UNIVERSITÀ DEL PIEMONTE ORIENTALE
"AMEDEO AVOGADRO"
Department of Science and Technological Innovation

PhD in "Chemistry & Biology"

Curriculum "*Energy, environmental and food sciences*"

XXXIII cycle 2017-2020

SSD: BIO/01

Study of the growth, artemisinin production and leaf volatilome of some micropropagated *Artemisia annua* clones inoculated or not with beneficial soil microorganisms

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Chapter 1

Introduction

The genus *Artemisia* is widely diffused, and it includes about 500 species of plants, commonly called "wormwood" (due to the traditional use for the treatment of intestinal worms), "mugwort", "sagebrush" or "tarragon" (1, 2), belonging to the *Asteraceae* family (3, 4). The name "*Artemisia*" derives from the Greek goddess "Artemis" (1). *Artemisia* species are perennial, biannual, or annual herbaceous plants and they can be used as ornamental, medicinal and aromatic plants (5, 6).

1 - *Artemisia*, artemisinin and malaria

Malaria is still today a big health problem in many regions of the world, particularly Africa, South America and South-East Asia, as reported by World Health Organization (7, 8). It was estimated to affect more than 225 million people worldwide with almost 429.000 victims, especially in the African Region, the majority of which (more than 50%) are represented by children under 5 years; about 3.3 billion people are at risk of infection and development of the disease (8). Malaria is caused by a blood protozoan parasite of the genus *Plasmodium* (9), whose life cycle involves both humans and a carrier insect represented by mosquitoes of the genus *Anopheles* (10, 11). Different *Plasmodium* species, such as *P. falciparum*, developed a multi-resistance to conventional drugs (12, 13). For this reason, for the past 15 years, the WHO recommended the use of artemisinin-based combination therapies as the best treatment currently available against malaria (8, 14, 15). Artemisinin is a bioactive molecule extracted from the *Artemisia annua* leaves (Figure 1; 2, 8). However, artemisinin content in *A. annua* plant is very low (0.01-1% of plant dry weight; 16-18), so different

strategies to enhance its concentration *in planta* have been explored: especially molecular, physiological and biochemical approaches (19-32). Unfortunately, biotechnological methods, such as *in vitro* cultivation hairy roots, plant cell cultures and fermentation with microbes, have not been found to be very effective (33-36), therefore the increase of artemisinin yield in cultivated plants remains an important research area.

1.1 - Artemisia annua L.: botanical characteristics and geographical distribution

A. annua L. is an annual aromatic herbaceous plant, and it is native from Hunnan region (China) but it is diffused in the temperate, cool temperate and subtropical zones of the world (37). The stem has an erect bearing, and it can be branchy from the base or monocaule; it is cylindrical and striated; it can grow to 40-100 cm, but the cultivated plants can reach a height of 200 cm.

Figure 1. The figure shows *A. annua* plant at the first stages of growth (**A**), at the full vegetative phase (**B**), and a botanic table (**C**) in which the flowers and shoot and root apparatus are also represented. (*Source: Vuyck L. (1906) Flora Batava. 22 Band. Vincent Loosjes, Haarlem. Tafel 1697).*

The leaves are alveolate-punctate-glandular, 3-5 cm long and 2-4 cm wide, ovate, thrice pinnately cut and their lobules are oblong-lanceolate and short-acuminate, the upper leaves are sessile, smaller and less composed (Figure 1 A, B). The

inflorescence consists of a terminal panicle of pendulous flower heads (15-20 cm), with imbricate bracts with a lanceolate shape, straw yellow in colour; the flowers are pentamers, hermaphrodites, composed of an actinomorphic tubular corolla (1.5 mm) of straw or dark yellow colour, with a five-lobed margin in the internal hermaphrodite flowers and a bilobate in the external female ones. The fruit is an elliptical-ovoid achen without pappus and seeds are numerous and very small in size. The root is taproot with many lateral secondary roots (Figure 1 C; 3, 4, 12).

1.2 - Artemisinin: localization and biosynthesis

Medicinal properties of *A. annua*, in the traditional Chinese medicine, have been well known for centuries (8, 12, 37). Artemisinin ([3R-(3α, 5αβ, 6β 8αβ, 9α, 12β, 12αR)]-octanohydro-3,6,9-trimetyl-3,12-epossi-12H-pirano[4,3-j]-1,2-benzodiossepin-10 (3H)-one) is the principal bioactive product of the *A. annua* plant (2, 6, 38) and, as mentioned above, it has an inhibitory action against *Plasmodium* species, which are multi-resistant to conventional drugs. Artemisinin is a sesquiterpene lactone that contains a peroxidic group, responsible for the inhibitory action on the *Plasmodium* (15, 21, 37, 39, 40). This molecule is synthesized in the biseriate glandular trichomes of the leaves (12, 41-44). Trichomes are small protrusions of epidermal origin present on the leaves, usually divided in two types: glandular and not glandular trichomes (18). Glandular trichomes can be small structures composed of few cells or big and complex structures that are differentiated in basal, median and apical secretory cells (18, 41). One of the most crucial features of trichomes is their capacity to synthetize, store and, in some cases, secrete considerable quantities of metabolites, included different classes of terpenoids (45). Artemisinin biosynthesis takes place inside multicellular glandular secretory trichomes, specifically *A. annua* glandular secretory trichome (Figure 2 A, B) which has a

biseriate structure formed by 10 cells: two basal cells, two median cells and three pairs of secretory cells; the cuticle of the secretory cells is separated from the cell walls to form a bilobed sac (41). Artemisinin and other sesquiterpenes are released and stored inside this subcuticular space (41, 42, 46, 47). The terpenoid pathway, that leads to the artemisinin biosynthesis, is a cytosolic pathway and needs isoprene units formed by five carbon atoms which are the basic carbonaceous skeletons. There are two important substrates: isopentenyl diphosphate (IPP) and dymethylallyl diphosphate (DMAPP; Figure 3; 4, 48).

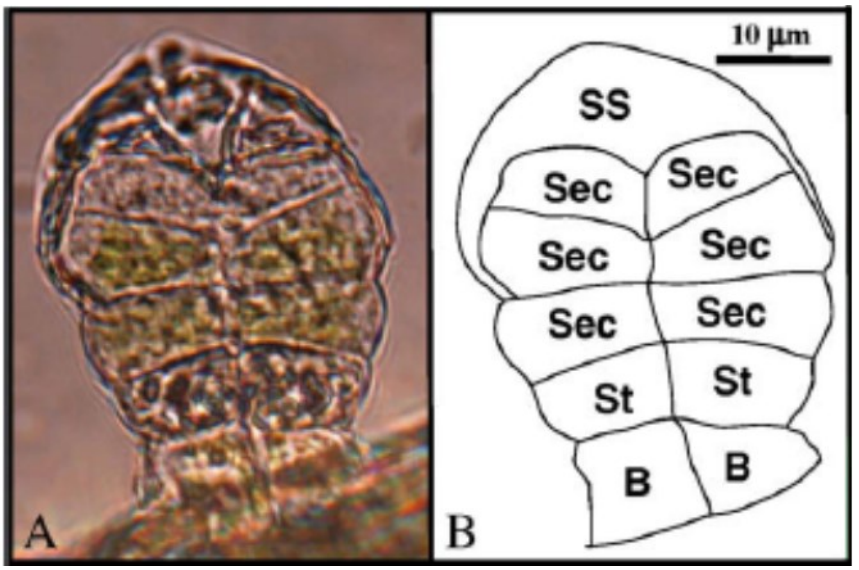

Figure 2. The figure shows the glandular secretory trichome of *A. annua* plant (**A**), and a graphic representation of B: basal cells; St: median cells; Sec: secretory cells; SS: subcuticular space (**B**). *Source: Olsson et al. (41*).

These compounds derive from two pathways: the mevalonate pathway (MVA; cytosolic) and the methyl erythrol phosphate (MEP; plastidial) (18, 47, 49). Mono- and diterpenes are synthesized inside the plastids from the geranyl- and geranylgeranyl diphosphate, whereas sesquiterpenes and triterpenes are produced in the cytosol from the farnesyl diphosphate (FPP) (4, 41, 47, 48). Biosynthesis of FPP, a compound of fifteen carbon atoms, is carried out through the union of

one isoprene unit from MEP and two isoprene units from MVA (4, 49, 50), thanks to the farnesyl diphosphate synthase enzyme (FPPS).

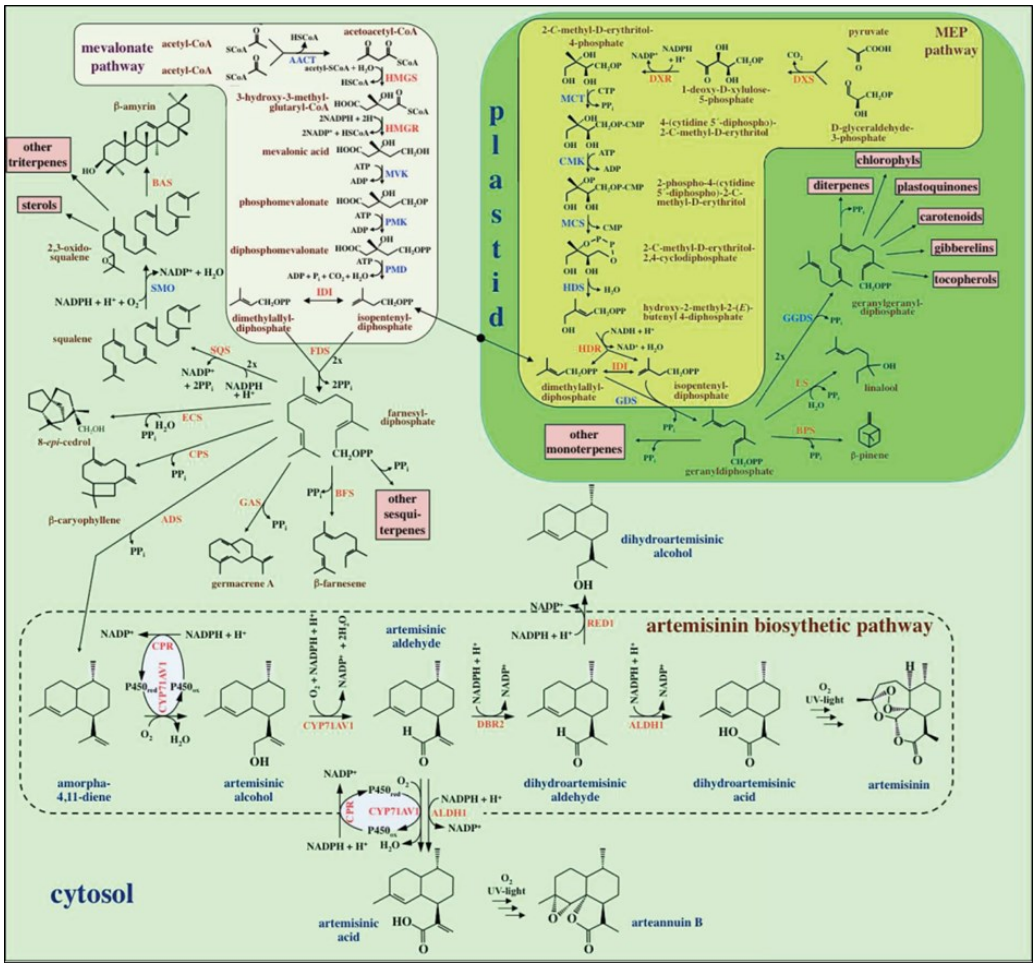

Figure 3. Global focus on terpenoid and artemisinin pathway. *Source: Olofsson et al. (48).*

The main artemisinin biosynthesis steps can be divided into different stages (Figure 4). In the first stage, the cyclization of the FPP into the amorpha-4,11-diene synthase (a bicyclic sesquiterpene) takes place by the amorpha-4,11-diene synthase (ADS; a sesquiterpene cyclase) (4, 46, 51-53). The second stage involves modifications on the isopropyldene of the amorpha-4,11-diene, which undergoes two consecutive oxidations by means of P450 cytochrome, CYP71V1

(CYP), until the formation of artemisinic alcohol and then of artemisinic aldehyde (34, 48, 54). Afterwards, the pathway is less known and many different products *in planta* have been isolated: artemisinic alcohol, artemisinic aldehyde, dihydroartemisinic acid (DHAA) and dihydroartemisinic aldehyde, thus suggesting that two interconnected pathways may be present (46, 52, 53, 55). Both pathways start from the artemisinic aldehyde (Figure 4), one leads to the arteannuin B synthesis and one leads to the artemisinin synthesis (4, 55). The first pathway concerns the oxidation of the artemisinic aldehyde to artemisinic acid (AA) by CYP or by aldehyde dehydrogenase enzyme (Aldh1), leading to the arteannuin B production (AB; 55, 56).

