Risparmio di Torino (RF – 2017.1966).

Informed Consent Statement: Not applicable.

Data Availability Statement: The sequences data presented in this study are available in GenBank under the accession numbers (MW162630-164623).

Acknowledgments: The authors acknowledged L. Allione (IPSP-CNR) for administrative support.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Amundson, R.; Berhe, A.A.; Hopmans, J.W.; Olson, C.; Sztein, A.E.; Sparks, D.L. Soil and human security in the 21st century. *Science* **2015**, *348*, 1261071. [CrossRef] [PubMed]
2. Jansson, J.K.; Hofmockel, K.S. The soil microbiome—From metagenomics to metaphenomics. *Curr. Opin. Microbiol.* **2018**, *43*, 162–168. [CrossRef] [PubMed]
3. Bahram, M.; Hildebrand, F.; Forslund, K.; Anderson, J.L.; Soudzilovskaia, N.A.; Bodegom, P.M.; Bengtsson-Palme, J.; Anslan, S.; Coelho, L.P.; Harend, H.; et al. Structure and function of the global topsoil microbiome. *Nature* **2018**, *560*, 233–237. [CrossRef] [PubMed]
4. Mahdi, S.S.; Hassan, G.; Samoon, S.; Rather, H.; Dar, S.A.; Zehra, B. Bio-fertilizers in organic agriculture. *J. Phytol.* **2010**, *2*, 42–54.
5. Kour, D.; Rana, K.L.; Yadav, A.N.; Yadav, N.; Kumar, M.; Kumar, V.; Vyas, P.; Dhaliwal, H.S.; Saxena, A.K. Microbial biofertilizers: Bioresources and eco-friendly technologies for agricultural and environmental sustainability. *Biocatal. Agric. Biotechnol.* **2020**, *23*, 101487. [CrossRef]
6. Castellano-Hinojosa, A.; Strauss, S.L. Impact of Cover Crops on the Soil Microbiome of Tree Crops. *Microorganisms* **2020**, *8*, 328. [CrossRef] [PubMed]
7. Du Jardin, P. Plant biostimulants: Definition, concept, main categories and regulation. *Sci. Hortic.* **2015**, *196*, 3–14. [CrossRef]
8. De Pascale, S.; Rouphael, Y.; Colla, G. Plant biostimulants: Innovative tool for enhancing plant nutrition in organic farming. *Eur. J. Hortic. Sci.* **2018**, *82*, 277–285. [CrossRef]
9. Lalitha, S. Plant growth-promoting microbes: A boon for sustainable agriculture. In *Sustainable Agriculture towards Food Security*; Dhanarajan, A., Ed.; Springer: Singapore, 2017; pp. 125–158. [CrossRef]
10. Rouphael, Y.; Franken, P.; Schneider, C.; Schwarz, D.; Giovannetti, M.; Agnolucci, M.; De Pascale, S.; Bonini, P.; Colla, G. Arbuscular mycorrhizal fungi act as biostimulants in horticultural crops. *Sci. Hortic.* **2015**, *196*, 91–108. [CrossRef]
11. Toscano, S.; Massa, D.; Romano, D.; Bulgari, R. Biostimulant applications in low input horticultural cultivation systems. *Italus Hortus* **2019**, *25*, 27–36. [CrossRef]
12. Bonfante, P.; Genre, A. Mechanisms underlying beneficial plant–fungus interactions in mycorrhizal symbiosis. *Nat. Commun.* **2010**, *1*, 48. [CrossRef] [PubMed]
13. Behie, S.W.; Bidochka, M.J. Nutrient transfer in plant-fungal symbioses. *Trends Plant. Sci.* **2014**, *19*, 734–740. [CrossRef] [PubMed]

14. Srivastava, P.; Saxena, B.; Giri, B. Arbuscular Mycorrhizal Fungi: Green Approach/Technology for Sustainable Agriculture and Environment. In *Mycorrhiza-Nutrient Uptake, Biocontrol, Ecorestoration*; Varma, A., Prasad, R., Tuteja, N., Eds.; Springer: Cham, Switzerland, 2017.
15. Lienhard, P.; Terrat, S.; Mathieu, O.; Levêque, J.; Prévost-Bouré, N.C.; Nowak, V.; Regnier, T.; Faivre, C.; Sayphoummie, S.; Panyasiri, K.; et al. Soil microbial diversity and C turnover modified by tillage and cropping in Laos tropical grassland. *Environ. Chem. Lett.* **2013**, *11*, 391–398. [CrossRef]
16. Lehmann, A.; Rillig, M.C. Arbuscular mycorrhizal contribution to copper, manganese and iron nutrient concentrations in crops—A meta-analysis. *Soil Biol. Biochem.* **2015**, *81*, 147–158. [CrossRef]
17. Pellegrino, E.; Gamper, H.A.; Ciccolini, V.; Ercoli, L. Forage Rotations Conserve Diversity of Arbuscular Mycorrhizal Fungi and Soil Fertility. *Front. Microbiol.* **2020**, *10*, 2969. [CrossRef]
18. Dodd, I.C.; Ruiz-Lozano, J.M. Microbial enhancement of crop resource use efficiency. *Curr. Opin. Biotechnol.* **2012**, *23*, 236–242. [CrossRef]
19. De Vries, F.T.; Wallenstein, M.D. Below-ground connections underlying above-ground food production: A framework for optimising ecological connections in the rhizosphere. *J. Ecol.* **2017**, *105*, 913–920. [CrossRef]
20. Fierer, N. Embracing the unknown: Disentangling the complexities of the soil microbiome. *Nat. Rev. Microbiol.* **2017**, *15*, 579–590. [CrossRef]
21. Ho, A.; Di Lonardo, D.P.; Bodelier, P.L.E. Revisiting life strategy concepts in environmental microbial ecology. *FEMS Microbiol. Ecol.* **2017**, *93*, fix006. [CrossRef]
22. Zanne, A.E.; Abarenkov, K.; Afkhami, M.E.; Aguilar-Trigueros, C.A.; Bates, S.; Bhatnagar, J.M.; Busby, P.E.; Christian, N.; Cornwell, W.K.; Crowther, T.W.; et al. Fungal functional ecology: Bringing a trait-based approach to plant-associated fungi. *Biol. Rev.* **2020**, *95*, 409–433. [CrossRef]
23. Wang, G.; Schultz, P.; Tipton, A.; Zhang, J.; Zhang, F.; Bever, J.D. Soil microbiome mediates positive plant diversity-productivity relationships in late successional grassland species. *Ecol. Lett.* **2019**, *22*, 1221–1232. [CrossRef] [PubMed]
24. Wei, Z.; Gu, Y.; Friman, V.-P.; Kowalchuk, G.A.; Xu, Y.; Shen, Q.; Jousset, A. Initial soil microbiome composition and functioning predetermine future plant health. *Sci. Adv.* **2019**, *5*, eaaw0759. [CrossRef] [PubMed]
25. Orgiazzi, A.; Ballabio, C.; Panagos, P.; Jones, A.; Fernández-Ugalde, O. LUCAS Soil, the largest expandable soil dataset for Europe: A review. *Eur. J. Soil Sci.* **2018**, *69*, 140–153. [CrossRef]
26. Hawksworth, D.L.; Lücking, R. Fungal diversity revisited: 2.2 to 3.8 million species. In *The Fungal Kingdom*; Heitman, J., Howlett, B.J., Crous, W., Stukenbrock, E.H., James, T.Y., Eds.; American Society of Microbiology: Washington, DC, USA, 2017; pp. 79–95. [CrossRef]
27. Tedersoo, L.; Bahram, M.; Põlme, S.; Kõljalg, U.; Yorou, N.S.; Wijesundera, R.L.C.; Ruiz, L.V.; Vasco-Palacios, A.M.; Thu, P.Q.; Suija, A.; et al. Global diversity and geography of soil fungi. *Science* **2014**, *346*, 6213. [CrossRef]
28. Berruti, A.; Desirò, A.; Visentin, S.; Zecca, O.; Bonfante, P. ITS fungal barcoding primers versus 18S AMF-specific primers reveal similar AMF-based diversity patterns in roots and soils of three mountain vineyards. *Environ. Microbiol. Rep.* **2017**, *9*, 658–667. [CrossRef]
29. Coller, E.; Cestaro, A.; Zanzotti, R.; Bertoldi, D.; Pindo, M.; Larger, S.; Albanese, D.; Mescalchin, E.; Donati, C. Microbiome of vineyard soils is shaped by geography and management. *Microbiome* **2019**, *7*, 140. [CrossRef]
30. Turrini, A.; Agnolucci, M.; Palla, M.; Tomé, E.; Tagliavini, M.; Scandellari, F.; Giovannetti, M. Species diversity and community composition of native arbuscular mycorrhizal fungi in apple roots are affected by site and orchard management. *Appl. Soil Ecol.* **2017**, *116*, 42–54. [CrossRef]
31. Praeg, N.; Seeber, J.; Leitinger, G.; Tasser, E.; Newesely, C.; Tappeiner, U.; Illmer, P. The role of land management and elevation in shaping soil microbial communities: Insights from the Central European Alps. *Soil Biol. Biochem.* **2020**, *150*, 107951. [CrossRef]
32. Brunner, I.; Frey, B.; Hartmann, M.; Zimmermann, S.; Graf, F.; Suz, L.M.; Niskanen, T.; Bidartondo, M.I.; Senn-Irlet, B. Ecology of Alpine Macrofungi—Combining Historical with Recent Data. *Front. Microbiol.* **2017**, *8*, 2066. [CrossRef]
33. Casazza, G.; Lumini, E.; Ercole, E.; Dovana, F.; Guerrina, M.; Arnulfo, A.; Minuto, L.; Fusconi, A.; Mucciarelli, M. The abundance and diversity of arbuscular mycorrhizal fungi are linked to the soil chemistry of screes and to slope in the Alpic paleo-endemic Berardia subacaulis. *PLoS ONE* **2017**, *12*, e0171866. [CrossRef]
34. Montagna, M.; Berruti, A.; Bianciotto, V.; Cremonesi, P.; Giannico, R.; Gusmeroli, F.; Lumini, E.; Pierce, S.; Pizzi, F.; Turri, F.; et al. Differential biodiversity responses between kingdoms (plants, fungi, bacteria and metazoa) along an Alpine succession gradient. *Mol. Ecol.* **2018**, *27*, 3671–3685. [CrossRef] [PubMed]
35. Giorgi, A.; Pentimalli, D.; Giupponi, L.; Panseri, S. Quality traits of saffron (Crocus sativus L.) produced in the Italian Alps. *Open Agric.* **2017**, *2*, 52–57. [CrossRef]
36. Manzo, A.; Panseri, S.; Bertoni, D.; Giorgi, A. Economic and qualitative traits of Italian Alps saffron. *J. Mt. Sci.* **2015**, *12*, 1542–1550. [CrossRef]
37. Aimo, S.; Gosetti, F.; D'Agostino, G.; Gamalero, E.; Gianotti, V.; Bottaro, M.; Gennaro, M.; Berta, G. Use of arbuscular mycorrhizal fungi and beneficial soil bacteria to improve yield and quality of saffron (Crocus sativus L.). *Acta Hortic.* **2009**, *850*, 159–164. [CrossRef]
38. Sharma, T.; Kaul, S.; Dhar, M.K. Diversity of culturable bacterial endophytes of saffron in Kashmir, India. *SpringerPlus* **2015**, *4*, 661. [CrossRef] [PubMed]