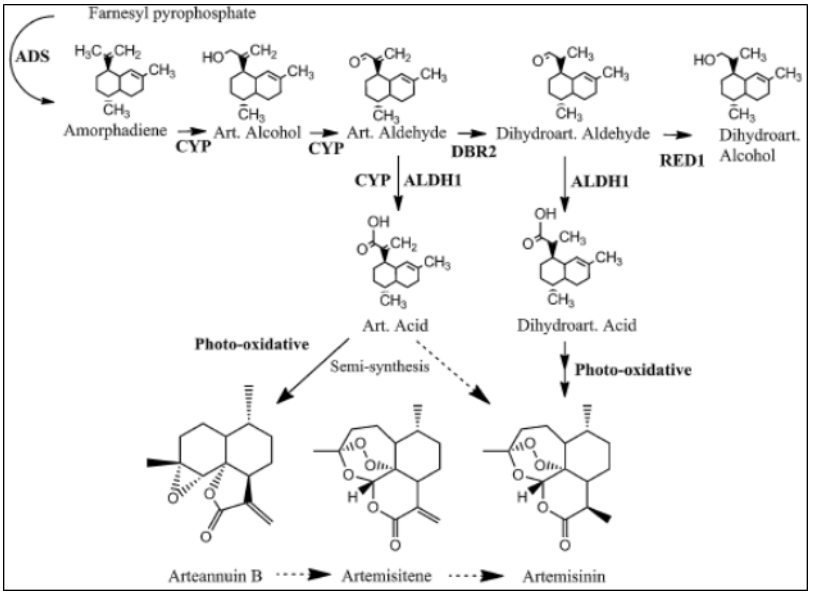

Figure 4. Key steps in artemisinin biosynthetic pathway. *Source: Nguyen et al. (55*).

Other authors suggested that AB may be converted into artemisitene (AT) and then into artemisinin (AN; 57), but this is true only *in vitro* cell systems but not *in vivo conditions*. Moreover, there are data that show a conversion of the artemisinic acid (AA) into artemisinin (AN) (47, 55, 58). In the other branch of the biosynthetic pathway, after the action of cytochrome P450, the artemisinic

aldehyde is reduced into dihydroartemisinic aldehyde by the artemisinic aldehyde reductase (DBR2; 59) and then oxidized, by Aldh1 enzyme, into DHAA (56). Finally, the DHAA is converted into AN as a result of not-enzymatic or foto- and self-oxidation reactions (4, 46, 48, 51-53, 60).

1.3 - Other secondary metabolites in A. annua plant

A. annua plant is a source of many other secondary metabolites, several of them are volatile compounds responsible for the characteristic aroma of this species (2, 6). Its essential oil is composed by terpenoids, phenylpropanoids, aliphatic compounds mainly produced in the aboveground part of the plants (61); in fact, it has been observed that mature leaf surface is covered by capitate glands containing terpenoic volatile compounds (62). The pre-flowering stage is the best harvesting time to have the best essential oil yield (49). However, a wide variability in the composition of essential oil has been registered, since the quantity and quality are strongly influenced by many factors such as harvesting time, season, fertilizers, soil pH, geographic location, subspecies or ecotypes, plant genotype and extraction method (62; Table 1). The main components in the leaves are artemisia ketone, 1,8-cineole, and camphor (2, 63-65), followed by other components such as alpha-pinene, camphene, β-pinene, myrcene, linalool, borneol, and β-caryophyllene (17).

Table 1 | Compounds (>4% of total compounds) isolated from essential oil of *A. annua* L. *Source: Bilia et al. (62)*.

Compound	Country	%
Artemisia alcohol	China (Cult)	7.5
	USA-CA	5.2
	Serbia	4.8
Artemisia ketone	Not stated	38.0
	France	52.5
	Serbia	35.7
	Egypt	13.9
	China	2.21
	Bosnia	30.7
	USA-CA	35.7
	China (Cult)	63.9
	USA-IN	68.5
	England	61.0
	Vietnam	0.1–4.4
	Indian (Cult)	58.8
	India (Cult)	11.5
	Turkey	22
Borneol	Not stated	20.0
	England	7.0
	China (Cult)	15.9
Camphene	Iran	7
Camphene hydrate	USA-IN	12.0
Camphor	Vietnam	21.8
	Serbia	4.2
	Egypt	5.08
	France	27.5
	China (Cult)	21.8
	Vietnam (Cult)	3.3
	Bosnia	15.8
	Iran	1.92
	Italy	17.6
	Indian (Cult)	15.75
	India (Cult)	8.4
	France	43.5
	Iran	48
	Turkey	31
Trans-Cariophyllene	Egypt	7.73
β-Caryo phyllene	Italy	9.0
	Vietnam (Cult)	5.6
	Vietnam	3.3–8.6
	China (Cult)	5.98
	India (Cult)	12.2
	France	8.9
Caryophyllene oxide	China	5.13
Chrysanthenone	Vietnam	1.1–7.3
	India (Cult)	10.19
1,8-Cineol	France	11.66
	Serbia	5.5
	Egypt	8.13
	Bosnia	4.8
	USA-IN	22.8
	USA-CA	31.5
	Iran	9.4
	Iran	11.4
	Turkey	10
β-Farnesene	Italy	10.2
	Vietnam	1.1–12.8
	Egypt	5.32
	China (Cult)	12.9
Germacrene D	Vietnam (Cult)	18.3
	Italy	21.2
	Vietnam	0.3–18.9
	China (Cult)	10.9
	France	15.6
α-Guaiene	China (Cult)	4.7
Linalool	Vietnam	0.1–4.2
	Iran	8.1
Linalool acetate	England	10.0
Myrcene	China (Cult)	5.1
	USA-CA	4.6
	Vietnam	0.1–8.5
α-Pinene	USA-CA	11.2
	USA-IN	16.0
	Serbia	16.5
(*Trans*)-Pinocarveol	France	10.9
	Serbia	4.8
Sabinene	France	9.4
Spathulenol	Iran	4.97
	Iran	4.9

In several reports it has been showed that phytogeographic origin can heavily influence metabolic profiles of *A. annua* leaves, showing great differences in the content of the aforementioned compounds, but also in the other produced mono- and sesquiterpenoids (62). In a study of Goel et al. (66) a poor quantity of essential oil (about 0.25% of weight) has been obtained also from roots of *A. annua* var. Jwarharti; it was rich in sesquiterpenes and oxygenated sesquiterpenes and had as its major constituents: cis-arteannuinic alcohol, (E)-β-farnesene, β-maliene, β-caryophyllene, caryophyllene oxide, and 2-phenylbenzaldehyde. All these abovementioned plant extracts have been used in several experiments on *Candida* sp. (67-69), *Aspergillus* sp. (64), *Staphylococcus aureus*, *S. pneumoniae*, *Escherichia coli*, *Haemophylus influenzae* and *Pseudomonas aeruginosa*, in order to test their antibacterial and antifungal activity, and, in some cases, a good effectiveness has been observed (6, 62).

2 - Clonal selection

The market demands of artemisinin cannot be met with current plant yields, due to low and variable production of artemisinin in cultivated plants (32, 70). In the last 15 years, the cultivar selection has increased artemisinin concentration in *A. annua* plant, but plants generated by seeds have a high variability in the artemisinin and biomass production due to genetic recombination (71-74). This led to a limited increase in the artemisinin plant production: in fact, the range in the commercial lines is among 0.5-1.4% of plant dry weight (75). Instead, clone selection has become a good method to reduce the genetic variability and to select specific plant traits (76), in particular by micropropagation that is a more rapid technique to select specific plant genotype (74).

2.1 - Clonal selection "in vitro": micropropagation technique

Plant biotechnology, and specifically plant tissue culture, started thanks to Gottlieb Haberlandt, an Austrian botanist who noticed the faculty “to culture isolated vegetative cells from higher plants in simple nutrient solutions” (77). Some of the plant tissue culture milestones were hormonal control of regeneration (78), specific knowledge on organogenesis and somatic embryogenesis (79), operative aspects of micropropagation and plant disease-free production (80) to mention just some of them. In the 1970, the mass production of *in vitro* plants became usable for ornamental and crop plants (81-83). During the following years, this technique has been widely used, and now most of plant species can be propagated *in vitro* on a commercial scale (84), becoming an important part of the plant industry (77, 85). *In vitro* plant cultures consist of cells, tissues or organs cultured in axenic and sterile conditions, on a specific medium as that created by Murashige and Skoog (known as MS medium; 86-88), in which plant pieces, derived from a mother plant, can express their potential due to the totipotency of plant cells (77, 89). MS medium is composed by agar, macro- and micronutrients, sugars and in some cases vitamins, amino acids and growth hormones (86). It must be taken into account that the medium will have a different composition, according to the used plant material and plant species (88). Micropropagation is a technique used as *in vitro* application of plant tissue culture, conducted in axenic or aseptic conditions, in order to obtain clonal propagation of many important silvicultural, horticultural and medicinal plants (90-92). The traditional clonal propagation techniques (seeds and cuttings) require long periods of time to obtain many new adult plants with many problems correlated to plant diseases that can dramatically reduce the number of produced plants (73, 93); furthermore, conventional techniques can be successful used on a small scale, but not for the production on a large scale. Therefore, in the last

case, more efficient methods are required, such as micropropagation (94, 95). Micropropagation has many advantages: it gives a rapid and mass multiplication of true-to-type, genetically identical plants in a short period of time; plants produced in this way are disease-free (77, 96, 97); the technique is particularly helpful for the propagation of those plants that are scarcely propagated by traditional techniques, for instance orchids (91, 98), and it is useful to select specific plant traits (76, 99).

Micropropagation leads to the regeneration of new plant individuals from plant explants exploiting the peculiar totipotency of plant cells (89), improving cell division in order to have the formation of a tissue named “callus”, consisting of undifferentiated cells (100). Successively, the callus proliferation or further differentiation events lead to the organ formation (shoots or roots) by a process known as organogenesis or to seed-like embryos from somatic cells, named somatic embryogenesis (77, 101). The cells of the plant explant are subjected to a dedifferentiation process, followed by a new activation of cell division through an increase of mitotic activity, induced by the exposure either to nutritional and hormonal medium constituents or to some parameters related to the growth conditions (e.g. light, temperature, humidity and so on; 102). Organogenesis and somatic embryogenesis depend on several processes of redifferentiation, such as cell cycle factors, hormonal and metabolic signalling, temporal and spatial activation of specific genes, cytoskeleton organization genes, followed by new shoot or root meristem organization or a bipolar cell division for somatic embryo growth (77; Figure 5), and these steps are very complicated and they are waiting for a better understanding.