39. Lone, R.; Shuab, R.; Koul, K. AMF association and their effect on metabolite mobilization, mineral nutrition and nitrogen assimilating enzymes in saffron (*Crocus sativus* L.) plant. *J. Plant Nutr.* **2016**, *39*, 1852–1862. [CrossRef]
40. Caser, M.; Victorino, Í.M.M.; Demasi, S.; Berruti, A.; Donno, D.; Lumini, E.; Bianciotto, V.; Scariot, V. Saffron Cultivation in Marginal Alpine Environments: How AMF Inoculation Modulates Yield and Bioactive Compounds. *Agronomy* **2019**, *9*, 12. [CrossRef]
41. Caser, M.; Demasi, S.; Stelluti, S.; Donno, D.; Scariot, V. *Crocus sativus* L. Cultivation in Alpine Environments: Stigmas and Tepals as Source of Bioactive Compounds. *Agronomy* **2020**, *10*, 1473. [CrossRef]
42. White, T.J.; Bruns, T.; Lee, S.; Taylor, J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A Guide to Methods and Applications*; Innis, M., Gelfand, D., Sninsky, J., White, T., Eds.; Academic Press Inc.: New York, NY, USA, 1990; p. 315.
43. Gardes, M.; Bruns, T.D. ITS primers with enhanced specificity for basidiomycetes—Application to the identification of mycorrhizae and rusts. *Mol. Ecol.* **1993**, *2*, 113–118. [CrossRef]
44. Ihrmark, K.; Bödeker, I.T.; Cruz-Martinez, K.; Friberg, H.; Kubartova, A.; Schenck, J.; Strid, Y.; Stenlid, J.; Brandström-Durling, M.; Clemmensen, K.E.; et al. New primers to amplify the fungal ITS2 region—Evaluation by 454-sequencing of artificial and natural communities. *FEMS Microbiol. Ecol.* **2012**, *82*, 666–677. [CrossRef]
45. Bolyen, E.; Rideout, J.R.; Dillon, M.R.; Bokulich, N.A.; Abnet, C.C.; Al-Ghalith, G.A.; Alexander, H.; Alm, E.J.; Arumugam, M.; Asnicar, F.; et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* **2019**, *37*, 852–857. [CrossRef] [PubMed]
46. Callahan, B.J.; McMurdie, P.J.; Rosen, M.J.; Han, A.W.; Johnson, A.J.A.; Holmes, S.P. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* **2016**, *13*, 581–583. [CrossRef] [PubMed]
47. McMurdie, P.J.; Holmes, S. phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS ONE* **2013**, *8*, e61217. [CrossRef] [PubMed]
48. Oksanen, J.; Blanchet, F.G.; Friendly, M.; Kindt, R.; Legendre, P.; McGlinn, D.; Minchin, P.R.; O'Hara, R.B.; Simpson, G.L.; Solymos, P.; et al. Vegan: Community Ecology Package. R Package Version 2.5-4. 2019. Available online: https://CRAN.R-project.org/package=vegan (accessed on 12 January 2021).
49. Wickham, H. *ggplot2: Elegant Graphics for Data Analysis*; Springer: New York, NY, USA, 2016.
50. Wickham, H. ggplot2. Wiley Interdisciplinary Reviews. *WIREs Comp. Stats.* **2011**, *3*, 180–185. [CrossRef]
51. Nguyen, N.H.; Song, Z.; Bates, S.T.; Branco, S.; Tedersoo, L.; Menke, J.; Schilling, J.S.; Kennedy, P.G. FUNGuild: An open annotation tool for parsing fungal community datasets by ecological guild. *Fungal Ecol.* **2016**, *20*, 241–248. [CrossRef]
52. Parray, J.A.; Azra, N.K.; Zaffar, A.R.; Rehana, H.; Raies, A.Q. Screening of beneficial properties of rhizobacteria isolated from Saffron (*Crocus sativus* L) rhizosphere. *Afr. J. Microbiol. Res.* **2013**, *7*, 2905–2910. [CrossRef]
53. Peay, K.G.; Kennedy, P.G.; Talbot, J.M. Dimensions of biodiversity in the Earth mycobiome. *Nat. Rev. Genet.* **2016**, *14*, 434–447. [CrossRef]
54. De Vries, F.T.; Griffiths, R.I.; Bailey, M.; Craig, H.; Girlanda, M.; Gweon, H.S.; Hallin, S.; Kaisermann, A.; Keith, A.M.; Kretzschmar, M.; et al. Soil bacterial networks are less stable under drought than fungal networks. *Nat. Commun.* **2018**, *9*, 3033. [CrossRef]
55. Orgiazzi, A.; Lumini, E.; Nilsson, R.H.; Girlanda, M.; Vizzini, A.; Bonfante, P.; Bianciotto, V. Unravelling Soil Fungal Communities from Different Mediterranean Land-Use Backgrounds. *PLoS ONE* **2012**, *7*, e34847. [CrossRef]
56. Egidi, E.; Delgado-Baquerizo, M.; Plett, J.M.; Wang, J.; Eldridge, D.J.; Bardgett, R.D.; Maestre, F.T.; Singh, B.K. A few Ascomycota taxa dominate soil fungal communities worldwide. *Nat. Commun.* **2019**, *10*, 1–9. [CrossRef]
57. Rubio-Moraga, A.; Gomez-Gomez, L.; Trapero, A.; Castro-Díaz, N.; Ahrazem, O.; Trapero-Mozos, A. Saffron corm as a natural source of fungicides: The role of saponins in the underground. *Ind. Crop. Prod.* **2013**, *49*, 915–921. [CrossRef]
58. Palmero, D.; Rubio-Moraga, A.; Galvez-Patón, L.; Nogueras, J.; Abato, C.; Gómez-Gómez, L.; Ahrazem, O. Pathogenicity and genetic diversity of Fusarium oxysporum isolates from corms of Crocus sativus. *Ind. Crop. Prod.* **2014**, *61*, 186–192. [CrossRef]
59. Wani, Z.A.; Mirza, D.N.; Arora, P.; Riyaz Ul Hassan, S. Molecular phylogeny, diversity, community structure, and plant growth promoting properties of fungal endophytes associated with the corms of saffron plant: An insight into the microbiome of Crocus sativus Linn. *Fungal Biol.* **2016**, *120*, 1509–1524. [CrossRef] [PubMed]
60. Wani, Z.A.; Kumar, A.; Sultan, P.; Bindu, K.; Riyaz-Ul-Hassan, S.; Ashraf, N. Mortierella alpina CS10E4, an oleaginous fungal endophyte of *Crocus sativus* L. enhances apocarotenoid biosynthesis and stress tolerance in the host plant. *Sci. Rep.* **2017**, *7*, 8598. [CrossRef]
61. Berruti, A.; Lumini, E.; Bianciotto, V. AMF components from a microbial inoculum fail to colonize roots and lack soil persistence in an arable maize field. *Symbiosis* **2016**, *72*, 73–80. [CrossRef]
62. Ceballos, I.; Ruiz, M.; Fernández, C.; Peña, R.; Rodríguez, A.; Sanders, I.R. The In vitro Mass-Produced Model Mycorrhizal Fungus, Rhizophagus irregularis, Significantly Increases Yields of the Globally Important Food Security Crop Cassava. *PLoS ONE* **2013**, *8*, e70633. [CrossRef]
63. Agnolucci, M.; Avio, L.; Pepe, A.; Turrini, A.; Cristani, C.; Bonini, P.; Cirino, V.; Colosimo, F.; Ruzzi, M.; Giovannetti, M. Bacteria Associated with a Commercial Mycorrhizal Inoculum: Community Composition and Multifunctional Activity as Assessed by Illumina Sequencing and Culture-Dependent Tools. *Front. Plant. Sci.* **2019**, *9*, 1956. [CrossRef]
64. Mardani, H.; Sekine, T.; Azizi, M.; Mishyna, M.; Fujii, Y. Identification of Safranal as the Main Allelochemical from Saffron (Crocus sativus). *Nat. Prod. Commun.* **2015**, *10*, 1934578X1501000519. [CrossRef]

65. Mirmostafaee, S.; Azizi, M.; Fujii, Y. Study of Allelopathic Interaction of Essential Oils from Medicinal and Aromatic Plants on Seed Germination and Seedling Growth of Lettuce. *Agronomy* **2020**, *10*, 163. [CrossRef]
66. Mardani, H.K.; Maninang, J.; Appiah, K.S.; Oikawa, Y.; Azizi, M.; Fujii, Y. Evaluation of Biological Response of Lettuce (Lactuca sativa L.) and Weeds to Safranal Allelochemical of Saffron (Crocus sativus) by Using Static Exposure Method. *Molecules* **2019**, *24*, 1788. [CrossRef]

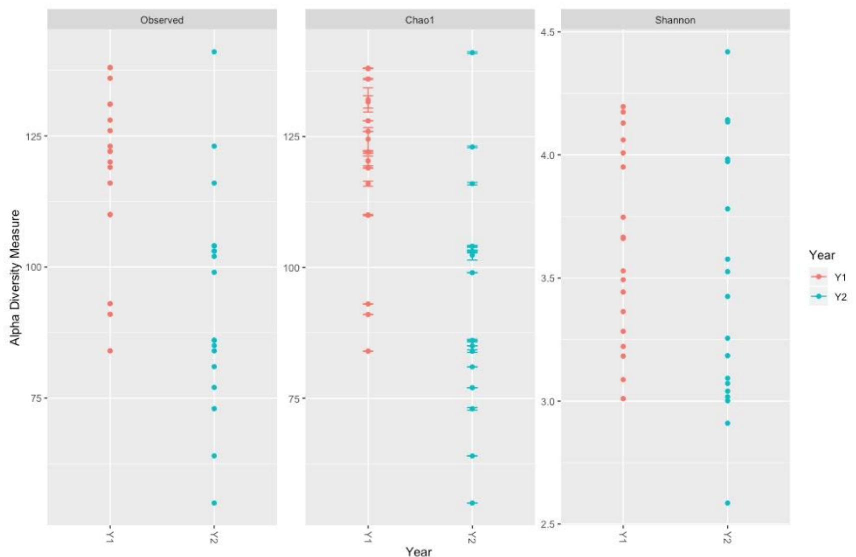


Fig.S2 Alpha diversity measure of Saffron fields: Observed OTUs, Chao 1 and Shannon.

Table S1. Taxonomic distribution, at Class level, of OTUs retrieved from the two sampled sites (Morgex: MG; Saint Christophe: SC) at Y1 (year 1) and Y2 (year 2). Data are expressed as percentage (mean ± standard deviation). Different letters indicate significant differences (Anova; Mann-Whitney pairwise comparison; Bonferroni corrected p values; p<0.05).

		Y1		Y2	
Phylum	**Class**	**MG**	**SC**	**MG**	**SC**
Ascomycota	**Archaeorhizomycetes**	0,00±0,00a	0,01±0,03a	0,00±0,00a	0,00±0,00a
	Dothideomycetes	12,22±3,53a	5,93±1,57b	7,87±2,19a	3,06±1,46b
	Eurotiomycetes	2,30±1,39a	1,14±0,52b	1,33±1,34a	1,32±1,02a
	Geoglossomycetes	0,00±0,00a	0,00±0,00a	0,04±0,11a	0,00±0,00a
	Laboulbeniomycetes	0,04±0,13a	0,01±0,02a	0,11±0,27a	0,00±0,00a
	Leotiomycetes	5,54±1,67a	3,69±2,07b	7,09±3,25a	3,33±1,59b
	Orbiliomycetes	0,32±0,45a	0,08±0,14a	0,28±0,25a	0,37±0,96a
	Pezizomycetes	1,00±0,95a	0,39±0,42a	1,41±1,46a	0,32±0,33b
	Saccharomycetes	0,37±0,51a	0,16±0,23a	0,12±0,29a	0,04±0,05a
	Sordariomycetes	8,94±2,05a	6,75±2,27b	9,66±2,63a	7,64±2,22a
	unidentified	0,33±0,40a	0,22±0,32a	0,89±0,70a	0,04±0,08b
Basidiobolomycota	**Basidiobolomycetes**	0,00±0,00a	0,02±0,04a	0,00±0,00a	0,00±0,00a
Basidiomycota	**Agaricomycetes**	25,20±4,37a	49,71±7,58b	31,13±9,18a	54,26±6,25b
	Agaricostilbomycetes	0,01±0,04a	0,00±0,00a	0,00±0,00a	0,01±0,02a
	Cystobasidiomycetes	0,01±0,03a	0,00±0,00a	0,10±0,29a	0,02±0,04a

	Dacrymycetes	0,15±0,24a	0,33±0,31a	0,08±0,19a	0,41±0,65a
	Exobasidiomycetes	0,00±0,00a	0,05±0,16a	0,08±0,15a	0,00±0,00a
	Geminibasidiomycetes	0,00±0,00a	0,03±0,08a	0,00±0,00a	0,00±0,00a
	Malasseziomycetes	0,00±0,00a	0,03±0,08a	0,00±0,00a	0,01±0,02a
	Microbotryomycetes	1,42±1,42a	0,92±0,54a	0,90±0,62a	0,88±0,71b
	Pucciniomycetes	2,10±1,09a	1,67±0,50a	0,67±0,54a	0,93±0,56a
	Tremellomycetes	16,02±4,93a	6,61±1,67b	13,63±3,53a	8,59±1,81b
	unidentified	0,14±0,20a	0,17±0,19a	0,20±0,24a	0,19±0,24a
	Ustilaginomycetes	0,08±0,13a	0,30±0,29a	0,06±0,19a	0,73±0,42b
Blastocladiomycota	**unidentified**	0,06±0,11a	0,00±0,01a	0,09±0,17a	0,00±0,01a
Chytridiomycota	**Chytridiomycetes**	0,00±0,00a	0,04±0,11a	0,00±0,00a	0,00±0,00a
	Lobulomycetes	0,00±0,00a	0,02±0,05a	0,00±0,00a	0,14±0,23a
	Rhizophlyctidomycetes	0,00±0,00a	0,01±0,03a	0,00±0,00a	0,07±0,18a
	Rhizophydiomycetes	0,06±0,12a	0,00±0,00a	0,10±0,21a	0,02±0,06a
	Spizellomycetes	0,06±0,07a	0,12±0,16a	0,01±0,03a	0,01±0,02a
	unidentified	1,34±0,90a	0,95±0,55a	1,91±1,20a	0,42±0,39b
Glomeromycota	**Archaeosporomycetes**	0,02±0,07a	0,00±0,00a	0,00±0,00a	0,00±0,00a
	Glomeromycetes	0,48±0,55a	0,18±0,19a	0,68±1,04a	0,29±0,31a
	Paraglomeromycetes	0,00±0,00a	0,03±0,06a	0,00±0,00a	0,00±0,00a
	unidentified	0,00±0,00a	0,01±0,04a	0,00±0,00a	0,00±0,00a
Kickxellomycota	**GS19**	0,09±0,21a	0,03±0,05a	0,10±0,25a	0,01±0,04a

	Kickxellomycetes	7,33±1,82a	7,06±0,75a	7,24±2,13a	9,34±1,49a
Mortierellomycota	**Mortierellomycetes**	2,92±1,30a	0,90±0,58b	5,63±3,41a	0,79±0,65b
	unidentified	0,00±0,00a	0,03±0,10a	0,00±0,00a	0,00±0,00a
Mucoromycota	**Mucoromycetes**	5,64±1,38a	3,42±1,38b	4,87±1,78a	1,32±1,08b
	unidentified	0,00±0,00a	0,01±0,02a	0,00±0,00a	0,00±0,00a
Olpidiomycota	**Olpidiomycetes**	0,44±0,36a	0,01±0,02b	0,00±0,00a	0,00±0,00a
Rozellomycota	**Rozellomycotina_cls_Incertae_sedis**	0,08±0,17a	0,01±0,04a	0,00±0,00a	0,01±0,02a
unidentified	**unidentified**	5,28±2,77a	8,94±4,82a	3,71±1,43a	5,43±5,78a

Chapter V

High-throughput sequencing platform on mycobiome diversity study and fungi identification

Preamble

After the publication of the two articles, the opportunity arose to participate in the writing of a book chapter on the molecular techniques used in the study of arbuscular mycorrhizal fungi and the subsequent Illumina MiSeq sequencing analysis.