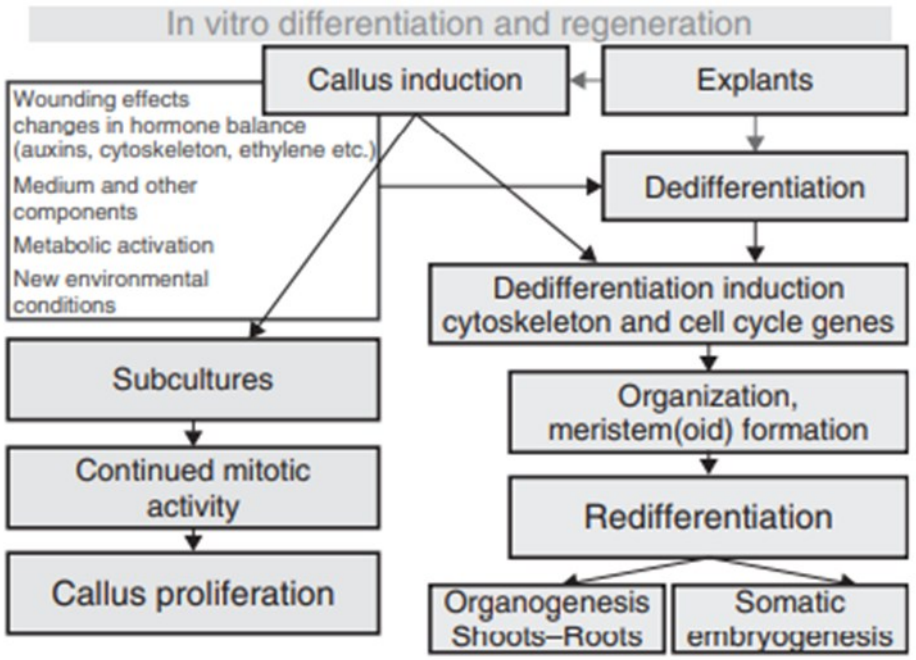

Figure 5. Principal steps of *in vitro* differentiation and regeneration of plant tissues. *Source: Loberant and Altman, (77).*

Micropropagation can start from three different plant material types: axillary buds, which can start off many new buds; direct regrowth of buds; or by somatic embryos, which gives rise to diploid seedling provided with shoot and root banded together by vascular system; indirect regrowth starting from callus of buds or somatic embryos (91, 95). If the number of required plants for the production is low, axillary bud regrowth *in vitro* is considered the better way for the micropropagation method (77, 102): this way does not require a callus stage, so it is less subjected to somaclonal variation (103), a phenomenon which will be successively examined in the text. Since a new bud regeneration is not used because bud meristems already exist in the leaf axils and in the tips of shoot, plant clonal traits are considered safer from genetic variations (100, 104). However, they do not grow in a whole plant due to apical control, and when small sections of apical meristems (like shoot tips) are cut and cultured in a medium containing

high cytokinin concentrations, many other quiescent axillary buds grow (77). Afterwards, shoots are divided into more cultures and relocated for rooting (100). Organogenesis leads to *de novo* development of shoots and roots thanks to a meristematic cell cluster, named meristemoid cells, whether it is an explant or a callus that grows up to be a shoot or a root meristem (102, 104). The formation of these two meristems is influenced by the type of explant, culture conditions and growth regulator ratio in the medium (77). Afterwards, shoots and roots go to an additional differentiation and growth until vascular connections between them are formed, thus resulting into a whole plant (105).

On the contrary, in the somatic embryogenesis, regeneration and organization are bipolar, and thus an initial cell gives concurrently origin to shoot and root meristems that leads to the formation of proembryonic masses, which are cluster of cells (106, 107). Also in this case, somatic embryo differentiation and organization occur starting from explant or from the callus, according to the medium composition; in fact an exposure of tissue culture to auxin-type regulators leads to pro-embryonic mass induction (102). Subsequently, the culture is transferred to a medium without auxin, in which somatic embryos can fully develop (108). Then, differentiation patterns are very conserved: 1) structures of globular embryos; 2) the heart stage, whereby shoot and root meristems are plainly identified at the embryo poles; 3) the torpedo stage, in which shoot and root elongation and vascular connection between these latter is made up (77), leading to the formation of the whole plantlet.

2.2 - Micropropagation technique: practical aspects

Usually, the micropropagation work-flow requires five different stages:

I. Mother plant selection

II. Aseptic culture initiation

III. Multiplication of shoot

IV. Rooting

V. Acclimatization

I. The physiological and phytosanitary status of the mother (or donor) plant, besides its genotype, strongly influence the explant responsiveness and quality (91, 107, 109, 110). Therefore, the selection and maintenance of plant source is very important to guarantee specific plant features: true-to-type characteristic of requested species and cultivar, disease- and contamination-free, and viable and vigorous plants (88, 111). The used explants can vary from small true meristems, dissected under the microscope, to bigger sections of stem, leaf or shoot tips; after the explant, these parts of the plant are surface sterilised with different detergents (Tween20), disinfectants (commercial bleach or alcohol), fungicide (Bavistin and Trimethoprim), and then rinsed with sterile water (77, 102, 112).

II. After dissection from mother plant and sterilization, the explant is usually placed in specific containers for in vitro cultures on an agar-based medium at room temperature (22-27 °C) for a period ranging between one week to 1-3 months, under fluorescent white lamps with a photoperiod in general of 16 h light/8 h dark at dark at a light intensity range of 20–100 $\mu E \cdot sec^{-1}$ m^{-2} (1 $\mu E \cdot sec^{-1}$ $\cdot m^{-2}$ = 6.02 × 10^{17} photons$^{-1}m^{-2}$ =

μmol·sec^{-1}m^{-2}; full sun is approximately 2000 μE·sec^{-1}m^{-2}; 77, 91, 102). The composition of the culture medium and growth regulator content are crucial and will change according to the plant species, multiplication method and type of used tissue (88). The MS medium is widely used, but for many plant species it is toxic and should be decreased to half strength or less (107). Moreover, it contains growth regulators, mainly cytokinins (6-benzylaminopurine, BAP, the more used), at a concentration range between 1 to 2 mg L^{-1} (89, 91, 102). The most used auxins are indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA), usually in a concentration from 0.1 to 1 mg L^{-1} (77).

III. Afterwards, many clonal propagules, deriving from tissue masses by sub-culturing in media that improve explant proliferation, are generated; this process is largely influenced by combinations of growth regulators in the medium (101). For instance, a high ratio of cytokinins encourages auxillary or adventitious shoot multiplication, whereas a higher auxin level leads to callus proliferation and to somatic embryogenesis (95). This stage can potentially last for an unlimited period of time, but commonly it keeps going from several months to 1-2 years, and the stock culture is continuously renewed to avoid the loss of regeneration potential of the culture (77, 97). During this phase the same medium composition, growth regulators and controlled conditions of the aseptic initiation culture stage are used.

IV. After many cycles of subculturing, the subculture is carried out for a screening of microbial contaminations and the plantlet is transplanted to the last *in vitro* stage: the rooting stage, in which it is stimulated the organization of a completely developed plant (95, 113). Sometimes *in vitro* shoots from the previous stage, can remain too short and have to be

exposed to an elongation step before rooting on a poor cytokinin medium (88, 91). A single shoot is transferred to a cytokinin-free medium containing auxin, reduced sugar levels and increased light intensity (77, 91, 95). Rooting stage follows a phase of induction characterized by cellular activation, orientation, organization and rooting that culminates with the formation of the first root (104). In order to reduce the rooting cost of micropropagation, it is used *in vivo* rooting in which micropropagated shoots are treated with commercial rooting powder (auxins) such as micro-cuttings and transplanted into a soil mixture (91, 114).

V. The transplantation of the plants in a soil mixture and acclimatization is another key stage for the success of micropropagation due to the fact that *in vitro* plants were under artificial conditions characterized by high levels of organic and inorganic nutrients, sucrose, hormones, high humidity, few gaseous exchanges and low light intensity (91). In these conditions, plants could grow well, but they suffer of many physiological and anatomical abnormalities, like a reduced ability to control water loss and heterotrophic nutrition mode (77, 115). Moreover, in conditions of high humidity, leaves have tiny wax deposition, less cuticle development and large stomata (89, 91, 102). For these reasons, the transplantation *in vivo* is needed to correct abnormalities (116, 117). *In vitro* plants harden for 4-6 weeks, then plants, from low light heterotrophic conditions and high humidity, move on to high light autotrophic conditions and low humidity (91, 108). Each micropropagated plant is pulled out from the culture medium, the roots are washed and seedlings are put into pots filled with a light soil mixture (118). Plantlets are covered with drilled plastic containers to maintain high humidity in low light conditions for about

twenty days until a complete acclimatization, usually when new roots and leaves are grown (95, 119).

2.3 - Micropropagation: advantages and disadvantages

In comparison to conventional vegetative propagation techniques, micropropagation provides many advantages in terms of economics and quantity and quality of the produced material. (77, 94). Some of the advantages can be the production of many large number of plant clones in a small space and in a short time (101), the possibility to eliminate bacterial, fungal and virus contamination from the newly produced plants (90, 120), the production of a big stock of true-to-type clonal plants assuring a high degree of plant characteristics like size, shape, flower colour, concentration and presence of specific metabolites, and in the end the possibility to breed and select new plant varieties with specific plant traits (118). On the other side, there are some disadvantages correlated with this technique, including frequent mutations, the difficulties to propagate woody plant species, internal infection, vitrification, toxic exudates, high levels of ethylene and CO_2 production, a high mortality during the transfer from *in vitro* to *in vivo* acclimatization (116), high costs of production (121). Surprisingly, most of the economic losses comes from endogenous contamination of plant cultures (77), but the main problem remains the somaclonal variation. This term is used to describe clonal micropropagated plants that show a wide range of genetic and epigenetic variations (97, 103, 122).

3 - Arbuscular Mycorrhizal Fungi (AMF)

The mutualistic symbiosis between some soil fungi and plant roots is known as "mycorrhiza"; it is classified in two types: ecto- and endomycorrhiza. Ectomycorrhizal fungi never penetrate inside root cells of the host plant (123), whereas endomycorrhizal hyphae go beyond the cell wall and enter in contact

with the plasmalemma of the plant cell (124). Endomycorrhizae are classified in five groups: arbutoid, ericoid, monotropoid, orchidaceae and arbuscular mycorrhizae. Arbuscular mycorrhiza represents the most widespread symbiosis in the whole world, involving about 90% of terrestrial plant species (125, 126) and fungi belonging to the subphylum *Glomeromycotina* (127). Arbuscular mycorrhizal fungi (AMF) coevolved along with plants for the last 450 million years (128) because of their crucial role in nutrient transfer toward plants, thus allowing land colonization by these latter (129). They cannot complete their life cycle without a host plant, and for this reason are considered obligate symbionts (130). AMF show several peculiar biological characteristics in addition to their obligate biotrophism, namely, coenocytic hyphae with thousands of nuclei inside their cytoplasm and spores; moreover, an uninucleate stage of life has been currently observed (129). A single spore can contain up to 35.000 nuclei (131) and these fungi would seem to have lost sexual reproduction (129). Spores, that are present in the soil, germinate because of specific exudates, secreted by plant root, stimulating the growth and hyphal branching (132-134). These compounds are usually released by roots when the plant is stressed by different conditions, particularly low bioavailability of phosphate in the soil (135). The pre-symbiotic mycelium perceives the host plant presence by these compounds, all of whom have a short distance effect before degrading themselves; they are named strigolactones, and stimulate fungal metabolism and hyphal branching (132, 134, 136). Hyphal growth is supported by the catabolism of spore lipids for few days (134, 137, 138), during which hyphae search the host plant because root colonization is crucial to complete the AMF life cycle (129, 134).