In the chapter is described a workflow for AMF identification by high-throughput sequencing through Illumina MiSeq platform of two DNA target regions: Small Subunit (SSU) and Internal Transcribed Spacer (ITS), for both soil and roots AMF communities.

This book chapter was published as:

Victorino, I.[a, b, c], Berruti, A.[a], Orgiazzi, A.,[d] Voyron, S.[b], Bianciotto, V.[a], Lumini, E.[a] (2020). High throughput sequence-based analysis of Arbuscular Mycorrhizal Fungal communities. In: Arbuscular Mycorrhizal Fungi: Methods and Protocols. pp. 99 – 116. Humana Press. https://doi.org/10.1007/978-1-0716-0603-2.

[a] Institute for Sustainable Plant Protection, National Research Council (CNR), Viale Mattioli 25, 10125 Torino.

[b] Department of Life Sciences and Systems Biology, University of Turin, Viale Mattioli 25, 10125 Torino, Italy.

[c] Biological Science Department, Science Faculty, Eduardo Mondlane University (UEM), Av. Julius.

[d] European Commission, Joint Research Centre (JRC), Directorate for Sustainable Resources, Land Resources Unit, Ispra, Italy.

Email address of corresponding author: rvictori@unito.it

Springer Protocols

Arbuscular Mycorrhizal Fungi

Methods and Protocols

Chapter 9

High-Throughput DNA Sequence-Based Analysis of AMF Communities

Íris Marisa Maxaieie Victorino, Andrea Berruti, Alberto Orgiazzi, Samuele Voyron, Valeria Bianciotto, and Erica Lumini

Abstract

Arbuscular mycorrhizal fungi (AMF) are obligate symbionts of most land plants. They have great ecological and economic impacts as they support plant nutrition and water supply, soil structure, and plant resistance to pathogens. Investigating AMF presence and distribution at small and large scales is critical. Therefore, research requires standard protocols to be easily implemented. In this chapter, we describe a workflow for AMF identification by high-throughput sequencing through Illumina MiSeq platform of two DNA target regions: small subunit (SSU) and internal transcribed spacer (ITS). The protocol can apply to both soil and root AMF communities.

Key words Arbuscular mycorrhizal fungi (AMF), DNA-based species characterization, High-throughput sequence, Illumina MiSeq, ITS primers, SSU primers

1 Introduction

Over the last decades, high-throughput sequencing has revolutionized microbial ecology. Indeed, we now have the ability to thoroughly characterize both bacterial and fungal communities in various environments [1]. Arbuscular mycorrhizal fungi (AMF) have been, no exception, one of the most deeply investigated groups of soil fungi [2, 3]. They are obligate symbionts colonizing roots of more than 200,000 plant species [4], exchanging soil nutrients and other services for plant carbon. Currently, there are about 300 morphologically defined [5] and over 1000 molecularly defined species of AMF [6, 7]. High-throughput sequencing, compared to Sanger methodology, has revealed a greater richness within AMF communities [8] as well as an increased understanding of their global distribution patterns [9, 10]. Due to their nature of obligate biotrophs, AMF cannot be isolated and quantified by in vitro culture. Therefore, AMF ecology largely relies on the use of DNA-based approaches for describing AM fungal guilds. Over

Nuria Ferrol and Luisa Lanfranco (eds.), *Arbuscular Mycorrhizal Fungi: Methods and Protocols*, Methods in Molecular Biology, vol. 2146, https://doi.org/10.1007/978-1-0716-0603-2_9, © Springer Science+Business Media, LLC, part of Springer Nature 2020

the last years, next-generation sequencing (NGS) has been extensively embraced to depict AMF populations in natural (e.g., grassland) and disturbed (e.g., cropland) ecosystems [11, 12]. However, it is currently known that NGS may lead to biases when interpreting microbial community patterns [13, 14]. This holds particularly true for AMF communities due to poor knowledge of basic biology and genetics of this fungal group [11, 15]. Therefore, when setting up a protocol for AMF characterization, the final research goal has to be clear in order to adopt the most fitting procedure.

Nowadays, high-throughput sequencing technology (i.e., Illumina MiSeq) ensures performance (up to 25 million reads per run) greater than ever before. This offers microbial ecologists the opportunity to carry out broad investigations encompassing the totality of fungi present in any type of environmental samples. Nonetheless, in some cases, an exclusive research interest on AMF communities may remain to specifically answer ecological questions associated to this group of organisms. Historically, two regions are considered when studying environmental AMF assemblages: the small subunit rDNA (SSU rDNA, 18S) and the internal transcribed spacer (ITS) (Fig. 1). Each of these shows strengths and weaknesses [11].

SSU gene is highly conserved and therefore considered useful in studying relatively distant organisms, and it is most often chosen due to the availability of primer pairs recovering the majority of AMF families. Furthermore, data wealth allows to easily assign SSU sequences to virtual taxa (VTs) from curated databases, such as "MaarjAM" (https://maarjam.botany.ut.ee/) [21], and, thus, facilitates comparisons across studies; however, it could fail to discriminate species belonging to some AMF taxonomic groups [22, 23].

ITS region is known as the universal DNA barcode for fungi [13, 24]. Despite the availability of AMF-specific primer sets [25], ITS region is less frequently targeted in AMF community surveys. This is due to technical reasons: (1) poor amplification of AMF DNA by the universal ITS fungal primer pairs [26, 27] and/or (2) high variability of ITS sequences within species, even within individuals, which hampers clade recognition [28]. Choosing ITS primer sets may be advantageous as they can reveal community-level response and interactions across the entire fungal kingdom without additional efforts and resources [12].

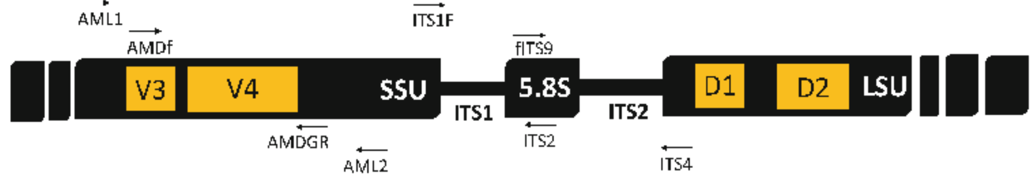


Fig. 1 Ribosomal RNA primer map. Major regions targeted by most used primers for fungal identification: ITS1F, ITS2, and ITS4 [16, 17], fITS9 [18], AMADF and AMDGR [19], and AML1 and AML2 [20]

A more reliable molecular identification of AMF species can be achieved by using a mixed primer pair designed by Krüger and collaborators [25]. This set amplifies a fragment of approximately 1500 base pairs covering a portion of SSU, the whole ITS, and a portion of LSU (large subunit, 28S). However, such a long amplicon is not suitable for the most commonly used platform (i.e., Illumina MiSeq), but it is compatible with other platforms (e.g., PacBio) [28].

All current approaches for AMF identification require trade-offs to be considered when choosing the DNA region to amplify. AMF-specific SSU primers may be able to retrieve more families and, thus, provide a wide, but exclusive, view on the AMF community [12, 19, 29]. Instead, universal fungal ITS primers could be taken in account when the aim is the assessment of soil treatment responses of only AMF dominant taxa, as long as a sufficient number of AMF sequences are obtained to ensure adequate detection of the existing diversity [19].

In this chapter, we propose a list of protocols for carrying out a high-throughput sequence-based analysis of AMF communities, from DNA extraction in environmental samples (i.e., soil and plant roots) to bioinformatics tools for analyzing DNA reads. Different workflows depending on final research aims and, thus, on primer set choice (SSU or ITS) are presented.

2 Materials

Before sampling, choose an appropriate area according to research objectives. Metadata collection is crucial and can be used for further investigation on drivers of AMF distribution. Therefore, it is impor tant to gather as many information as possible: land use, land cover, irrigation, signs of grazing, stoniness, and burned areas. Depending on the type of land, natural or cultivated, approaches to sample may differ. In natural field, whenever possible, collect samples from locations least disturbed by human activities [7]. In cultivated areas, be sure to know whether it is a monoculture or a rotation crop field (additional metadata); moreover check and take note of the presence of weeds, cover crops, or litter [30]. According to [31], on each study site, collect samples in at least two plots 30 × 30 m (or 15 × 15 m), each with similar vegetation type.

2.1 Plant

2.1.1 Plant Sampling and Processing

1. Entire and/or root segments on the base of plant species.
2. 95% ethanol.
3. Sterile gloves.
4. Zip locks of different sizes.
5. Permanent marker.

6. Shovel.
7. Box with coolers for sample storage and transport.
8. Scalpel.
9. Tweezers.

2.1.2 Plant Root Genomic DNA Extraction

1. DNeasy® Plant Mini Kit (Qiagen).
2. TissueLyser.
3. Clean and sterile 1.5 and 2 ml microcentrifuge tubes.
4. Pipettes (10–1000 μl).
5. Disposable pipette tips.
6. Microcentrifuge (20,000 × *g*).
7. Vortex.
8. Personal protection equipment (e.g., lab coat, disposable gloves, protective goggles).

2.2 Soil

2.2.1 Soil Sampling and Processing

1. Rhizospheric and/or bulk soil.
2. 95% ethanol.
3. Sterile gloves.
4. Soil corer.
5. Zip locks of different sizes.
6. Permanent marker.
7. A polystyrene box with freezer blocks or similar.

2.2.2 Soil Core Genomic DNA Extraction: NucleoSpin® Soil Kit (Macherey-Nagel) (See **Note 1**)

1. NucleoSpin® soil kit (Macherey-Nagel).
2. Clean and sterile 1.5 ml and 2 ml microcentrifuge tubes.
3. Pipettes (10–1000 μl).
4. Disposable pipette tips.
5. Microcentrifuge (20,000 × *g*).
6. Vortex.
7. Personal protection equipment (e.g., lab coat, disposable gloves, protective goggles).

2.2.3 Soil Core Genomic DNA Extraction: DNeasy PowerSoil® Kit (Qiagen) (See **Note 1**)

1. DNeasy PowerSoil® Kit (Qiagen).
2. Clean and sterile 1.5 ml and 2 ml microcentrifuge tubes.
3. Pipettes (10–1000 μl).
4. Disposable pipette tips.
5. Microcentrifuge (20,000 × *g*).
6. Vortex.

7. Vortex adapter tube holder.
8. Personal protection equipment (e.g., lab coat, disposable gloves, protective goggles).

2.3 DNA Quantification

2.3.1 Gel Electrophoresis

1. 200 ml Erlenmeyer flask.
2. Microwave.
3. Spatula.
4. Pipettes (10–1000 μl).
5. Disposable pipette tips.
6. Casting tray.
7. 100 bp or 1 kb Plus DNA Ladder (depending on the size of amplicons).
8. Gel loading buffer.
9. Electrophoretic apparatus.

2.3.2 NanoDrop (Thermo Fisher Scientific)

1. NanoDrop 1000 spectrophotometer and V 3.7 software.
2. Pipettes (10 μl).
3. Disposable pipette tips.

2.3.3 Qubit® (Invitrogen)

1. Qubit® 2.0 Fluorometer.
2. Qubit® thin-wall clear 0.5 ml PCR tubes.
3. Pipettes (2–200 μl).
4. Disposable pipette tips.

2.4 PCR Amplification

1. Pipettes (2–1000 μl).
2. Disposable pipette tips.
3. Thin-wall 0.2 ml PCR tubes.
4. PCR Master Mix (2×) (containing high-fidelity DNA polymerase).
5. PCR thermal cycler.
6. Primers solutions: First prepare stock primers solution at 100 μM. Then, prepare primers working solution at 10 μM and store primer stocks and working solutions at −20 °C. Primers sequences are reported in Table 1. Use positive (DNA extractions successfully used in previous assays) and negative (water) controls.

2.5 PCR Products Purification

1. PCR purification kit (Promega).
2. Microcentrifuge.
3. Eppendorf collection tubes 1.5 ml.

Table 1
Primers list

Primer pair	Nucleotide sequence (5′–3′)	Amplicon size (bp)
Forward AML1 [20] Reverse AML2 [20]	ATCAACTTTCGATGGTAGGATAGA GAACCCAAACACTTTGGTTTCC	~800
AMADF [19] AMDGR [32]	GGGAGGTAGTGACAATAAATAAC CCCAACTATCCCTATTAATCAT	~420
Forward ITS1F [16, 17]	TTGGTCATTTAGAGGAAGTAA	~400
Forward fITS9 [18] Reverse ITS4 [16, 17]	GAACGCAGCRAAIIGYGA TCCTCCGCTTATTGATATGC	~350
Forward Illumina overhang (FIo)	TCGTCGGCAGCGTCAGATGTGTA TAAGAGACAG	–
Reverse Illumina overhang (RIo)	GTCTCGTGGGCTCGGAGATGTGTA TAAGAGACAG	

3 Methods

A clean working bench should be used, and unless stated otherwise, the water and all other reagents used should be of molecular biological grade. For waste disposal, diligently follow all regulations. Do all centrifugation steps at room temperature (RT = 15–25 °C). When working with small amounts of DNA and using nested PCR protocols, it is necessary to work in a contamination-free environment. Use separate rooms for pre- and post-PCR steps and never expose the samples to an environment where, e.g., target DNA-carrying plasmids were extracted or PCR products handled. Make sure that all solutions are prepared and kept uncontaminated; work under UV light decontaminated benches for initial sample preparation and in strictly UV decontaminated PCR cabinets. Use clean pipettes and pipette tips that are only used for DNA extraction and separate pipette sets for the first PCR and the nested PCR, respectively.

In the following sections, one procedure for root DNA extraction and two procedures (two different commercial kits) for soil DNA extraction are described.

3.1 Processing Plant Material

1. Wear sterile gloves and use your hands to carefully remove any weed from the area.
2. Excavate and collect each plant by removing a block of approximately 15 cm of diameter and depth [30]. This may vary depending on the type of plant and root length. You may decide to transport it as it is in zip locks to the laboratory or to carefully remove the entire root system without any soil.

3. When arriving at the laboratory, wash the plant roots with tap water to remove any adherent soil particles and organic debris, quick dry with a tissue paper, and then cut the entire root system from the plant with a scalpel. To avoid cross-contamination, process each sample using a new scalpel. Alternatively, between one sample and another, sterilize the scalpel blade by flame.
4. According to [33], cut 10–20 pieces of sizes between 0.5 and 1 cm length (use an amount of approximately 20-cm long root segment), wrap in tissue paper, and place it in zip locks containing silica gel. If it is possible, freeze-dry roots as follow: put 200 mg in a 1.5 ml Eppendorf tube, sonicate to remove any remaining soil particles, and then freeze the roots in liquid nitrogen and lyophilize. Store at −20 °C.

3.1.1 DNeasy® Plant Mini DNA Extraction Kit (Qiagen) Protocol

Buffer AP1 and buffer AW1 solutions may form precipitates upon storage. If necessary, warm to 65 °C to redissolve (before adding ethanol to buffer AW1). Do not heat buffer AW1 after ethanol has been added. Buffer AW2 and buffer AW1 are supplied as concentrates. Before using for the first time, add the appropriate amount of ethanol (96–100%) as indicated on the bottle to obtain a working solution.

1. Disrupt samples (≤150 mg fresh weight or ≤20 mg of lyophilized root segments) using TissueRuptor, TissueLyser (24 Hz for 2 s, repeat once), or a mortar and pestle.
2. Add 400 μl of AP1 buffer and 4 μl RNase A. Remember not to mix AP1 buffer and RNase A before use.
3. Vortex and incubate for 10–15 min at 65 °C. Do not forget to invert the tube every 2 min during incubation.
4. Add 130 μl P3 buffer and mix well by pipetting. Incubate for 5 min on ice. This step precipitates detergent, proteins, and polysaccharides.
5. Centrifuge for 5 min at 20,000 × *g* (14,000 rpm).
6. Pipet the lysate into a QIAshredder spin column placed in a 2 ml collection tube and centrifuge for 2 min at 20,000 × *g* (14,000 rpm).
7. Transfer the flow through fraction from step e into a new 2 ml tube without disturbing the cell debris pellet.
8. Add 1.5 volumes of AW1 buffer and mix by pipetting. Transfer 550 μl of the mixture it into a DNeasy® Mini spin column placed in a 2 ml collection tube.
9. Centrifuge for 1 min at 6000 × *g* (≥8000 rpm) and discard the flow-through. Reuse the collection tube.
10. Repeat **step 8** with remaining sample. Discard flow-through and collection tube.

11. Place the spin column into a new 2 ml collection tube, add 500 μl of AW2 buffer, and centrifuge for 1 min at 6000 × *g* (≥8000 rpm).
12. Discard the flow-through, reassemble the column in the same collection tube, and add 500 μl of AW2 buffer and centrifuge at 20,000 × *g* (14,000 rpm).
13. Discard the flow-through and centrifuge for 2 min at 20,000 × *g* (14,000 rpm) to dry the membrane from residual ethanol that may interfere with subsequent reactions. If it is possible, let the lid of the centrifuge open in order to maximize the ethanol evaporation.
14. Carefully remove the spin column from the collection tube avoiding any contact with the residual ethanol.
15. Transfer the spin column to a new 1.5 ml microcentrifuge tube and add 35–100 μl of AE buffer (10 mM Tris–HCl; 0.5 mM EDTA; pH 9.0) or nuclease-free water to elute the DNA. Leave for 5 min at RT and centrifuge for 1 min at 6000 × *g*. Add again 35–100 μl of AE buffer, leave for 5 min at RT, and centrifuge for 1 min at 6000 × *g*. Note that the choice of elution buffer depends from the next required analyses.

3.2 Processing Soil Core Samples

When aiming at soil DNA community analysis, gather minimum five to nine topsoil (0–20 cm) subsamples and mix to form a single composite sample [31]. Collect soil samples in a pre-established georeferenced (GIS) location. Once chosen the first sampling point, collect other four to eight soil subsamples on a plot (30 × 30 m or 15 × 15 m) having the GIS point as central focus.

1. Wear sterile gloves and remove all signs of vegetation residues from the point.
2. Collect soil sample through soil corer and place it into a zip lock.
3. Keep the soil as fresh as possible (e.g., polystyrene box with freezer blocks) until arrive at the laboratory.
4. In laboratory, sieve each soil sample on a 2-mm stainless steel sieve, aliquot samples (250 mg) into 2 ml microcentrifuge tubes, and store at −20 °C.
5. Clean sampling material with ethanol before processing samples from other locations.

3.2.1 NucleoSpin® Soil DNA Extraction Kit (Macherey-Nagel) Protocol

Before starting, check if lysis buffers SL1 and SL2 show sign of precipitation; in this case, use a water bath at 40 °C for 10 min to dissolve it; shake the bottle every 2 min. All the procedures have to be done at RT.

1. Put the ceramic beads into a NucleoSpin® Bead Tubes Type A and add 250 mg of fresh sample material paying attention not to exceed the 1 ml mark (0.6–0.8-mm ceramic beads are recommended for soil and sediments).
2. Add 700 μl of buffer SL1 to the NucleoSpin® Bead Tubes Type A and 150 μl of Enhancer SX. (Enhancer SX ensures the highest possible DNA yield.) Place the NucleoSpin® Bead Tubes Type A horizontally on a vortex at full speed for 5–10 min at RT. Note that longer vortex time could improve DNA extraction. For more than ten samples is recommended to augment the vortex time to 20 min.
3. Centrifuge for 2 min at 11,000 × *g* (≥9000 rpm) and repeat this step collecting the supernatant into a new 2 ml collection tube.
4. Add 150 μl of buffer SL3 and vortex briefly. Incubate for 5 min on ice and then centrifuge at 11,000 × *g* (≥9000 rpm) for 1 min (this step precipitates contaminants).
5. Use the NucleoSpin® Inhibitor Removal Column to filter the lysate by placing the column into a new 2 ml collection tube and then add 800 μl of the supernatant from **step 4**. Centrifuge for 1 min at 11,000 × *g*.
6. Repeat **step 5** with the remaining supernatant of **step 4**.
7. Discard the NucleoSpin® Inhibitor Removal Column. Add 250 μl of buffer SB and vortex briefly to adjust binding conditions.
8. Place a NucleoSpin® Soil Column in a new 2 ml collection tube, and load 550 μl of sample onto the column.
9. Centrifuge the NucleoSpin® Soil Column 1 min at 11,000 × *g* (≥9000 rpm), and discard the flow-through.
10. Repeat **step 9** loading the remaining sample onto the column, and discard the flow-through. And place the column back into the collection tube.
11. To wash and dry silica membrane, add 500 μl of buffer SB to the NucleoSpin® Soil Column and centrifuge for 1 min at 11,000 × *g* (>9000 rpm).
12. Place the MB spin column into a new 1.5 ml clean tube, add 35–100 μl of buffer C6 and centrifuge again for 30 s at 10,000 g (>=9000 rpm). Discard the MB spin column and keep the flow-through.
13. Add 650 μl of SW2 buffer to the NucleoSpin® Soil Column. Vortex briefly prior to centrifuge for 1 min at 11, 000 × *g* (≥9000 rpm). Discard the flow-thorough and place the column back into the collection tube and centrifuge for 2 min at 11,000 × *g* (≥9000 rpm). If it is possible, let the lid of the centrifuge open in order to maximize the ethanol evaporation.

14. Put the NucleoSpin® Soil Column into a new microcentrifuge 1.5 ml tube. Add 50 μl of buffer SE (5 mM Tris–HCl, pH 8.5) to the column and incubate 1 min at RT with the lid open. Close the lid and centrifuge for 1 min at 11,000 × *g* (≥9000 rpm).

3.2.2 DNeasy PowerSoil® DNA Extraction Kit (Qiagen) Protocol

Before starting, use a water bath at 60 °C to dissolve C1 solution if precipitation occurs. All the centrifugations have to be done at RT.

1. Put 250 mg of soil into PowerBead Tube. Vortex briefly and add 60 μl of buffer C1. Invert the tube from time to time and vortex the tubes positioned horizontally for 10 min (if you process more than 12 samples, vortex for 20 min). Note that might be necessary to increase the soil amount up to 500 mg for soil with high sand content.
2. Centrifuge tubes at 10,000 × *g* (≥9000 rpm) for 1 min and then transfer the supernatant to a clean 2 ml collection tube.
3. Add 250 μl of buffer C2 and vortex briefly. Incubate for 5 min on ice and then centrifuge tubes for 1 min at 10,000 × *g* (>9000 rpm).
4. Avoiding the pellet, transfer up to 600 μl of supernatant to a clean 2 ml collection tube.
5. Add 200 μl of buffer C3, vortex briefly, and then incubate for 5 min on ice. Centrifuge for 1 min at 10, 000 × *g* (≥9000 rpm). Without disturbing the pellet, transfer total amount of supernatant to a clean 2 ml collection tube.
6. Mix by shaking C4 buffer, take 1200 μl, and add it to the supernatant. Vortex briefly.
7. Carefully load 600 μl onto an MB spin column and centrifuge at 10,000 × *g* (≥9000 rpm) for 1 min.
8. Discard the flow-through and repeat the previous step until no sample is left.
9. Add 500 μl of buffer C5 onto an MB spin column and centrifuge for 1 min at 10,000 × *g* (≥9000 rpm). Discard the flow-through and centrifuge once more for 2 min at 10,000 × *g* (≥9000 rpm). This step will dry the membrane.
10. Carefully place the MB spin column into a 1.5-ml clean collection tube.
11. Add of 35 μl of buffer C6 (10 mM Tris–HCl, pH 8.0) in the center of the white filter membrane and centrifuge for 1 min at 10,000 × *g* (>9000 rpm).
12. Place the MB spin column into a new 1.5 ml clean tube, add 35–100 μl of buffer C6 and centrifuge again for 30 s at 10,000 × *g* (>=9000 rpm). Discard the MB spin column and keep the flow-through.

3.3 DNA Quantification

To prepare sample for gel electrophoresis, add 1 μl of 6× loading buffer in 5 μl of DNA sample.