However, it is well known that AMF are also active during the pre-symbiotic stage (134): in fact, a cross-talk communication between the partners precedes root colonization (139, 140). This molecular dialogue takes place through small lipophilic bioactive molecules, known as “Myc factor” (129, 141). These signals

are perceived by plants even if there is not a physical contact between the two symbionts (134, 142, 143). The plant responses to Myc factors range from molecular to organ level, that reprogram and predispose the plant development for the mycorrhizal symbiosis (134). Some comparative studies showed a symbiotic signalling pathway, named SYM, which involves essential genes for the symbiosis (144, 145). This pathway is shared with another symbiosis between root plant and rhizobia, whereby Nod factors are involved (146). In this direction, there is huge evidence that the same genes are responsible for different parasitic, pathogenic and symbiotic relationship (147). The symbiosis pathway seems to be controlled by the most widespread second messenger in the eukaryotic cells, calcium ion (Ca^{2+}), in fact after the exposure to strigolactones it has been observed an increase of the Ca^{2+} concentration in the cytoplasm of the fungus (148; Figure 6).

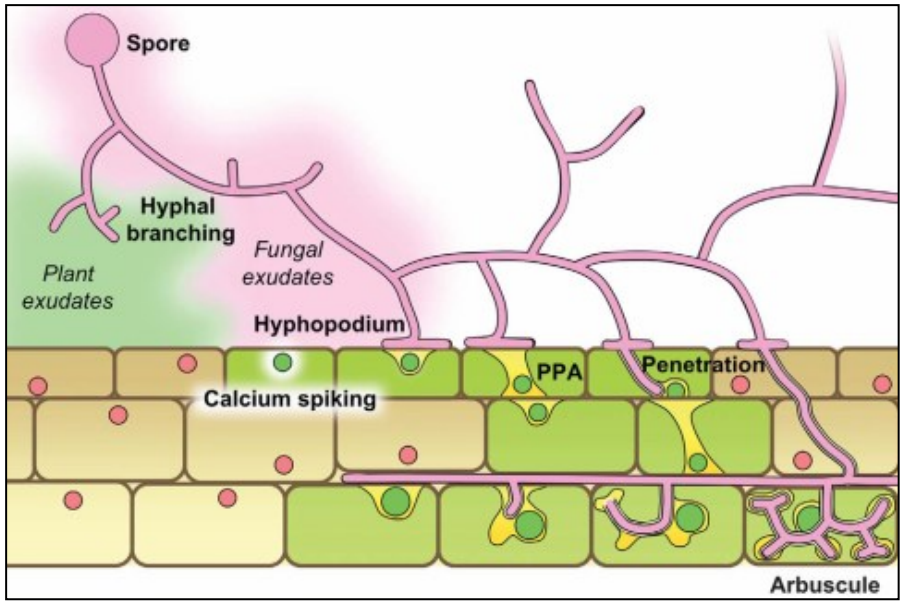

Figure 6. Representation of spore growth, fungal penetration, and arbuscule formation in root cells, underlining calcium variations after root contact with fungal hyphae. *Source: Lanfranco et al. (129*).

However, some variations in calcium concentration in the root epidermal cells after contact with the fungus hyphae have also been observed (129, 149), but strigolactone receptors of the fungus remain unknown yet (150). Probably, when the hyphopodium touches the epidermidis surface of the root, additional exchanges of chemical signals occur (140). When a hypha gets in touch with a root surface the pre-symbiotic phase ends, then it can swell, flatten on the cell wall and develop a hyphopodium (151). Young lateral roots are the primary colonization site of AMF (134, 150). The epidermal cells, which are in contact with the hyphopodium, assemble the compartment where the fungus will penetrate; cytoplasm develops a route of hypha across the cell (134, 152). The fungus penetrates from root epidermidis to the cortical parenchyma of the root without occupying the conduction tissues of the plant (151, 152). Inside the epidermal cells of the root, a structural reorganization takes place: the nucleus migrates near the contact point of the hyphopodium with clusters of actin strands and endoplasmic reticulum cisterns are organized around it (129, 144). Concurrently, the nucleus migrates to an opposite direction leaving behind a column-like of cytoplasmatic material formed by microtubules, actin strands and endoplasmic reticulum cisterns: the Pre-Penetration Apparatus (PPA). It has the function to surround and isolate the entry-hypha from the cytoplasm of the cell (151, 152). Once the fungal hypha has crossed the epidermal cell, it colonizes the cortical parenchyma of the root forming a structure named “arbuscule”, a typical AMF structure that degenerates about three days after maturity (153). Arbuscule can occupy most of the cellular space without compromising the integrity of the plasmatic membrane (151), but the cortical cells envelope the arbuscule into a specialized membrane named “periarbuscular membrane” responding to the fungus invasion (154). The wide interface formed between the arbuscular membrane of the fungus and the periarbuscular membrane of the plant is the active site for the exchanges occurring between the two symbionts (155, 156):

plant supplies photosynthates to the fungus, while the latter improves water and mineral nutrient (especially phosphorus and nitrogen) uptake to the plant (129, 157-160). The most investigated and understood function of AMF is the transfer of phosphate taken up by extraradical hyphae (161) and delivers it to the plant in exchange for the carbon compounds derived from photosynthesis (138, 161; Figure 7).

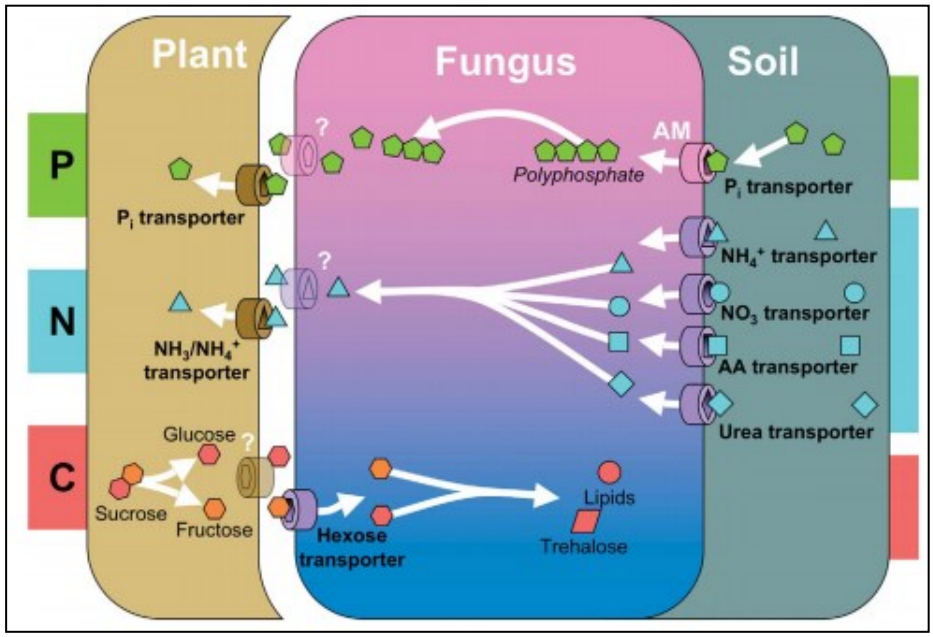

Figure 7. Schematic representation of plant-fungus nutrient trade-off. The figure shows the principal transporters of the main essential macronutrients from plant (carbonious compounds) to fungus, and vice versa (phosphorous and nitrogen). *Source: Bitterlich et al. (161*).

Thus, plants can generally acquire this element through a direct pathway (DP), transferring phosphate ions from the soil near the roots, or through a mycorrhizal symbiotic pathway (MP; 162). Therefore, phosphate availability is limited by a rapid immobilization in the form of free cations (163). Afterwards, a depletion area near the roots is generated, reducing the supply of available phosphate for plant uptake (163, 164). The network of AMF hyphae elongates over the depletion zone, gaining access to an extensive area of soil for phosphate uptake

(163, 165; Figure 8). So, the mycorrhizal symbiosis may improve the use of phosphate by plant (166).

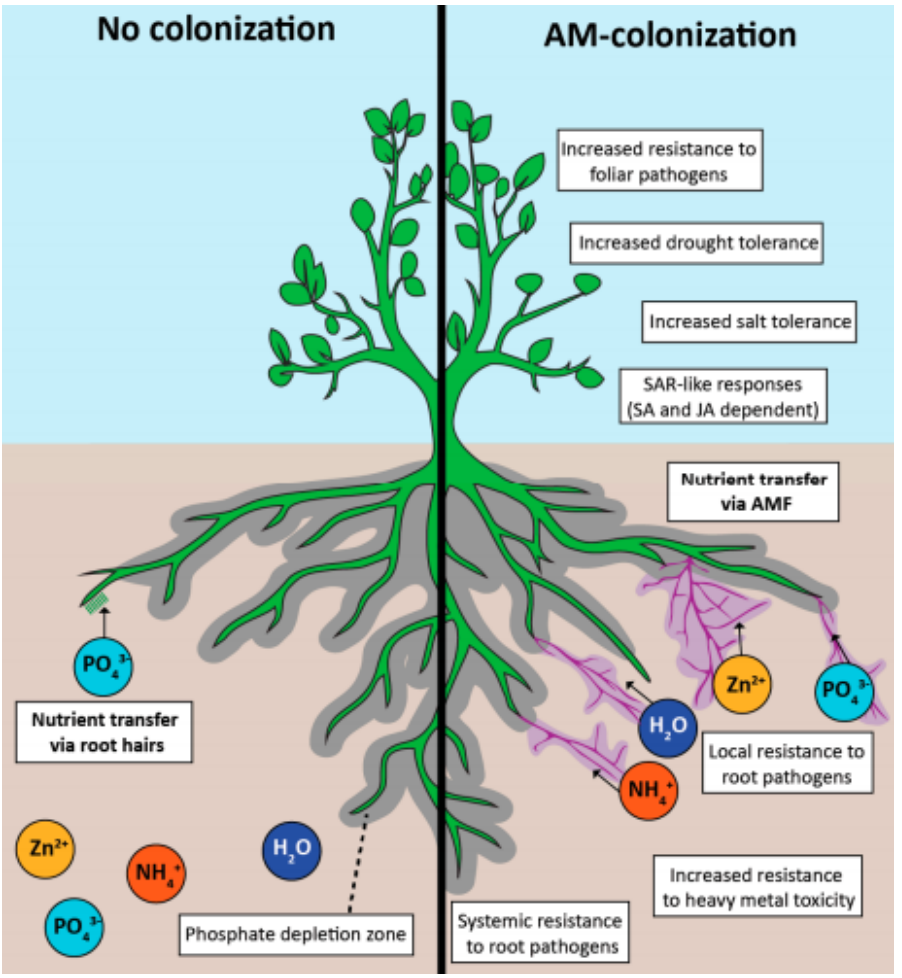

Figure 8. The figure shows the limited nutrient uptake by plant root, causing also by the phosphate depletion zone in non-mycorrhizal plants (left side), and the enhanced uptake of nutrients from the soil in mycorrhizal plants due to hyphal elongation beyond the depletion zone (right side). *Source: Jacott et al. (163*).