3.3.1 Gel Electrophoresis

1. Using an Erlenmeyer flask, weigh 1 g of agarose and add 100 ml of 0.5× TAE buffer.
2. Put the Erlenmeyer flask the microwave oven to dissolve completely and gently mix the solution. Let it cool down and then add 2 μl of ethidium bromide at a concentration of 10 mg/ml.
3. Pour the solution into a casting tray, place a comb (or two depending on the number of samples to run), and let it become solid.
4. Load the samples and the DNA ladder into the wells, connect the electrodes to the power supply (negative electrode, south, and positive electrode, north), and let the gel electrophoresis run with 100 V for 30 min (or more depending on the gel size and voltage).

3.3.2 NanoDrop Quantification

To clean the instrument, open the sampling arm and load 1.5 μl onto the lower pedestal and follow the instructions provided by the software.

1. Make a "blank" measurement using the same buffer used for DNA elution (this buffer is different for the different extraction kits).
2. Once completed the blank measurement, open the sampling arm, and with a towel paper, wipe the upper and the lower pedestals and load the sample to read.
3. Close the arm and initiate the measurement pressing the "start" button on the operating software on the PC V 3.7 software.
4. Once completed the measurement, open the sampling arm, and with a towel paper, wipe the upper and the lower pedestals and carry onto the next sample.

3.3.3 Qubit® (Invitrogen) Quantification

Store the dye, DNA standards at 4 °C, and the buffer at RT. Prior to begin, leave all the reagents at RT for 30 min.

1. Prepare two tubes for the standards and one tube for each sample.
2. Dilute Qubit® reagent 1:200 in Qubit® buffer—working solution—using this working solution, prepare 200 μl of the working solution for each standard and for each sample.
3. Prepare a thin-wall clear 0.5 ml PCR tubes as follows:

	Standard tubes	Sample tubes
Volume of working solution from **step 2** to add	190 μl	180 199 μl
Volume of standard from kit to add	10 μl	–
Volume of sample to add	–	1–20 μl
Total volume in each tube	200 μl	200 μl

4. Briefly vortex all tubes and then incubate for 2 min at RT.
5. Put the tubes into the Qubit® 2.0 Fluorometer and start the readings.

Before the setup of the PCR reactions, adjust the genomic DNA concentration of each sample to 2 ng/μl.

3.4 SSU rDNA Amplification

3.4.1 First PCR

1. Prepare a final volume of 25 μl of the mixture for PCR reaction using:

PCR Master Mix (2×)	10 μl
10 μM forward primer (AML1)	0.5 μl
10 μM reverse primer (AML2)	0.5 μl
DNA (2 ng/μl)	1 μl
PCR-grade water	13 μl

2. Use as PCR conditions for 96-well thermocycler for each primer set the following:

Temperature	Time	Repeat
95 °C	15 min	
95 °C	60 s	×35
57 °C	60 s	×35
72 °C	60 s	×35
72 °C	7 min	
4 °C	Hold	

3.4.2 Second (Nested) PCR

PCR products from the first PCR should be used in the hemi-nested PCR with primer AMADF/AMDGR with added Illumina overhang adapter sequences (*see* **Note 2**).

1. Prepare a final volume of 25 μl of the mixture for PCR reaction using:

PCR Master Mix (2×)	10 μl
10 μM forward primer (AMADF)	0.5 μl
10 μM reverse primer (AMDGR)	0.5 μl
DNA (1 ng/μl)	1 μl
PCR grade water	13 μl

2. Use as PCR conditions for 96-well thermocycler for each primer set the following:

Temperature	Time	Repeat
95 °C	15 min	
95 °C	40 s	×30
58 °C	40 s	×30
72 °C	45 s	×30
72 °C	7 min	
4 °C	Hold	

3.5 ITS rDNA Amplification

3.5.1 First PCR (See Note 3)

1. Prepare a final volume of 25 μl of the mixture for PCR reaction using:

PCR Master Mix (2×)	10 μl
10 μM forward primer (ITS1F)	0.5 μl
10 μM reverse primer (ITS4)	0.5 μl
DNA (2 ng/μl)	1 μl
PCR-grade water	13 μl

2. Use as PCR conditions for 96-well thermocycler for each primer set the following:

Temperature	Time	Repeat
95 °C	5 min	
94 °C	30 s	×35
54 °C	45 s	×35
72 °C	1mins	×35
72 °C	10 min	
4 °C	Hold	

3.5.2 Hemi-Nested PCR or Direct PCR

PCR products from the first PCR should be used in the seminested PCR with primer fITS9/ITS4 with added Illumina overhang adapter sequences (*see* **Note 2**).

1. Prepare a final volume of 25 μl of the mixture for PCR reaction using:

PCR Master Mix (2×)	10 μl
10 μM forward primer (fITS9)	0.5 μl
10 μM reverse primer (ITS4)	0.5 μl
DNA (1 ng/μl)	1 μl
PCR grade water	13 μl

2. Use as PCR conditions for 96-well thermocycler for each primer set the following:

Temperature	Time	Repeat
95 °C	5 min	
95 °C	35 s	×30
56 °C	35 s	×30
72 °C	45 s	×30
72 °C	7 min	
4 °C	Hold	

It is recommended to obtain enough PCR products to be purified and sequenced and to do hemi-nested or nested PCR in triplicate. PCR products must be checked on agarose gel electrophoresis.

3.6 PCR Products Purification (Promega)

Prior to purification, pool the three identical replicates coming from hemi-nested PCR reactions.

1. Add an equal volume of membrane binding solution to each sample and mix by pipetting.
2. Transfer the solution from point 1 into a SV Minicolumn inserted in a collection tube and incubate 1 min at RT.
3. Centrifuge the SV Minicolumn assembly at 16,000 × *g* for 1 min and then discard the flow-through.
4. Place back the SV Minicolumn into the collection tube and add 700 μl of membrane washing solution. Centrifuge for 1 min at 16,000 × *g*.

5. Repeat **step 4** with 500 μl of membrane washing solution and centrifuge for 5 min at 16,000 × *g* and discard the flow-through.
6. Place back the SV Minicolumn in the collection tube and centrifuge for 2 min at 16,000 × *g* to allow residual ethanol evaporation. If it is possible, let the lid of the centrifuge open in order to maximize the ethanol evaporation.
7. Transfer the SV Minicolumn into a new 1.5 ml microcentrifuge tube. Add 35–50 μl of nuclease-free water. Make sure to place it in the center of the membrane and incubate at RT 1 min. Centrifuge at 16,000 × *g* for 1 min and discard the SV Minicolumn and store the purified DNA at −20 °C.

3.7 Sequencing

1. Prepare samples for the Illumina MiSeq sequencing (250–300 bp paired-end) according to your sequencing provider (in a 96-well plate or in single tubes "upon request").
2. Ship them with ice packs.

3.8 Bioinformatics Pipeline for Analyzing Illumina MiSeq Reads

Treat 18S rRNA gene or ITS2 DNA raw sequencing data with the freeware Mothur v1.42 [34] in order to clean and cluster your datasets. Here, our pipeline is briefly described. For more detailed information, please refer to Mothur v1.42 Standard Operative Procedures (available online at https://www.mothur.org/wiki/MiSeqSOP).

1. After making contigs with the make.contigs() command, de-multiplex with the trim.seqs() command if needed; filter raw sequences, using the screen.seqs() command, based on the following parameters: desired fragment length range, absence of ambiguous nucleotides, and maximum homopolymers' length.
2. Cluster sequences based on 100% similarity using the unique. seqs() command and remove singletons. We suggest to carry out a de novo chimera check on the sequences using the chimera, vsearch() command with the dereplicate parameters set as TRUE.
3. Carry out OTU picking using the default cluster() command which uses the OptiClust algorithm [35] set at 3% dissimilarity (although this distance cutoff is arbitrary and can be considered controversial, it was chosen on the basis of the fact that the choice of OTU delineation method has been demonstrated to negligibly affect the interpretation of NGS-based AMF community patterns by [36].
4. Select the most abundant unique sequence of each OTU as the representative one using the get.oturep() command; prune out OTUs with low numbers of sequences (<5–10, according to

the dataset size) on a per-sample basis (an OTU that is common in one sample may occur as a low-abundant contaminant in other samples due to cross-contamination).

5. Compute individual rarefaction curves in order to assess the sequencing effort provided per sample. For 18S rRNA gene sequences, conduct a search for similar sequences with local Blast v2.2.29 [37] on the latest release of the MaarjAM AMF Virtual Taxa online database (available at https://maarjam.botany.ut.ee/) [21] integrated with the SSU Silva database (available at https://www.arb-silva.de/) [38], after removal of AMF sequences. Remove unwanted lineages (plant DNA, protists' DNA, metazoan DNA, etc.). Affiliate sequences to AMF when they have at least ~90% homology with a VT reference sequence and ~97% coverage. Alternatively, assign taxonomy to the sequences of each OTU using the RDP classifier [39] and a 60–80% confidence threshold. Then, compute consensus taxonomy for each OTU with the classify.otu() command. For ITS2 DNA sequences, the same approach but a different reference database can be used (UNITE+INSD online database, available at https://unite.ut.ee/repository.php) [40].
6. Prior to statistical analyses, as a normalization step to reduce bias associated with different sequencing depths, subsample all samples down to the size of the smallest sample that has a reasonable size for correctly describing the inferred diversity of the sample type under study.
7. Compute sample rarefaction curves in order to assess the level of diversity coverage provided by the experimental design sampling effort.

4 Notes

1. Different soil extraction kits may have provided different yield and DNA quality. Therefore they must be chosen on the basis of soil features.
2. PCR products from the first PCR (AML1/AML2 or ITS1F/ITS4) might be diluted from 1/10 up to 1/500.
3. fITS9/ITS4 can be applied directly if the DNA is well amplifiable; however the whole ITS fragment could be amplified in a first PCR (with primers pair ITS1F/ITS4) in order to perform following hemi-nested PCR with either ITS1F/ITS2 and/or fITS9/ITS4 primers to cover both ITS1 and ITS2.

Acknowledgments

The described work was possible thanks to the financial support of the Fondazione Cassa di Risparmio di Torino (n. 2017.1966) to the project "SaffronAlp" (Italy), the FIAM bilateral project titled "MYCOTTON" (Mozambique), and the European LUCAS project.

References

1. Montagna M, Berruti A, Bianciotto V et al (2018) Differential biodiversity responses between kingdoms (plants, fungi, bacteria and metazoa) along an alpine succession gradient. Mol Ecol 27:3671–3685
2. Taylor T, Remy W, Hass H, Kerp H (1995) Fossil arbuscular mycorrhizae from the early Devonian. Mycology 87:560–573
3. Lichtfouse E (2017) Sustainable agriculture reviews, 1st edn. Springer International Publishing, Marseille
4. Brundrett M (2009) Mycorrhizal associations and other means of nutrition of vascular plants: understanding the global diversity of host plants by resolving conflicting information and developing reliable means of diagnosis. Plant Soil 320:37–77
5. Öpik M, Davison J (2016) Uniting species and community-oriented approaches to understand arbuscular mycorrhizal fungal diversity. Fungal Ecol 24:106–113
6. Kivlin SN, Hawkes CV, Treseder KK (2011) Global diversity and distribution of arbuscular mycorrhizal fungi. Soil Biol Biochem 43:2294–2303
7. Öpik M, Davison J, Moora M, Zobel M (2014) DNA-based detection and identification of Glomeromycota: the virtual taxonomy of environmental sequences. Botany 92:135–147
8. Öpik M, Metsis M, Daniell TJ et al (2009) Large-scale parallel 454 sequencing reveals host ecological group specificity of arbuscular mycorrhizal fungi in a boreonemoral forest. New Phytol 184:424–437
9. Stürmer SL, Bever JD, Morton JB (2018) Biogeography of arbuscular mycorrhizal fungi (Glomeromycota): a phylogenetic perspective on species distribution patterns. Mycorrhiza 28:587–603
10. Davison J, Moora M, Öpik M et al (2015) Global assessment of arbuscular mycorrhizal fungus diversity reveals very low endemism. Science 349:970–973
11. Hart M, Aleklett K, Chagnon P et al (2015) Navigating the labyrinth: a guide to sequence-based, community ecology of arbuscular mycorrhizal fungi. New Phytol 207:235–247
12. Lekberg Y, Vasar M, Bullington LS et al (2018) More bang for the buck? Can arbuscular mycorrhizal fungal communities be characterized adequately alongside other fungi using general fungal primers? New Phytol 220:971–976
13. Lindahl BD, Nilsson RH, Tedersoo L et al (2013) Fungal community analysis by high-throughput sequencing of amplified markers—a user's guide. New Phytol 199:288–299
14. Vasar M, Andreson R, Davison J et al (2017) Increased sequencing depth does not increase captured diversity of arbuscular mycorrhizal fungi. Mycorrhiza 27:761–773
15. Corradi N, Brachmann A (2017) Fungal mating in the most widespread plant symbionts? Trends Plant Sci 22:175–183
16. Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes—application to the identification of mycorrhizae and rusts. Mol Ecol 2:113–118
17. White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M, Gelfand D, Sninsky J, White T (eds) PCR protocols: a guide to methods and applications. Academic Press Inc, New York, p 315
18. Ihrmark K, Bodeker I, Cruz-Martinez K et al (2012) New primer to amplify the fungal ITS 2 region-evaluation by 454-sequencing of artificial and natural communities. FEMS Microbiol Ecol 82:666–677
19. Berruti A, Desirò A, Visentin S et al (2017) ITS fungal barcoding primers versus 18S AMF-specific primers reveal similar AMF-based diversity patterns in roots and soils of three mountain vineyards. Environ Microbiol Rep 9:658–667
20. Lee J, Lee S, Young JPW (2008) Improved PCR primers for the detection and identification of arbuscular mycorrhizal fungi. FEMS Microbiol Ecol 65:339–349

21. Öpik M, Vanatoa A, Vanatoa E et al (2010) The online database MaarjAM reveals global and ecosystemic distribution patterns in arbuscular mycorrhizal fungi (Glomeromycota). New Phytol 188:223–241
22. Van Geel M, Busschaert P, Honnay O, Lievensv B (2014) Evaluation of six primer pairs targeting the nuclear rRNA operon for characterization of arbuscular mycorrhizal fungal (AMF) communities using 454 pyrosequencing. J Microbiol Methods 106:93–100
23. Stockinger H, Kruger M, Schußler A (2010) DNA barcoding of arbuscular mycorrhizal fungi. New Phytol 187:461–474
24. Schoch C, Seifert K, Huhndorf S et al (2012) Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi. Proc Natl Acad Sci 109:6241–6246
25. Krüger M, Stockinger H, Krüger C, Schußler A (2009) DNA-based species-level detection of Glomeromycota: one PCR primer set for all arbuscular mycorrhizal fungi. New Phytol 183:212–223
26. Tedersoo L, Bahram M, Polme S et al (2015) Response to comment on 'global diversity and geography of soil fungi. Science 349:936
27. Tedersoo L, Tooming-Klunderud A, Anslan S (2018) PacBio metabarcoding of fungi and other eukaryotes: errors, biases and perspectives. New Phytol 217:973–976
28. Thiéry O, Vasar M, Jairus T et al (2016) Sequence variation in nuclear ribosomal small subunit, internal transcribed spacer and large subunit regions of Rhizophagus irregularis and Gigaspora margarita is high and isolate-dependent. Mol Ecol 25:2816–2832
29. Berruti A, Bianciotto V, Lumini E (2018) Seasonal variation in winter wheat field soil arbuscular mycorrhizal fungus communities after non-mycorrhizal crop cultivation. Mycorrhiza 28:535–548
30. Lumini E, Vallino M, Alguacil M et al (2011) Different farming and water regimes in Italian rice fields affect arbuscular mycorrhizal fungal soil communities. Ecol Appl 21:1696–1707
31. Fernández Ugalde O, Orgiazzi A, Jones A et al (2018) LUCAS 2018—SOIL COMPONENT: sampling instructions for surveyors, EUR 28501 EN. https://doi.org/10.2760/023673
32. Sato K, Suyama Y, Saito M, Sugawara K (2005) A new primer for discrimination of arbuscular mycorrhizal fungi with polymerase chain reaction-denature gradient gel electrophoresis. Grassl Sci 51:179–181
33. Öpik M, Zobel M, Cantero J et al (2013) Global sampling of plant roots expands the described molecular diversity of arbuscular mycorrhizal fungi. Mycorrhiza 23:411–430
34. Schloss PD (2009) A high-throughput DNA sequence aligner for microbial ecology studies. PLoS One 4:e8230
35. Westcott S, Schloss P (2017) OptiClust, an improved method for assigning amplicon-based sequence data to operational taxonomic units. mSphere 8:2–11
36. Lekberg Y, Gibbon S, Rosendahl S (2014) Wild different OUT delineation methods change interpretation of arbuscular mycorrhizal fungal community patterns? New Phytol 202:1101–1104
37. Zhang Z, Schwartz S, Wagner L, Miller W (2000) A greedy algorithm for aligning DNA sequences. J Comput Biol 7:203–214
38. Yilmaz P, Parfrey L, Yarza P et al (2014) The SILVA and "all-species living tree project (LTP)" taxonomic frameworks. Nucleic Acids Res 42:D643–D648
39. Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 73:5261–5267
40. Abarenkov K, Nilsoon H, Larson K et al (2010) The UNITE database for molecular identification of fungi recent updates and future perspectives. New Phytol 186:281–285

Chapter VI

Final remarks and Conclusions

6.1. Final remarks and Conclusions

Saffron (*Crocus sativus*) is an important and well-known medicinal plant due to its valuable bioactive compounds. Its cultivation is widely explored in diverse habitats, more specific in Mediterranean open fields but, unlike other horticultural crops, the information relating to its cultivation in the soilless system is poorly documented. The use of arbuscular mycorrhizal fungi as biostimulants has been extensively described in the literature. However, only recently the focus on the use of AMF has changed from only the improvement of cultivation traits to the improvement of secondary metabolites as well (Sibile et al. 2021). For this purpose, AMF have been applied to all possible crops great success, including some horticultural crops – *Zingiber officinale* L., *Curcuma longa* L., *Amorphophallus commutatus* Engler and *Pueraria tuberosa* (Shuab et al, 2016); *Eclipta prostrata* L. (Hong Duc et al, 2021); *Valeriana officinalis* L. (Amanifar and Toghranegar, 2020); *Thymus vulgaris* L. and *Thymus daenensis* Celak (Bistgani et al, 2019); *Rosmarinus officinalis* L. (Seró et al, 2019). During my PhD thesis, I have investigated the use of AMF in soilless cultivation of *Crocus sativus* and I was able to verify and report, for the first time, that microorganisms as the AMF can improve overall plant traits leading to production of ISO 3632-1:2011 category I saffron spice. The cultivation in a soilless system has proved to be suitable for saffron cultivation, without interfering with the flowering process, which is a very important process as the main target are the stigmas from which saffron powder is produced. To date, a few biostimulants formulas have been produced and although AMF ubiquitous distribution, for some areas evidence of AMF efficacy is still scant, incomplete or lacking altogether (Hart et al., 2017). According to same author, a list of factors can be assumed as the one contribution to certain inocula success or unsuccess, propagule type, existing niche or dosage just to mention a few (Klironomos and Hart, 2002; Varga et al., 2015; Owen et al., 2015; Nielsen et al., 2016). In this study it was observed that inocula did increased saffron yield and productivity. Similarly, Mohebi-Anabat and collaborators (2015) showed that AMF inoculation caused significant increase of the above and below ground growth of saffron plants but still, more studies are encouraged. Saffron is known to grow better in Mediterranean areas (Alizadeh, 2006) but recently other environments are also being explored for successful cultivation of saffron. One example is the northern alpine region in Italy where saffron is produced by local farmers who were involved in our

project aimed to investigate the AMF symbiosis in open field conditions and its effects on saffron plant growth, productivity, and bioactive compounds content during two cultivation cycles. As expected, both single and multispecies inoculated saffron plants were colonized, similarly to what was described in Aimo et al. (2010) and Lone et al. (2016) reports. Colonization levels however were much lower when compared to pot trials using the same inoculants and same plant species (Caser et al., 2019b). Flower number and saffron yield between treatments greatly increased, in particular when multispecies inocula were used. Berruti et al (2017) previously mentioned that multispecies inocula greatly influence crop performance when compared to single species inocula. The number of replacement corms, the weight of the corms and the antioxidant activity, on the other hand, were not influenced by the inoculation in the open field. Previous data suggested that sometimes undesirable effects of AMF inoculation also may occur in systems where the AMF community is already established. Janouskova et al. (2013) reported similar results after inoculation of fields with pre-established AMF community suggesting that there might be an optimal level of AMF propagule density and composition in soil, and that changes in these levels can lead to increased competition between AMF species and decreased AMF community potential to promote plant growth. The ecological aspect of competition between inocula and native species has been neglected in inoculation studies, but should however be further explored. Similarly to saffron produced in soilless systems (with and without AMF) the saffron produced in open fields is also ranked in ISO 3632-1:2011 category I.

Soil is a super exploited ecosystem and a source of microorganisms acting as decomposers, plant mutualists and pathogens. Fungi from soil are known to strongly influence ecosystem structure and functioning, playing a key role in many ecological services. Over the last decades, with the advance of metagenomics studies, knowledge regarding soil fungi biodiversity has increased into a whole new level with discovery of many new species, in habitats ever explored before (Hart et al., 2017) and another thousand expecting to be discovered according to high throughput data predictions. Agricultural practices are known to severely interfere with soil, both physical and chemical properties but also with above and belowground microbial communities' abundance, diversity and activity (Lienhard et al., 2013). Most common primers used to describe soil fungi are ITS primers but they have proved not to be fail proof. Bias can be

introduced and cause changes in the interpretation of fungal community patterns and as a way to face this problem over the last year's international projects as the Earth Microbiome Project is being used to compare soil microbial diversity at a large scale (Hawksworth et al., 2017). From the experience I have acquired during my PhD period I was able to see, by using Illumina MiSeq metabarcoding technique, that agricultural practices showed no evident impact on resident soil fungal communities associated with *Crocus sativus* cultivation fields. AMF inoculation instead has increased crop yield and productivity proving to be a "safe" addition to inoculate bulbs in marginal areas farms such as alpine sites for profitable gains. As a recommendation I would suggest further investigating the possibility to co-inoculate saffron bulbs with other endophytes (i.e bacterial PGPRs) to be co-inoculated on saffron bulbs, and/or to isolate and to multiply microorganisms directly recruited from the same soils, that could offer further advantages, as for example increased tolerance to biotic and/or abiotic stresses.

I would add as main conclusions that:

1. AMF were able to colonize saffron roots on both soilless systems and open field;
2. AMF act as biofertilizer improving not only yield, overall growth, and crop performance but also secondary metabolites production;
3. when using Illumina MiSeq metabarcoding technique or any other similar platform the experimental design must be done with as much accuracy and attention to details as possible, from sampling, choosing replicates number, DNA extraction, primer set, PCR conditions or even platform to use;
4. being able to establish collaborations is essential either with scientists or with local farmers. With scientists as a way of being able to access as much data as possible and to analyze it in all degrees of expertise (molecular biologists, statisticians, bioinformaticians, taxonomists, and ecologists). With local farmers as a way of having accurate information of agricultural management that is being applied to the field, as to approximate more advanced science to actual application in the field;

In my PhD I was also able to exploit the skills acquired during my stay in Italy and applied them in different experiments carried out at the laboratories of Biology Science

Department of Eduardo Mondlane University (DCB-UEM, Mozambique), as well as in a field experiment aimed to investigate soil fungal biodiversity associated with cotton cultivation in the north of Mozambique. These last experiences were carried out in the frame of a bilateral collaboration project between Mozambique and Italy (FIAM Project "MYCotton: Microbial Resources for agriculture: arbuscular mycorrhizal fungi in cotton and their potential use as biofertilizer" Applied and Multi-Sectorial Research Fund to Eduardo Mondlane University (UEM) Mozambique. (2017-2020). Results obtained from this collaboration were presented as posters and were the subject of an extended abstract on the FAO proceedings (2021). Posters and Extended abstract are enclosed as supplementary attached material.