In support of what has been previously described, the periarbuscular membrane is equipped with specific transporters of phosphate, for instance STPT3 in potato plant (167) and MtPT4 in *M. truncatula* (168), by absorbing phosphate released into the periarbuscular space by the AMF (163). However, the fungus represents a remarkable cost in terms of carbon for the plant, and this aspect should not be

underrated (161, 169). In exchange for photosynthates, AMF provide mineral nutrients and water to the plant (138, 170). Moreover, fungal hyphae are more capable to stick into small pores of the soil compared to plant roots, due to their thinness (171). In this way, inorganic macronutrients (as P and N), micronutrients and water, can be transferred from the soil to the plant by the extending hyphae of the root outside, and the fungus receives carbon from the plant (172-174). As it has been formerly said, arbuscules are the exchange site of nutrients between the two partners (170, 175), so in case of scant nutrient supply that leads to a limited plant growth, AMF can ameliorate this limiting condition (176). Consequently, AMF are accounted to be the movers of soil elements to the plant, often immobile elements such as phosphorus, copper and zinc, which would not be available for plant uptake (177). In these limited conditions, plants can strongly profit by this symbiotic relationship (174, 178). AMF can also aid plants to grow under abiotic stress conditions such as drought (179-181), salinity (182-183), heavy metal contamination (185, 186) and in the presence of biotic stress such as plant pathogens (174, 187). This latter phenomenon is probably due to the activation of an immune plant system thanks to the fungal presence in the roots, as observed in a study on tomato plant attacked by *Phytophthora* ssp., where it was underlined a systemic effect of mycorrhizal symbiosis on plant resistance to pathogens (187-190).

Even if the arbuscular mycorrhizal symbiosis is rather aspecific, some differences in the colonization among the different AMF and plant species were observed (181, 184, 191). In fact, the recognition process between the two partners, and the following starting of the symbiosis, may be considered as a compatibility due to genetic factors (181, 192-195). AMF colonization can also be influenced by scion type (196), and different responses to different mycorrhizal fungus species were reported in several plant cultivars (181, 197-200). Moreover, many other factors can influence symbiosis, from climate conditions (180, 196) to the levels

of available nutrients in the soil (196, 201, 202); mainly high phosphate concentrations in the growth substrate can strongly inhibit root colonization by the fungus (177, 196, 203).

3.1 - Effects of AMF on plant growth

As previously mentioned, mycorrhizal symbiosis is built on a strong exchange of nutrients between the two symbionts: the fungus provides many mineral nutrient supplies to the plant that gives it the possibility to improve its own plant growth (163, 204). In many cases, it has been observed an improvement in plant growth with different plant and AMF species, the principle is based on a better uptake of nutrients, particularly phosphate, an element that is involved in many biological processes in plants (205). A higher availability of high energy compounds (like ATP) and substrates into plant cells can lead to an activation of the primary metabolism, which results in an increased biomass production and consequently to better plant growth performances (166). Many studies, on different plant species, showed that AM symbiosis can enhance host growth, for instance, in plant of *Medicago truncatula* (206), *Solanum licopersicum* (207), *Linum usitatissumum* (208), *Cucumis sativus* (204), *Triticum aestivum* (209), *Capsicum spp.* (210), and *Arundo donax* (211), if compared to non-mycorrhizal ones. In order to better explain the spreading advantages of mycorrhizae for plant growth, a recent paper has reviewed data of different plant species inoculated with AMF from 127 articles and 47 different scientific journals, published in the last 15 years (170). The experiments aided to observe the AMF effects in several growth conditions on species belonging to 43 plant families, including *Fabaceae*, *Asteraceae*, *Poaceae* and *Solanaceae*, sometimes even under abiotic stress, evaluating the mycorrhizal colonization in the roots, the root and shoot biomass enhancement, plant nutrition and yield increase. As a result, in the majority of the studies, a significant root colonization degree in comparison to non-

mycorrhizal plants was observed in 93% of the experiments. In the same way, an increase of root and shoot biomass in about 73% and 80% of the studies, respectively, in the inoculated plants were registered; and finally, mycorrhizal plants showed an enhancement in yield and plant nutrition after inoculation in 84% and 92% of the experiments, respectively (170, 212). Also studies on different aromatic plants, like basil (*Ocimum basilicum*; 213), oregano (*Origanum vulgare*; 214), mint (*Mentha piperita*, *Mentha arvensis*; 215, 216), sage (*Salvia officinalis*; 217) reported the positive effects of AMF on plant growth, leading to significant variations in plant biomass production (212). Regarding *A. annua* plant, the *Asteraceae* family is very responsive to mycorrhizal colonization, and in most of the available studies, an increase in plant biomass in presence of AMF was observed (19, 20, 23, 24, 30); whereas in other studies a neutral effect on *A. annua* biomass production when colonized by AMF was recorded (218). Likewise, in several studies AMF colonization did not lead to an improvement of plant biomass production and plant growth (163); highlighting different growth responses of plants colonized by AMF, in which neutral or negative growth variations can occur (163, 208, 219). For instance, in a study on wheat plants inoculated with ten different AMF species, a diminished growth in presence of all the AMF species was reported (220); this negative effect on plant growth was already noticed in other research on wheat (221, 222), barley (223) and tobacco (163). However, in some experiments, mycorrhizal plants showed a decreased growth in the early phases of development, but they still resulted to be able to complete their reproductive cycle (224). Instead, in other trials on tobacco plants, inoculated with AMF, the growth reduction was quantifiable in 50% comparing with the respective non-mycorrhizal plants, suggesting a trade-off between host-plant responses and fungal colonization (163, 225). For it is widely recognised that the symbiosis is based on the exchange of plant photosynthetic products for fungal phosphate (152), therefore it is

plausible that the amount of carbon supply in respect to the phosphate acquisition is crucial for the host fruition (163, 226). Perhaps the reduction in plant growth appears whether the cost of plant carbon derived from photosynthesis oversteps the advantage in growth obtained from the enhancement of phosphate uptake (227). It has been formerly stated that the MP way gave additional phosphate to mycorrhizal plants and the DP way was not subjected to the colonization influence (228). However, phosphate uptake through these two ways would be not completely additive (208, 219). Throughout the fungal colonization, the DP contribution to the phosphate uptake would be lessened and, in relation to the available phosphate amount and to the species and genotype of the plant and AMF, the MP way could not be enough to supply advantages to the plant and, as a consequence, there would be a complete reduction of phosphate uptake (208, 222, 228, 229). Moreover, in some studies, the phosphate transporters of DP way would be downregulated during the mycorrhizal symbiosis (230, 231), whilst in other studies it would not happen (223, 232). This conflicting interrelation, between DP and MP ways, could partially give an explanation to the decreased plant growth due to the AMF colonization, so highlighting some fungal species which do not provide suitable amount of phosphate in exchange for carbon produced by plant (233-235).

A number of studies showed that AMF ameliorate the nutrient reservoir of several vegetable crops, and this ability is governed by the plant and fungus genotypes (187); as it was broadly verified in a study on *Allium cepa*, where a significant enhance of phosphate uptake in inoculated plants, in comparison with uninoculated plants, was observed according to the different used species of AMF (187, 236). Even if the positive growth responses are ascribed to an enhanced phosphate uptake via MP, it could also emerge from an improving uptake of other limiting nutrients, for instance nitrogen (159, 162). In fact, nitrogen uptake through fungal hyphae was confirmed in several experiments

(237-239), and specific fungal transporters of ammonium were characterized in *Sorgum bicolor* (240) and *Lotus japonicus* (241). However, the importance of mycorrhizal symbiosis on plant uptake of nitrogen is not well understood like as the phosphate one, but the involvement of AMF in nitrogen plant nutrition would vary to a great extent depending on several conditions (129, 159, 242, 243). Therefore, growth promotion of the plants, due to mycorrhizal inoculation, has been observed in different species and cultivars (187, 244), but a non-responsiveness after the fungal colonization can also appear, like in cucumber (*Cucumis sativus*) and pea (*Pisum sativum*; 138). Instead, some crops like tomato (*Solanum lycopersicum*) showed a growth promotion, and the same host species can be likewise either non-responsive toward AMF colonization or suppressed in their growth (159, 187). These observations can be related to a different "symbiosis efficiency" (208, 245), intended as the capacity of the fungus to transport essential nutrients, like phosphate, from the extraradical mycelium (ERM) to the intraradical mycelium and to transfer them into the plant (155, 245), but also the capacity of the ERM to solubilize and absorb nutrients from the soil (246).

Results of some studies underlined that AMF showed a species-dependent and genetic-dependent host specificity (177). Several fungal isolates from diverse soils and geographic origins were partially re-sequenced in order to study the functional involvement of genetic diversity in AMF populations and to select further fruitful AMF for plants (170, 247). Other two aspects, which should not be underrated, are plant species and plant genotype that vary considerably in response to mycorrhizal inoculation (159, 170, 248, 249). This has been confirmed in a study on different cultivar of *Cucumis sativus*, where different effects on plant growth in respect to the same AMF species were observed (250), underlining the importance of the genetical compatibility aspects in this relationship. It has been observed that plant productivity increases in mycorrhizal

plants, as in tomato plants where an increase of fruit yield was recorded together with an earlier flowering and fruiting time compared to non-mycorrhizal plants (251-253). These latter effects on the plant phenology seem to be due to modifications of phytohormone levels, such as abscisic acid, jasmonic acid (254), auxin, ethylene and salicylic acid (255), throughout the AMF colonization (129). These hormones are involved in the establishment and functioning of the mycorrhizal symbiosis (256, 257). Therefore the modification of hormonal equilibrium and transcriptional profile in plants colonized by AMF, could likewise influence plant responses toward stresses (biotic and abiotic; 129, 258, 259). In the same way, symbiosis can decrease the negative effects of soil pathogens (260) and its effect suggests that the protective role of mycorrhizal fungi would not be plainly caused by an increased mineral nutrition, but an activation of systemic defence reactions (129, 261, 262). This latter hypothesis is strongly supported by studies in which an up-regulation of genes related to stress- and defence-responses, in mycorrhizal plants, endowed plants with high tolerance to shoot pathogens (263).

Root colonization by AMF also leads to a modification of the root architecture (264, 265). The total root extension can enhance, as observed in *Vitis vinifera* (266), or not, as in *Solanum lycopersicum* (267). Also, the length and the number of roots can change according to the symbiotic associations, with more frequently modifications in the lateral roots than in the main root (268). In fact, an increase in the development of the lateral roots is a common effect due to the mycorrhizal colonization (267, 269, 270) maybe to improve the AMF penetration sites (271, 272). Consequently, the root apparatus is highly branched, as noticed in a study on *Allium porrum* (273). The crucial role of mycorrhizal symbiosis in the development of lateral roots, was confirmed in studies on maize plants (*Zea mays*) deprived of the gene responsible for lateral root formation (231); while in *Medicago truncatula* plants, lateral root formation was induced by the AMF

spore germination (274). Further effects involve the root apical meristem (275), into the colonized tissues stele and the cortical tissue differentiation takes place near the apical tissues, so that a more rapid senescence pattern in the root apexes is observed, in comparison with not-colonized roots (273, 276). Moreover, meristematic cells show a fast mitotic cycle when the mycorrhizal colonization increases (277). An enlargement of the root apex, that leads to a thinner root (275), an improvement of root dry weight, and an enhancement of root branching (264) are all other effects due to the AMF root colonization (269). The causes liable to this event can be direct, including the fungal exudate action, and indirect, relating to a better mineral nutrition and hormone level balancing, both in mono- and dicots (268). In fact, it is widely recognised that mineral nutrients, like phosphorus and nitrogen, can strongly influence root morphogenesis, even if the contribution of AMF toward nitrogen nutrition is currently poor understood (159, 268). As previously described in the text, a precise exchange of signals between plant and fungus leads to the symbiosis (278), and these compounds also have a function as plant growth regulators, able to change root growth (279); as broadly demonstrated in *M. truncatula* plants treated with exudates derived from germinating spores of *Gigaspora margarita*, *G. rosea*, *R. irregularis* and *R. intraradices*, stimulated the development of lateral roots (274, 279). Furthermore, an increased supply of sucrose toward the root system was also reported in mycorrhizal plants (280, 281), highlighting that these effects can be ascribed to the fungus signals in order to obtain carbon from the plant (138, 281). Many plant hormones change in their own levels during the establishment of the symbiosis, suggesting a regulatory role in this relationship (254, 255, 282) and an involvement in root morphogenesis (283-286). Data on these related-AMF changes in plant hormonal concentrations are few, sometimes, diametrically opposed to poorly correlated with root morphogenesis, and the involved molecular pathway is until now unknown (268). Auxin, for instance, has a

positive role in the regulation of root apical meristem size through promoting cell division, opposing to cytokinins, and in process of cell elongation along with ethylene (287); furthermore, it is also the chief governor throughout every step which succeeds one another during the lateral root development (288, 289). Given that mycorrhizal colonization causes an enhancement in root branching, the root architecture organization in roots colonized by AMF can be modified by an implication of auxin (255, 290, 291). This was broadly confirmed in many studies on different plant species in which modifications in root architecture in presence of AMF were positively correlated with increased concentrations of auxins, like indole-3-acetic acid (IAA; 273, 292) and indole-3-butyric acid (IBA; 293-296). Moreover, the auxin transport in plant and its regulation have an important role in the morphogenesis of mycorrhizal roots, involving different molecules that can modify auxin and PIN protein distribution and synthesis, as the following: sucrose (284), ethylene, citokinins, strigolactones (297), gibberellins (298), jasmonate (299), abscisic acid (300), nitric oxide (301) and flavonoids (302). Strigolactones deserve a separated speech, beyond their role in promoting the establishment of AMF symbiosis (129, 282, 303), it has been observed that they can contribute to modify root development, but they also can inhibit branching in the shoot (297, 204, 305). Both in monocots and dicots some genes involved in strigolactone synthesis have been isolated, and a reduced flux of auxin under the optimum needed for the formation of lateral roots has been ascribed to influence the development of these latter (304, 306).

3.2 - Effects of AMF on plant metabolism

Plants can produce a wide range of compounds that apparently do not seem related to its growth, named secondary metabolites (307), which derive from products of primary metabolism and have an important function for the adaptation of the species, such as protection to parasites, attractive features for

pollinators and seed dispersers, in plant-plant competition and plant microorganism symbioses (as formerly mentioned). Verpoorte (308) reported: "Secondary metabolites are compounds which act as a defensive role in the interaction of the organism with its environment for survival in the ecosystem and are restricted to particular taxonomic group". AM association can lead to a physiology alteration and variation of metabolic composition in plant leaves and roots (211, 309). AMF symbiosis noticeably modifies plant metabolism, both primary and secondary one, in the colonized roots (310); for this causing specific physiological variations in plant cells (311). It is an increase of nucleus size, and the chromatin inside these latter decondenses, due to an improved transcriptional activity (312); moreover, mitochondria become more numerous, migrating to the arbuscule structures (313; 314). Plastids move nearby arbuscules, rise in number, and stromules become more numerous, thus making a net structure around the fungus (314). Therefore, metabolic changes in the cortical cells of the root are triggered by these physiological variations, inasmuch more plastids and mitochondria can give rise to enhanced production of energy (from the TCA cycle) and plastid metabolites, like amino acids, fatty acids, carotenoids and terpenoids (260, 314). Likewise, in order to support the exchange between the arbuscule and the plant cell, sugar content in the cytosol improves, thanks to the increased photosynthetic activity in the aboveground of the plant (315-317). When these metabolic shifts have occurred, mineral nutrient (especially P) are exchanged from the fungus to the plant cell for amino acids, sugars (fructose and glucose) and fatty acids (315). For instance, in rosemary plants, it has been observed that some physiological responses to the AMF colonization were interrelated to plant metabolome modifications in the root (318). This highlighted that plant compounds with antioxidant purposes (polyphenols; 319) were correlated with rosemary plants inoculated with AMF, whereas other polyphenols were more affected in non-inoculated plants (318, 320). The same

former observation was found in Rivero et al. (321), whereby metabolite profile changes in tomato roots colonized by AMF were registered, highlighting the possibility of a potential bioactive metabolite increase in consequence of AMF symbiosis establishment (322). Therefore, in mycorrhizal plants, a series of changes in the amino acid production, oxylipid pathway activation, plant hormones, fatty acids, secondary metabolites, and sugar metabolism were globally reported (311, 317, 321, 323).

It is well known that root colonization by AMF can affect the production of secondary metabolites, such as alkaloids (324), phenolic compounds (325) and isoprenoids (19, 20, 30, 218, 326), but also vitamins, chlorophylls and carotenoids (327). Thus, secondary metabolism of the shoot strongly varies: AMF may lead to an enhanced biosynthesis of health-promoting phytochemicals (polyphenols, carotenoids, flavonoids, phytoestrogens) and a higher activity of antioxidant enzymes (30, 325, 328-330). In some studies, an enhancement of chlorophyll and carotenoid levels in mycorrhizal plants was observed, thanks to an increase of the photosynthetic activity (327, 331, 332). This event not only improves host-plant photosynthetic activity through the enhanced content of chlorophylls, but it also affects the development of the photosynthetically tissue (333). On the contrary, some studies revealed no differences in the photosynthetic apparatus in plants inoculated with AMF (334), so it could be presumed that mycorrhizal colonization alone does not directly impact on the above-mentioned photosynthetic system. In other studies, higher carotenoid concentrations (lycopene and β-carotene) in fruits of tomato plants inoculated with *Glomus* sp., were considered due to the above-mentioned photosynthetic activity enhancement (327, 335). AMF colonization can also increase the nutritional fruit values by modifications of plant metabolomic profile, as reported in many papers (336-339). Furthermore, the use of AMF inocula to improve the production of secondary metabolites in plants is favourable because it contributes to sustainable

agriculture, reducing the use of chemical fertilizers (340). Mycorrhizal plants show different altered metabolite profiles, both quantitatively and qualitatively, in comparison to non-mycorrhizal plants (341-346). In a study on *Coriandrum sativum*, for instance, inoculation with AMF increased the monoterpene α-pinene in a significant manner, if compared to non-mycorrhizal coriander plants (326); whereas in another study on two species of *Mikania* genus (*M. laevigata* and *M glomerate*), belonging to the *Asteraceae* family, a four-time increase of some terpenoids (such as diterpenes) in plants inoculated with the fungus *R. irregularis* was observed, but the effects varied according to the plant species (345). Regarding this latter aspect, specific secondary metabolites can be subjected to an increase of their own levels according to plant species and plant genotype (311).

Speaking of this last assertion, Schweiger et al. (347) noticed that different plant species, such as *Poa annua*, *M. truncatula*, *Plantago lanceolate*, *P. major* and *Veronica chamaedrys*, inoculated with *R. irregularis* had different metabolome responses and showed a species-specific enhancement of some secondary compounds. On the other hand, different AMF species may also result in diverse effects on plant secondary metabolite levels, as it was reported in experiments in which *Funneliformis mosseae* induced a high degree of metabolic changes comparing to *R. irregularis* (321). Many data are available in regard to AMF effects on the secondary metabolites production in medicinal and aromatic plants (20, 214, 217, 326, 348-354). Members of the *Asteraceae* family, like *A. annua*, easily establish symbiosis with AMF (345, 355-358). Concerning *A. annua* plant, few studies were reported, yet the vast majority of them reported an increase in artemisinin production (19, 20, 23, 29-31) and antioxidant enzymes (30); whereas, in some studies, significant changes in terpenoid and artemisinin content were not observed (23, 359). Moreover, it has been observed a correlation between an increase of reactive oxygen species (ROS) and an improvement of

secondary metabolite production, suggesting that in *A. annua* the dihydroartemisinic acid had a scavenger function converting itself to artemisinin (360). However, ROS enhancement has been also observed in mycorrhizal roots (361), so that artemisinin improvement could be due to a plant defensive response against the fungus (20, 190). Nevertheless, mycorrhizal symbiosis can potentially negatively influence terpenoid yield reducing shoot biomass, as it has been observed in Rapparini et al. (218) in which fungal colonization had a neutral effect on terpenoid concentrations, otherwise reducing plant shoot biomass. Terpenoid accumulation in consequence of AMF symbiosis establishment was associated with different processes: plant morphology modifications (19, 24, 344), phosphorus bioavailability (362), and terpenoid pathway related-genes (24, 344, 363). Plant morphology can be altered by mycorrhizal colonization because this latter is associated with changes in density of leaf glandular trichomes (351, 364), and since terpenoids are stocked inside these structures, a positive correlation with the increase of terpenoid contents in the leaves has been observed (19, 24). In Kapoor et al. (19) a close correlation between trichome density and artemisinin contents in the *A. annua* leaves has been showed, so this factor could be significantly crucial to contribute terpenoids accumulation due to AMF; yet the increased trichome density is subjected to the specificity and compatibility between the two symbionts (19, 364). Furthermore, only some terpenoids are contained into trichomes (327, 365), instead carotenes and diterpenes are stocked into leaf (327), fruits (336) or roots (365).

Mycorrhizal symbiosis can result in an improvement of phosphorus bioavailability, and this could be an important mechanism that leads to a major terpenoid accumulation (362). In fact, in order to build isoprenoid precursors (IPP and DMAPP), phosphorus is needed, likewise phosphorus related-factors as adenosine triphosphate (ATP), acetyl-CoA, and nicotinamide adenine dinucleotide phosphate (NADPH; 366). The increase of phosphorus plant

concentration was observed in many mycorrhizal plants (362, 367, 368) and, in some studies, a positive relationship between terpenoid and phosphorus content was reported, both in quantitative and qualitative terms (24, 336, 350). It has been reported that mycorrhizal colonization positively influences the uptake and flow of phosphorus, but also the P transporter expression (369). However, phosphatic nutrition alone is not sufficient to comprehend and explain the enhanced accumulation of terpenoid compounds in plants colonized by AMF (362). In fact, in a study on *Coleus forskholii,* which produces forskolin (a diterpene used for lowered intraocular pressure; 370), inoculated with two different *Glomus* sp., an increase of forskolin was observed, but it was correlated to an improved phosphorus concentration in plants inoculated with one fungus species, while with the second fungus species it did not happen (368). Therefore, other phosphorus independent mechanisms could be involved in the AMF related-terpenoid enhancement, highlighting the possibility that isoprenoid precursor production is controlled by a multiplicity of processes independently to the phosphatic nutrition (371, 372). As formerly mentioned, in order to synthesise IPP and DMAPP from the MEP pathway two enzymes are needed: 1-deoxy-D-xylulose-5-phosphate synthase (DXS) and 1-deoxy-xylulose-5-phosphate reductoisomerase (DXR; 363, 373) and, in many cases, improvements in transcription of these two enzyme genes associated with plant terpenoid enhancement were registered (24, 363, 374-376). It has been demonstrated that genes, involved in the isoprenoid pathway, are upregulated in consequence of mycorrhizal colonization and interrelated with improved terpenoid plant production (24, 344). This increased gene expression induced by AMF has been ascribed to a higher availability of mineral nutrients (344) and a higher jasmonic acid levels in the plant (24, 377). MEP gene expression can be influenced by several range of factors: photoperiod, light intensity, temperature, abiotic and biotic stress, and circadian cadences (378, 379). Furthermore, it is a metabolic

cross talk between the plastidic (MEP) and the cytosolic (MVA) isoprene pathways, that can partly influence the isoprene biosynthesis (380). Considering the wide range of influences which the MEP pathway can undergo, the existence of different DXS isoforms has been inferred, resulting in the overproduction of various terpenoids (24, 363).