6.2. References

- Aimo, S., Gosetti, F., D'Agostino, G., Gamalero, E., Gianotti, V., Bottaro, M., Gennaro, M., Berta, G. 2010. Use of arbuscular mycorrhizal fungi and beneficial soil bacteria to improve yield and quality of saffron (*Crocus sativus* L.). Acta Hortic. 850. 159–162.
- Alizadeh A. 2006. Irrigation. In: Kafi M , Koocheki A , Rashed M.H , Nassiri M. (Eds.), Saffron (*Crocus sativus*) Production and Processing. Science Publishers. Enfield. 79–90.
- Amanifar, S., and Toghranegar, Z. 2020. The efficiency of arbuscular mycorrhizal for improving tolerance of Valeriana officinalis L. and enhancing valerenic acid accumulation under salinity stress. Ind. Crops Prod. 147. DOI: 10.1016/j.indcrop.2020.112234.
- Bistgani, Z., Hashemib, M., DaCostab, M., Crakerb, L., Maggic, F., and Morshedloo, M. 2019. Effect of salinity stress on the physiological characteristics, phenolic compounds and antioxidant activity of *Thymus vulgaris L.* and *Thymus daenensis Celak*. Ind. Crops Prod. 135. 311–320. DOI: 10.1016/j.indcrop.2019.04.055.
- Caser, M., Demasi, S., Victorino, I., Donno, D., Faccio A., Lumini E., Bianciotto V., Scariot V. 2019b. Arbuscular Mycorrhizal Fungi Modulate the Crop Performance and Metabolic Profile of Saffron in Soilless Cultivation. Agronomy 9. 232; DOI: 10.3390/agronomy9050232.
- Hart, M., Antunes, P., Chaudhary, V. and Abbott, L. 2017. Fungal inoculants in the field: Is the reward greater than the risk? Functional Ecology. pgs 126-135. DOI: 10.1111/1365-2435.12976.
- Hong Duc, N., Trung Vo, A., Haddidi, I., Daood, H. and Posta, K. (2021) Arbuscular Mycorrhizal Fungi Improve Tolerance of the Medicinal Plant *Eclipta prostrata (L.)* and Induce Major Changes in Polyphenol Profiles Under Salt Stresses. Front. Plant Sci. 11. DOI: 10.3389/fpls.2020.612299.
- Hawksworth, D. and Lücking, R. 2017. Fungal diversity revisited: 2.2 to 3.8 million species. In The Fungal Kingdom; Heitman, J., Howlett, B.J., Crous,W., Stukenbrock, E.H., James, T.Y., Eds.; American Society of Microbiology: Washington, DC, USA. 79–95. DOI: 10.1128/microbiolspec.FUNK-0052-2016.
- Janouskova, M., Krak, K., Wagg, C., Storchova, H., Caklova, P. and Vosatka, M. 2013. Effects of Inoculum Additions in the Presence of a Preestablished Arbuscular Mycorrhizal Fungal Community. Applied and Environmental Microbiology. 79 (20). 6507–6515.
- Klironomos, J. and Hart, M. 2002. Colonization of roots by arbuscular mycorrhizal fungi using different sources of inoculum. Mycorrhiza, 12, 181–184. DOI: 10.1007/s00572-002-0169-6.
- Lone, R., Shuab, R., Koul, K. 2016. AMF association and their effect on metabolite mobilization, mineral nutrition and nitrogen assimilating enzymes in saffron (*Crocus sativus* L.) plant. J. Plant Nutr. 39. 1852–1862.
- Lienhard, P., Terrat, S., Mathieu, O., Levêque, J., Prévost-Bouré, N.C., Nowak, V., Regnier, T., Faivre, C., Sayphoummie, S., Panyasiri, K., Tivet, F., Ranjard, L. and Maron, P. 2013. Soil microbial diversity and C turnover modified by tillage and cropping in Laos tropical grassland. Environ. Chem. Lett. 11. 391–398. DOI: 10.1007/s10311-013-0420-8.
- Mohebi-Anabat, M., Riahi, H., Zanganeh, S., Sadeghnezhad, E. 2015. Effects of arbuscular mycorrhizal inoculation on the growth, photosynthetic pigments and soluble sugar of *Crocus sativus* L. (saffron) in autoclaved soil. Int. J. Agron. Agric. Res. 6. 296–304.
- Nielsen, K., Kjøller, R., Bruun, H., Schnoor, T. and Rosendahl, S. 2016. Colonization of new land by arbuscular mycorrhizal fungi. Fungal Ecology, 20. 22–29. https://doi.org/10.1016/j.funeco.2015.10.004.
- Owen, D., Williams, A., Griffith, G. and Withers, P. 2015. Use of commercial bio-inoculants to increase agricultural production through improved phosphorus acquisition. Applied Soil Ecology, 86. 41–54. https://doi.org/10.1016/j.apsoil.2014.09.012.

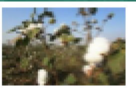
- Seró, R., Núñez, N., Núñez, O., Camprubí, A., Grases, J. M., Saurina, J., Moyano, E., and Calvet, C. 2019. Modified distribution in the polyphenolic profile of rosemary leaves induced by plant inoculation with an arbuscular mycorrhizal fungus. J. Sci. Food Agric. 99. 2966–2973. DOI: 10.1002/jsfa.9510.
- Shuab, R., Malla, N., Ahmad, J., Lone, R. and Koul, K. 2016. Arbuscular mycorrhizal fungal symbiosis with saffron (*Crocus sativus* L.) plant. Journal on New Biological Reports. 5 (1) 59-67.
- Sibile, C., Seebauer, J., Below, F. 2021. Plant Biostimulants: A Categorical Review, Their Implications for Row Crop Production, and Relation to Soil Health Indicators. Agronomy 11, 1297. https://doi.org/10.3390/agronomy11071297.
- Varga, S., Finozzi, C., Vestberg, M., and Kytöviita, M. M. 2015. Arctic arbuscular mycorrhizal spore community and viability after storage in cold conditions. Mycorrhiza, 25. 335–343. DOI: 10.1007/s00572-014-0613-4

6.3. Supplementary material

Victorino, I., Martins, C., Ventura, S., Quilambo, O., Girlanda, M., Voyron, S., Berruti, A., Bianciotto, V., Lumini, E. 2018. Microbial resources for agriculture: arbuscular mycorrhizal fungi in cotton and their potential use as biofertilizer. Poster. X Eduardo Mondlane Scientific Conference.

MICROBIAL RESOURCES FOR AGRICULTURE: ARBUSCULAR MYCORRHIZAL FUNGI IN COTTON AND THEIR POTENTIAL USE AS BIOFERTILIZER

Victorino I[1,4], Martins C.[1], Ventura S.[1], Quilambo O.[1], Girlanda M.[2], Voyron S.[2], Berruti A., Bianciotto V.[3], Lumini E.[3]



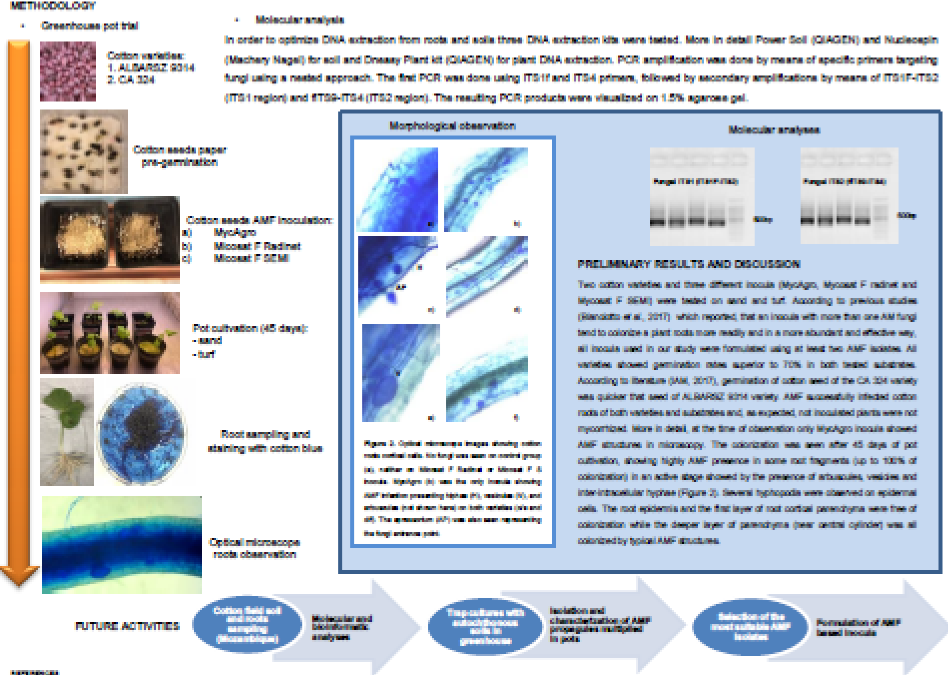
BACKGROUND

Cotton (Gossypium spp.) is a shrub (Figure 1) with soft fiber that grow on a protective case – boll, brought to African continent in the 19th century. In Mozambique cotton detain 97% of the national investment and in central and northern regions it's one of the main sources of income for rural families. With decades of civil war, the country's agricultural sector fell drastically and cotton cultivation was not an exemption, having also been characterized by environmental harmful due to use of agrochemicals and low productivity due to presence of insects, weeds and low fertility soils. The endophytic fungi that form arbuscular mycorrhizae (AM) have recently gained notoriety, representing a viable and self-renewable alternative to agrochemicals along with benefits for the environment. AM fungi (AMF) are obligate root fungal symbionts that facilitate plant growth by capturing nutrients in depleted soils becoming a sustainable alternative to cotton fertilization and protection against diseases (Quilambo, 2003). Advances in molecular biology and biotechnology have resulted in the development of powerful tools and techniques that have assisted the evolution in crop improvement. AMF influence on cotton cultivation is scarcely reported (Eskandari et al., 2017). In 2017 a project entitled "Microbial Resources for Agriculture: Arbuscular Mycorrhizal Fungi in Cotton and Their Potential Use as Biofertilizer (MYCOTTON)", between DCB/FC(UEM) and IPSP(CNR) and DBIOS(UNITO) has been funded by FIAM (Fundo Para Investigação Aplicada e Multissectorial) aiming isolate and molecularly characterize AMF to formulate a cotton-tailored biofertilizer inoculum.

OBJECTIVES

The main goal of MYCOTTON Project is to develop functionally and efficient AMF based inocula for cotton biofertilization. In this context the aims of this poster are: 1) to test two cotton varieties commonly used in Mozambique in order to verify their ability to be mycorrhized; 2) to set up reliable molecular protocols to extract and amplify AMF DNA from soil and roots sampled in Mozambique cotton fields.

METHODOLOGY



FUTURE ACTIVITIES

Voyron S., Victorino I., Mondlane Milisse A., Ventura Guilundo S., Martins C., Bianciotto V., Quilambo O., Lumini E. Metabarcoding targeting the ITS2 region to profile the AMF Communities in Mozambican cotton cultivated fields. 6th International Conference on Microbial Diversity (MD) 2021, 15- 16 December 2021.

FAO. 2021. Keep soil alive, protect soil biodiversity – Global Symposium on Soil Biodiversity 19–22 April 2021. Proceedings. Pg 906-912. Rome. https://doi.org/10.4060/cb7374en.

Microbial resources for Mozambican agriculture: use of arbuscular mycorrhizal fungi as a sustainable alternative to chemical input in cotton production

Victorino I[1-4*], *Mondlane Milisse A.*[4], *Martins C.*[1], *Ventura S.*[1], *Quilambo O.*[1], *Voyron S.*[2], *Girlanda, M.*[2]., *Faccio A.*[3], *Lumini E.*[3], *Bianciotto V.*[3]

[1]Biology Science Department of Eduardo Mondlane University (DCB-UEM). rvictori@unito.it
[2]Department of Life Sciences and Systems Biology of Turin University (DiBIOS-UNITO)
[3]Institute for Sustainable Plant Protection of National Research Council (IPSP-CNR)
[4]Biotechnology Centre of Eduardo Mondlane University (CB-UEM)

Abstract

Mozambique is a sub-Saharan country subsisted by familiar agriculture in which the use of pesticides and chemical fertilizers are common. Local farmers however poorly understand the harmful effect of chemical inputs, either for themselves or soil biodiversity. For decades cotton have been one of the major cash crops levying around 80 percent of the national investment. Constraints to cotton cultivation were greatly presence of insects and weeds and soil nutrients deficit. Arbuscular mycorrhizal fungi (AMF) arisen representing a viable and self-renewable alternative to agrochemicals along with benefits for the environment. AMF are obligate root fungal symbionts that facilitate plant growth, by capturing nutrients in depleted soils like the Mozambican one's, fact that makes them a possible solution to limit chemical input. In the present work two cotton varieties (CA 324 and ALBARSZ 9314) were pot cultivated with previously tested commercial inocula in order to evaluate whether these fungi could be able to infect these particular varieties. It was verified that AMF infected cotton roots of both varieties, being the commercial inoculum from MycAgro Lab the one that showed best infection/typical structures presence ratio. Secondarily we are studying soil native fungal communities present in cotton cultivated field in Nampula province (Mozambique).

Keywords: arbuscular mycorrhizal fungi, cotton, soil fungal communities, biofertilizers

Introduction, scope and main objectives

Cotton (*Gossypium* spp.) is a shrub with soft fibre that grow on a protective case – boll (Figure 1), brought to African continent in the 19th century. In Mozambique cotton detain 97 percent of the national investment and in central and northern regions it's one of the main sources of income for rural families (Tombez and Bokusheva, 2011). With decades of civil war, the country's agricultural sector fell drastically, and cotton cultivation was not an exemption, having also been characterized by environmental harmful due to use of agrochemicals and low productivity due to presence of insects, weeds and low fertility soils.