In some cases, the mycorrhizal colonization can negatively influence the terpenoid production through the expression of DXS isoform genes that lead to decreased levels of specific related compounds, as reported in a study on *Medicago truncatula* in which the inoculation with *R. irregularis* improved the expression of DXS2, a DXS isoform that was negatively correlated with carotene content (363). However, in *Ipomoea batata* colonized by the same fungus, an improvement of carotene content comparing to non-mycorrhizal plants was reported (365). Surprisingly, in Giovannetti et al. (336) the lycopene content was not associated with DXS2 expression, and significantly increased in tomato plants inoculated with the former fungus according to a significant improvement in phosphorus content. So that it is not completely clear if higher contents of these compounds are ascribed to a better plant nutrition or to gene expression switches; alternatively, it has been considered that different AMF species could have a range of broadly different effects depending on the plant species (353, 362). Recently, an influence on genes that encode for enzymes downstream of the MEP pathway in consequence of AMF-plant symbiosis has been observed (344), and this could support a theory in which a strength specificity between the two partners is needed (20, 23). Regarding this latter aspect, a great genetic fluctuation related to the origin of AMF has been already shown through metagenomic analysis (381). In fact, the different AMF genotypes derived from different climatic and environmental conditions and their use can have limited effectiveness far from their own ecological niche, resulted in reducing capacity to establish symbiosis with non-native plant species (245, 362). Divergences

among the transcriptional profiles of AMF species in several taxa were recorded (206, 245, 382); so that terpenoid improvement can be simultaneously connected with a better plant nutrient uptake and variations in the gene transcription levels of the terpenoid biosynthetic pathway (24, 344). The differences in terpenoid production due to mycorrhizal colonization would result from several genetic differences among the genotypes of AMF species (383). Therefore, plant species and genotypes, climate and environmental conditions, AMF species and genotypes, and the host plant-fungus genetic compatibility can be key factors in the AMF-related accumulation of terpenoids (362). Recently, some studies have showed a species-specificity in the metabolic responses of the leaf related to the used AMF (330, 347).

4 - Plant Growth Promoting Bacteria (PGPB)

Plant growth promoting bacteria (PGPB) are a group of rhizospheric bacteria able to improve plant growth (384, 385). In the soil there are naturally present microorganisms, and it was estimated that bacteria represent the vast majority of them (about 95%; 386), but only a few soil bacterial cells are cultivable *in vitro*, about 1% (387). Soil conditions, like moisture, salt, other chemical substances, temperature, number and type of plants in the site can strongly influence the number and the type of bacteria in a specific soil (388-390). Rhizosphere is the part of soil near the plant roots and this zone has a large number of bacteria, from 10 to 100 times higher than that found in bulk soil (391, 392). Around the roots, a high concentration of soil bacteria is found due to the root exudates released by plants (393-396). The main compounds in root exudates are sugar (fructose), organic acids (citric, lactic, succinic, malic, oxalic, pyruvic, aliphatic and aromatic acids), photosynthates (397, 398), polyamine (399) and a wide range of insoluble substances (cellulose, protein, lignin) derived by root cell exfoliation (397, 400). Root exudates are used by microorganisms as a nutrient source for its

own growth (384, 401) and play a key role in indirect plant-bacteria interactions (387). On the other hand, organic matter decomposition, essential element recycling, plant growth regulators, root growth stimulation, soil fertility, plant pathogen biocontrol, degradation of organic pollutants, mineral nutrient solubilization and vegetation changes are all mechanisms involved in the plant growth promotion by PGPB (386, 402, 403). The use of PGPB as biofertilizer could be a good opportunity to reduce the amount of chemical fertilizers and thus economically advantageous, beneficial for the environment and convenient to switch toward a sustainable agriculture (404-407). PGPB include either bacteria that live inside plants, directly exchanging with them nutrients and metabolites, or free-living bacteria which live surrounding plant roots (384). They have been classified in intercellular-PGPB and in extracellular-PGPB (408). The first ones are endophytes and mostly reside in the intercellular spaces of the host plant and can also penetrate into plant cells forming specialized structures in the roots, named nodules (387, 390). They belong to the *Rhizobiaceae* family that includes different genera, such as *Allorhizobium*, *Bradyrhizobium*, *Mesorhizobium* and *Rhizobium*, endophytes and species like *Frankia* (409). The second ones are present in the rhizosphere or in spaces between root cell cortex, and they are represented by the genera *Agrobacterium*, *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Caulobacter*, *Chromobacterium*, *Erwinia*, *Flavobacterium*, *Micrococcus*, *Pseudomonas* and *Serratia* (410).

4.1 - Effects of PGPB on plant growth

It is widely recognised and proven that PGPB inoculation result in an improvement of plant growth on a wide range of plant species (384, 411, 412) even in the presence of several stressful conditions (387, 413). These treatments improve seed germination percentage, seedling vigour, root and shoot growth, plant biomass, leaf area, chlorophyll content, weight of seeds, flowering, grain

and fruit yield (414-418). For instance, seed bacterization of different plants (ornamental and crop plants) led to increased plant growth and to resistance to disease (419). Many studies highlighted this phenomenon, in vegetable crops (both in greenhouse and field experiments). PGPB inoculation led to an enhancement of plant productivity (420). For example, in broccoli plants inoculated with *P. fluorescens* (strain MTCC103) in greenhouse conditions or with *Brevibacillus reuszeri* and *R. rubi* in field conditions, an increase of broccoli yield, plant growth and productivity was recorded (421, 422). In lettuce plants, *Lactuca sativa*, an early seed germination, increase of leaf dry weight, leaf area, number of leaves, seedling height and root length were observed when inoculated with *A. brasilense*, also when plants were grown in the presence of sodium chloride (423-425). An enhancement of root and shoot weights, stem diameter, root length was recorded also in experiments on pepper plants inoculated with different *Bacillus* (426) and *Pseudomonas* (427) strains, even under severe drought conditions (428). Instead on fruit crops, as reported in several studies either in greenhouse or field conditions, the most used strains of PGPB belong to *Pseudomonas* and *Bacillus* genera that determined the best positive effects, like enhanced yield, weight and quality of fruits, especially on apple (429-431), apricot (432, 433), banana (434), cherry (435), grape (436), hazelnut (437), kiwifruit (438), and strawberry plants (253, 328, 339, 439). Moreover, PGPB can positively influence flower and ornamental plant growth, including members of *Asteraceae*, like *Chrysanthemum*, *Dahlia* (440)*, Zinnia* (441); *Solanaceae*, like *Petunia* (442); *Iridaceae*, like *Gladiolus* (443, 444); *Geraniaceae*, such as *Pelagornium* (440); and *Oleaceae*, like *Jasmine* (445). The major effect of PGPB inoculation has been an enhancement in the flower number per plant, but in some cases even an increase of shoot and root weights was observed (446, 447). Furthermore, PGPB can be used to reduce the transplantation stress in ornamental plants, causing a significant reduction of necrosis, leaf abscission and more

tolerance toward drought stress (387, 448). PGPB have positive effects also on the root system, resulting in morphological changes that lead to root elongation and proliferation improving the plant capacity to find water and nutrients in the soil (449). However, different effects on plant growth have been observed, as reported in a study on two cultivars of *Oryza sativa* var. *japonica* (450) inoculated with two PGPB strains of *Azospirillum* sp: the plant growth responses varied according to the cultivar and bacterial strain. On one hand, these observations are supported by other studies in which differential varietal response in many crops (e.g. corn, sorghum, rice and wheat) has been reported (451-453). On the other hand, after inoculation of one cultivar with several PGPB strains, different effects on plant growth were also reported (454). According to these works, the results of the bacterium-plant interaction depend on the plant cultivar and PGPB strain combination (450, 455). Also in medicinal and aromatic plants, PGPB inoculation can positively affect plant productivity (352, 456-458); nevertheless, few reports on *A. annua* plant are present in the literature. In Awasthi et al. (23) *A. annua* plants showed a higher productivity of biomass and nutrient uptake when inoculated with two PGPB species, such as *Bacillus subtilis* and *Stenotrophomonas* spp., compared with non-inoculated plants. Moreover, in different studies of Arora et al. (25, 26), on plants of *A. annua* inoculated with *A. chroococcum*, an increase in plant height, plant biomass production (both in root and shoot), and either in fresh or dry weights was observed, also in severe soil salinity conditions (200 mM NaCl; 459).

4.2 - PGPB mechanisms of action

PGPB can generally ameliorate plant growth in a direct way, often due to their capacity to improve plant nutrient uptake and/or influencing plant hormone balances, or in an indirect way through the diminishing of the injurious effects of plant pathogens, thus playing a role as biocontrol agents (Figure 9; 386, 409).

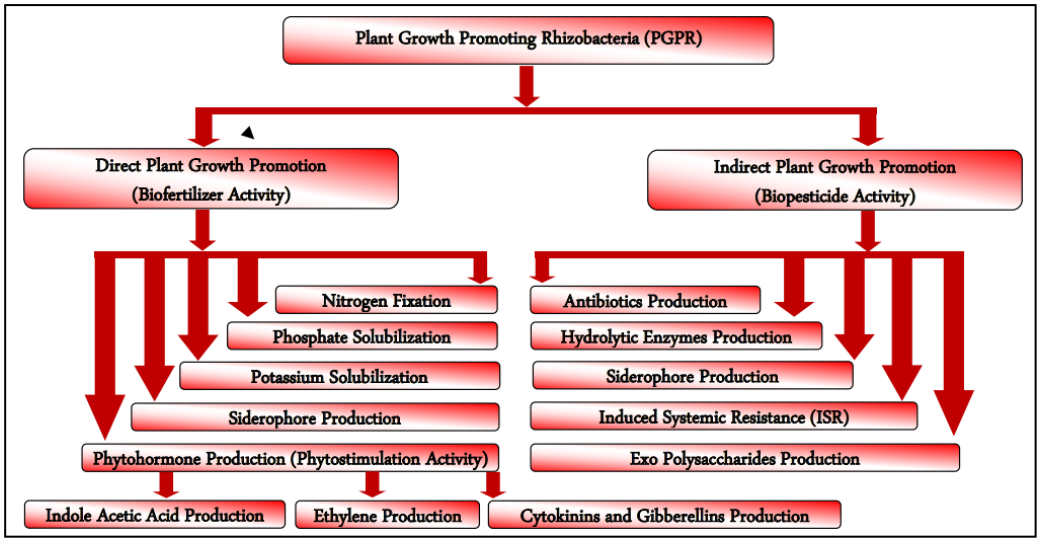

Figure 9. Schematic representation of the principal direct and indirect mechanisms of action with which PGPB affect plant growth. *Source: Gupta et al. (409)*.

4.2.1 - Direct mechanisms

An improvement of nutrient availability and uptake for plants, is a common direct mechanism in consequence of PGPB plant inoculation (407), due to the capacity of these microorganisms to do solubilization of mineral nutrients (460), nitrogen fixation (461, 462), mineralization of organic compounds and phytohormone production (463, 464). Plant growth and productivity strongly depend on an essential element, nitrogen (N), and even if it represents the most abundant element in the atmosphere (78%), it is not available for plant uptake (164). Some microorganisms are able to fix atmospheric nitrogen to ammonia, a useful form for plants, using a specific bacterial enzyme named nitrogenase (384, 465). PGPB can supply fixed nitrogen to plants through two different relationships: one is symbiotic nitrogen fixation, and it is a mutualistic symbiosis between plant and bacteria whereby these latter penetrate inside the root and form nodules in which fixation of nitrogen takes place (387, 410, 466), as previously reported. Also free-living diazotroph bacteria can promote plant growth, as it has been observed in

radish and rice, and in this case we are talking about non-symbiotic nitrogen fixation (124, 420, 467).