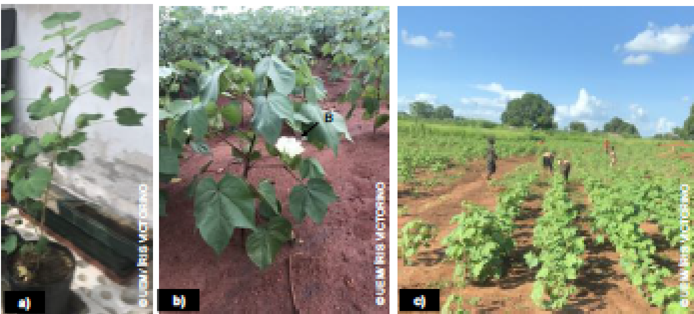


Figure 1: Cotton plants

a) grown in green house, b) grown in open field (Nampula – Mozambique) showing boll (B), c) cotton cultivated field in Nampula province (Mozambique)

Since a long time, the endophytic fungi that form arbuscular mycorrhizae (AM) have gained notoriety representing an eco-friendly alternative to agrochemicals. AM fungi can symbiotically interact with plant roots facilitating plant growth by capturing nutrients in depleted soils and, by this, becoming a sustainable alternative to cotton fertilization and protection against diseases (Quilambo, 2003). Advances in molecular biology and biotechnology have resulted in the development of powerful tools and techniques that have assisted the evolution in crop improvement. AMF influence on cotton cultivation is scarcely reported (Eskandari *et al.*, 2017). In 2017 a project entitled "Microbial Resources for Agriculture: Arbuscular Mycorrhizal Fungi in Cotton and Their Potential Use as Biofertilizer (MYCOTTON)", between DCB/FC (UEM) and IPSP (CNR) and DBIOS (UNITO) has been funded by FIAM (Fundo Para Investigação Aplicada e Multisectorial) aiming to isolate and molecularly characterize AMF to formulate a cotton-tailored biofertilizer inoculum. The main objective of MYCOTTON Project (2017-2020) is to develop functionally and efficient AMF based inocula for cotton biofertilization. In this context, the aims of this work are: 1) to test two cotton varieties, commonly used in Mozambique, in order to verify their ability to be mycorrhized; 2) to extract and amplify AMF DNA from soil and roots, sampled in cotton cultivated field, in Nampula province (Mozambique).

Methodology

Biological materials

Two cotton varieties, ALBARSZ 9314 and CA 324, were pre-germinated in Petri dishes and the obtained seedlings were then transferred into pots filled with a sterilized substrate. Each pot from treated group were inoculated with three different commercial inocula (MycAgro Lab, Micosat F Radinet, Micosat F SEMI) containing AMF (Figure 2) previously tested by Caser *et al.* (2019), while a set of plants non-

inoculated were used as control. All the plants were maintained several months in greenhouse.

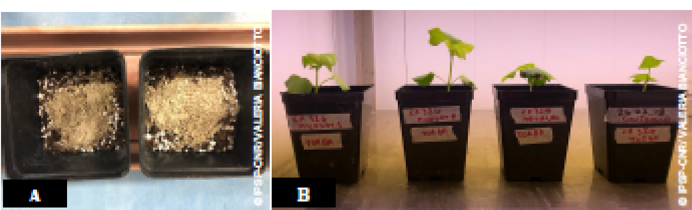


Figure 2: Display of pots in greenhouse

A - inocula in pots containing sterilized substrate, B - treated pots with different inocula + control pot

AMF Evaluation

During the vegetative phase of both control and treated cotton plants groups, roots were harvested, rid of topsoil, cleaned and stained with 0.1 percent (w/v) cotton blue in 80 percent lactic acid overnight, then distained 3 times with lactic acid for 18 h, cut into 1-cm-long segments and placed on microscope slides for the morphological analysis. Approximately 25 fragments were observed under light microscope for each sample. Fungal colonization was determined and calculated as described by Trouvelot, Kough and Gianinazzi-Pearson (1986).

For the preparation of the roots for further observations in light and transmission electron microscopy we randomly selected fragments of cotton mycorrhized roots. Root segments were excised under a stereomicroscope and quickly fixed in 2.5 percent glutaraldehyde in 0.1 M cacodilate buffer (pH 7.2) for 2 hours at room temperature and overnight at 4 °C. The samples were then post-fixed in 1 percent OsO_4 in the same buffer and dehydrated in an ascending series of ethanol to 100 percent, incubated in two changes of absolute acetone and infiltrated in Epon-Araldite resin. The resin was polymerized for 24 h at 60 °C. Semi-thin (1 micron) sections were then stained with 1 percent toluidine blue and ultra-thin (70 nm) sections were counter-stained with uranyl acetate and lead citrate and used for electron microscopy analyses under a transmission electron microscope.

Molecular analysis

In order to optimize DNA extraction from soils and roots, collected in cotton cultivated field in Nampula province (Mozambique), two kits were tested: DNeasy Power Soil kit (QIAGEN) for soil and DNeasy Plant kit (QIAGEN) for eukaryotic DNA extractions. PCR amplification was done by means of specific primers targeting fungi. The resulting PCR products were visualized on 1.5 percent agarose gel.

Results

Greenhouse cotton roots morphological observation and mycorrhizal evaluation

Two cotton varieties treated with three different inocula (MycAgro Lab, Mycosat F Radinet and Mycosat F SEMI) were tested in pot. AMF successfully infected cotton roots of both varieties and, as expected, not inoculated plants were not mycorrhized. The colonization was seen after 45 days of pot cultivation in greenhouse, showing highly AMF presence in some root fragments, detected by the presence of arbuscules, vesicles and inter-intracellular hyphae (Figure 3). MycAgro Lab inoculum was the best inoculum in terms of ability to colonize, as treated plants were able to form all AMF typical structures mentioned before.

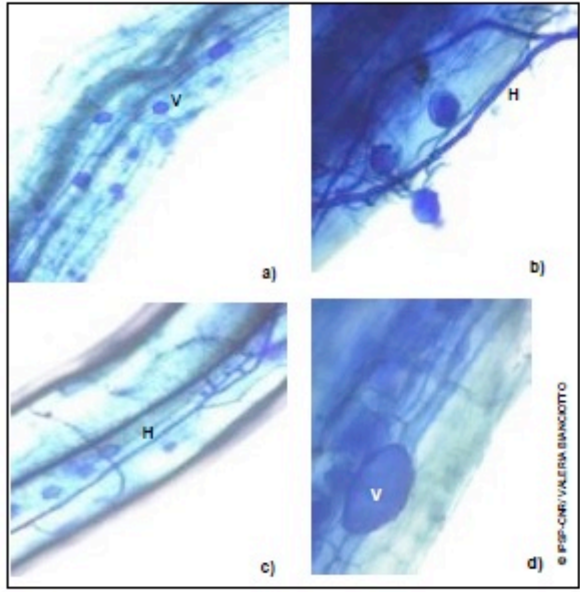


Figure 3: Optical microscope images showing cotton roots cortical cells

In the pictures above (a-d) are presented cotton roots colonized by MycAgro Lab inoculum showing hyphae (H), vesicles (V), on ALBARSE 9314 variety.

Under light and transmission electron microscopes by observing semithin and thin sections hyphopodia were observed on epidermal cells. The root epidermis and the first layer of root cortical

909

parenchyma were free of colonization while the deeper layer of parenchyma (near central cylinder) was all colonized by typical AMF structures. At this time of pot cultivation very few mycorrhizal structures were detected in roots treated with Mycosat F Radinet and Mycosat F SEMI inocula.

Open field soil and roots DNA extraction and amplification

Using DNeasy Power Soil kit (QIAGEN) and DNeasy Plant kit (QIAGEN) for soil and cotton roots DNA isolation, we were able to successful extract template DNAs for amplification and subsequent NGS. Previously extracted DNAs resulted amplifiable by fungal specific primers.

The characterization of fungal communities associated to soil and cotton roots samples, from cultivated field in Nampula province (Mozambique), will be carried out with paired-end Illumina MiSeq data, cover the full ITS2, a suitable target for environmental fungal community assessment. In the meanwhile trap cultures, with autochthonous soils, will be set up in greenhouse experiments in order to isolate and characterize "local adapted" AMF inocula that will be tested as tailored-biofertilizer for cotton.

Discussion

This study presents, for the first time, experimental evidence for directly inoculation of two cotton varieties cultivated in Mozambique. The AMF are soil microorganisms able to establish a mutualistic interaction with most land plants (Berruti *et al.*, 2016), thus as expected al three used inocula were able to spread into the root systems of almost all infected plants after 45 days in greenhouse. Mycorrhizal fungi from all three commercial inocula were able to infect cotton, MycAgro commercial inocula however, showed the best performance confirming that an inocula with more than one AM fungi readily colonized plant roots fact that was stated previously by Bianciotto et *al.* (2016). All three inocula differently infected cotton roots (data not shown). This fact can be explained because each inoculum (AMF isolate) may act differently both ecologically and functionally, depending on the plant species (Janous˘kova *et al.*, 2017).

Commercial DNA extraction kits are widely available and have become favourable because they are often faster than traditional extraction methods (Mahmoudi, Slater and Fultrorpe, 2011). It is noteworthy that successful DNA extractions, characterized by high quantity and integrity of dsDNA and low quantity of contaminants that absorb at 230 and 280 nm, are the first important step for a future reliable characterization of microbial communities associated to soil and plant samples and Next-Generation Sequencing (NGS) analyses.

Conclusions

AMF commercial inocula can infect cotton plants and can be a great alternative in cotton cultivation by reducing the use of agrochemicals. Inocula with more than one AM fungi tend to readily

infect cotton plant roots as previously described for many other plant species. For this reason, all the research efforts to select and improve local AMF production and use are welcome.

Acknowledgements

We gratefully acknowledge FIAM for funding this bilateral project and all colleagues from UEM, CB-UEM, UNITO, IPSP-CNR for their contribution. The PhD School in Biology and Applied Biotechnology of the University of Torino are acknowledged for funding Iris Victorino's PhD (2016-2020).

References

Berruti, A., Lumini, E., Balestrini R. & Bianciotto, V. 2016. Arbuscular Mycorrhizal Fungi as Natural Biofertilizers: Let's Benefit from Past Successes. *Frontiers in Microbiology*. 6: 1559.

Bianciotto, V., Victorino, I., Scariot, V. & Berruti, A. 2016. Arbuscular mycorrhizal fungi as natural biofertilizers: Current role and potential for the horticulture industry. Presented in III International Symposium on Woody Ornamentals of the Temperate Zone, pp. 207-216. https://doi.org/10.17660/ActaHortic.2018.1191.29

Caser, M., Victorino, I., Demasi, S., Berruti, B., Donno, D., Lumini, E., Bianciotto, V. & Scariot, V. 2019. Saffron Cultivation in Marginal Alpine Environments: How AMF Inoculation Modulates Yield and Bioactive Compounds *Agronomy* 9: 1-14.

Eskandari, S. Guppy C., Knox O., Backhouse D. & Haling R. 2017. Mycorrhizal Symbioses of Cotton Grown on Sodic Soils: A Review from an Australian Perspective. *Pedosphere*, 27:1015-1026.

Janousˇkova, M., Krak, K., Vosátka M., Puˇschel, D. & Sˇtorchová, H. 2017. Inoculation effects on root-colonizing arbuscular mycorrhizal fungal communities spread beyond directly inoculated plants. *PLoS ONE*, 12(7): 1-21.

Mahmoudi, N., Slater, G. & Fultrorpe, R. 2011. Comparison of commercial DNA extraction kits for isolation and purification of bacterial and eukaryotic DNA from PAH-contaminated soils. *Canadian Journal of Microbiology*. 57: 623-628.

Quilambo, O. 2003. The vesicular-arbuscular mycorrhizal symbiosis – review. African *Journal of Biotechnology*, 2:539-546

Trouvelot, A, Kough, J & Gianinazzi-Pearson, V. 1986. Mesure du taux de mycorhization VA d'un système radiculaire. Recherche de méthodes d'estimation ayant une signification fonctionnelle. In: V. Gianinazzi-Pearson & S. Gianinazzi, eds. Paper presented at the European symposium on mycorrhizae, pp 217-221, 1-5 July 1985, Paris, INRA Press.

Tombez, G., & Bokusheva, R. 2011. *Evaluation of socio-economic feasibility of pilot micro-insurance schemes for cotton producers in Mozambique*. Zürich, Institute for Environmental Decisions (IED)

6.5. Acknowledgements

Here all the people I want to thank:

Prof Valeria Bianciotto: super mom, very wise woman, the woman and professional I am now I owe it all to you. Keep shinning dear ‘nonna’ Vale!!

Prof Erica Lumini: my mentor, my rock, forever my dearest speedy Gonzalez!

Dr Andrea Berruti: the quietest water.

Dr Samuele Voyron: specialist.

Dr Matteo Caser: semper FI!!!

Dr Antonietta Mello, Dr Raffaella Balestrini, Dr Stefano Ghignone, Dr Sergio Favero, Dr Silvia Perotto, Dr Stefania Daghino, MSc Sónia Guilundo, Prof Celia Martins, Prof Orlando Quilambo and all the scientists I met during my abroad time: teachers.

All lab guys (dwellers and passengers): friends.

Prof Paola Bonfante: farsighted, most networked scientist. I hope more can benefit of the pleasure of being in the same environment as you.

Dr Ivete Maquia: BFF.

Dr Mauro Colombo and Dr Luís Neves: thanks for investing and believing in me.

My family: many thanks couldn’t have done it without each and every one you. Mom I hope you are proud!!

Nuno: my dear younger brother, the most awesome person I have ever met.

My Italian friends: support!!

My Mozambican friends: you are the best.

Ítalo: my weakness, my strength, most loved and life forever mate…

May God bless you all.