Another key element for plants is phosphorus (P), formerly reported in the text, which takes place in most metabolic processes, like respiration, energy transfer, signal transduction, biosynthesis of macromolecules, photosynthesis (164, 468). However, the widely diffused form of P in nature is the insoluble form, that is not absorbed by plants (205, 469), because plants can uptake only the soluble form: monobasic (H_2PO_4) and dibasic (HPO_4^{2-}) phosphate (164). In this outline, PGPB also have the capacity to solubilize phosphate, thus increasing bioavailability of this nutrient for plant uptake (387, 384, 460), as reported in a study on rice with several *Pseudomonas* species (470). The mechanisms used by PGPB to solubilize phosphate involve the secretion of compounds, such as proton hydroxyl ions, organic acid anions (471), that can dissolve insoluble phosphate (407, 469). It can be divided in biochemical mineralization of phosphate through releasing of extracellular enzymes, and biological mineralization of phosphate throughout substrate degradation (386, 472). Among different genera of bacteria there are phosphate solubilizers: *Arthrobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Microbacterium*, *Pseudomonas*, *Rhizobium*, *Rhodococcus* and *Serratia* (473). Furthermore, solubilization and mineralization of phosphate capability can be present in the same bacterial strain (407), and when these bacteria are co-inoculated with bacteria that have different physiological abilities, for instance nitrogen fixation, the positive effects are more evident (386, 474, 475).

PGPB are also able to render available potassium (K), the third macro-element important for plant growth and development (409, 476). In general, it is immobilized into rocks and silicate minerals (477), and it is solubilized through the secretion of organic acids released from the bacteria (478, 479) belonging to

the genera *Acidothiobacillus*, *Bacillus*, *Burkholderia, Paenibacillus* sp. and *Pseudomonas* (476, 480).

Bacteria have acquired specific mechanisms by which they can bind to iron, in order to survive in a limited iron supply environment, in molecules with lower mass (about 400-1500 Da) and high affinity toward ferric ion, named siderophores (481-483). Iron is an important micro-nutrient for all the organisms, even if it is broadly distributed in the whole earth, it is not easily assimilated by both plants and bacteria because of ferric ion (Fe^{3+}), that is moderately soluble and thus poorly available for assimilation by the above-mentioned organisms (164, 484). Siderophores have been classified in three families, hydroxamates, catecholates and carboxylates, according to the presence of a specific functional group, leading to the identification of more than 500 diverse siderophore types (481, 485). They are involved in both direct and indirect benefits, as it will be later shown. The direct benefits are registered on plant growth as reported in several studies, in which plants, cultivated in a substrate with radiolabelled ferric-siderophores as the only source of iron, were able to absorb the iron-marker (386); or when bean plants inoculated with a *Pseudomonas* strain that produced siderophores, were grown in a limiting condition of iron and they showed decreased chlorotic symptoms and a high chlorophyll concentration in comparison with uninoculated bean plants (486, 487); or in *Arabidopsis thaliana* plants, in which an improvement in iron concentration into tissues and in plant growth were observed, as a consequence of plant inoculation with *P. fluorescens* strain that produced iron-pyoverdine complex (488). Moreover, siderophores can reduce the injurious stress on plants related to heavy metal pollution in the soil (387, 489). Many genera of bacteria can produce siderophores, such as *Aeromonas*, *Azadirachta*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Pseudomonas*, *Rhizobium*, *Serratia* and *Streptomyces* sp. (409).

PGPB can stimulate or influence plant cell proliferation, and thus plant growth, by the production of phytohormones, like auxins, cytokinins, gibberellins and ethylene (384, 482, 490). Auxin has a regulatory role in most plant development and growth processes (462, 491, 492), and the most famous and active form is indole-3-acetic acid (IAA) (493, 494), which is naturally present in plants influencing plant growth and development (495). About the 80% of soil bacteria can produce IAA near or inside the roots (496), and it has been proposed that, together with the endogenous IAA, plant growth could be significantly modulated (497, 498). The plant can respond in a different way to IAA, according to the plant type and the involved tissue. The ideal IAA level in the root, for instance, is about five times lower than in the shoot in order to support plant growth (499), and the endogenous IAA of plant can change through the obtaining of IAA that is released by PGPB (386). If on the one hand, in the presence of low exogenous IAA levels the length of primary root increases, on the other hand high IAA levels lead to a decreased primary root elongation, improved formation of root hairs and lateral roots (494). It can also control vegetative growth, responses to light, gravity, fluorescence, pigment formation, biosynthesis of several metabolites, and resistance against stressful conditions (500, 501). Furthermore, bacterial IAA play an important role in the interactions between plant and bacteria, as reported in several studies in which an enhancement of auxin levels in plants was crucial for nodule development in roots colonized by most of all *Rhizobium* strains which have been observed to produce IAA (386, 502). The bacterial IAA is produced starting from tryptophan, usually located in the root exudates (503), the biosynthesis involves the indole-3-pyruvic acid and indole-3-acetic aldehyde pathways in most of bacteria such as *Pseudomonas*, *Agrobacterium*, *Enterobacter*, *Klebsiella*, *Rhizobium* and *Bradyrhizobium* (504), and also by free-living bacteria like *Acetobacter dizotrophicous*, *Alkaligenes faecalis*, *Enterobacter cloacae*, *Azospirillum* sp.,

Pseudomonas and *Xanthomonas* sp. that are all interrelated to low amount of released IAA (386, 409).

Many PGPB, like *Azotobacter sp.*, *Bacillus subtilis*, *Paenobacillus agglomerans*, *Pantoea agglomerans*, *Pseudomonas fluorescens*, *Rhizobium sp.*, *Rhodospirillum rubrum*, can synthesise and release cytokinins and gibberellins, alone or both, that stimulate plant growth (462, 505-507). Gibberellins (GA) influence many processes in plants, such as seed germination (508), growth of leaf and stem (509), flowering, fruiting (384) and shoot elongation (510, 511), as reported in a study on tomato plants inoculated with a gibberellin-producing bacterium (512). Instead, cytokinins mainly affect plant cell division (513), development and differentiation of vascular cambium, root hair growth (514), but impede the growth of primary root and the development of lateral roots (515, 516). Moreover, some phytopathogens can synthesize cytokinins too, but in higher levels than PGPB and, for this reason, the phytopathogens have an inhibitory effect on plant growth (386).

Ethylene, another important plant hormone, has a regulatory role in the plant growth cycle, like leaf abscission, fruit ripening (462). When its concentration is high, it inhibits root and shoot growth leading to senescence (517) and, thus, to low crop productivity (409, 518). Ethylene is biosynthesized by plants from 1-aminocyclopropane-1-carboxylate (ACC) in response to several stressful conditions, like drought, flooding, cold, pathogens and heavy metals (384). PGPB can degrade ACC, due to ACC deaminase enzyme, reducing the negative effects of high concentrations of ethylene in plants (519). So, in this way ACC deaminase bacterial strains can increase plant growth particularly under stressful conditions (87, 498). Different genera of bacteria show ACC deaminase activity, such as *Azospirillum*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, *Ralstonia*, *Serratia* and *Rhizobium* (409), only to mention some of them.

4.2.2 - Indirect mechanisms

Similarly to direct mechanisms, the indirect ones can lead to a better plant growth due to other PGPB capacities, such as antibiotic production, siderophores, volatile organic compound (VOC) production, hydrogen cyanide (HCN) or hydrolytic enzymes (520, 521). One of the major understood biocontrol mechanisms of PGPB against phytopathogens is antibiotic production (476). Many different antibiotics, such as amphisin, 2,4-diacetylphloroglucinol (DAPG), phenazine, oomycin A, tropolone, tensin, pyrrolnitrin, cyclic lipopeptides have been found in *Pseudomonas* genus (522), and others in *Bacillus*, *Streptomyces* and *Strenotrophomonas* genera, like xanthobaccin, oligomycin A, and kanosamine (523). These compounds are mainly effective against plant pathogens and, for instance, it has been reported that DAPG produced by *Pseudomonas sp*. reduces the disease related to the fungus *Gaeumanomyces graminis* in *Triticum aestivum* (524). Furthermore, some soil bacteria are also able to produce HCN, a volatile compound that can strongly contribute to biocontrol of pathogens, as reported in a study on tobacco plants attacked by *Thielaviopsis basicola* (525) or in another study on canker of tomato (526). VOCs released by PGPB, including several genera (*Bacillus*, *Pseudomonas*, *Serratia*, *Arthrobacter* and *Stenotrophomonas*) can significantly improve plant growth (384, 527) because they could operate like a biopesticides (528, 529). Some bacteria can directly destroy cell walls of pathogenic fungi, like *Botrytis cinerea*, *Sclerotium rolfsii*, *Fusarium oxysporum*, *Phytophtora sp*., *Rhizoctonia solani*, *Pythium ultimum* (530, 531) thanks to their ability to produce a wide range of enzymes, consisting of chitinases, cellulases, $\beta\beta$-1,3 glucanases, proteases, lipases (532). Recently, it has been observed that the use of a multi-strain inoculum was more effective to protect plants from pathogen infection, due to the synergistic effects of the used PGPB strains (533). In fact, in the absence

of sufficient amounts of available iron, fungal pathogens cannot survive (386). So since PGPB siderophores have a higher affinity toward iron, in comparison to phytopathogens (534), the latter are unable to proliferate owing to iron lack (516, 535). On the other side, plant growth is not negatively influenced by iron lack in the soil due to PGPB siderophores, probably because plants can grow at a lower iron concentration compared to microorganisms (164) and, moreover, they can utilize PGPB siderophores as an iron source (536).

Another indirect PGPB-related mechanism that positively influences plant growth is the activation of the Induced Systemic Resistance (ISR), generally defined as "a physiological state of enhanced defensive capacity elicited in response to specific environmental stimuli and consequently the plant's innate defences are potentiated against subsequent biotic challenges" (521). A significant damage reduction in plants attacked by fungal, bacterial and viral infections or by insects and nematodes, in the presence of PGPB has been observed (537). Some studies underlined that this resistance probably involves plant endogenous ethylene and jasmonate, that activate defence responses against many several plant pathogens (386, 462). Moreover, different bacterial components elicited the ISR, like siderophores, DAPG, homoserine lactones, acetoin, flagellar proteins, lipopolysaccharides (LPS) and cyclic lipopeptides, pyoverdine, chitin, and salicylic acid (538). Instead, components as exo-polysaccharides (EPS) can form a biofilm surface, mainly on the roots, protecting them against stress like desiccation (539), or plant defensive response to microbes (540). The former polysaccharides are produced by several PGPB, and are important as signal molecules (541), or in binding sodium cations (Na^+) into salinity stress conditions (482). Despite the above discussed mechanisms through which PGPB ameliorate plant growth, few reports have been attempted to explain the different effects of PGPB on plant growth, probably these organisms could be highly specific to plant genotypes, cultivars and species and/or influenced by

temperature, water content, oxygen, pH, soil and environmental conditions (418, 483, 542). It has been hypothesised that this specificity could be due to the components of root exudates (sugars, defence compounds, amino acids, vitamins and organic acids) which can either up- or downregulate the expression of useful bacterial gene (543, 544). So, both PGPB and plant mutually regulated the release of bioactive compounds: PGPB secretions can promote plant growth and plant exudates can drive growth, colonization and gene expression of PGPB (483).

4.3 - Effects of PGPB on plant metabolism

It is well known that secondary plant metabolism can be modulated by PGPB inoculation (545-547), even if the precisely involved mechanisms are not completely understood. Plant benefits, which have been previously reported in the text, are related to these microbes through direct and indirect mechanisms of action and that they can also elicit the ISR (458). Therefore, microbial colonization of plant tissues (internal or external) can induce plant metabolism shifts (547). Many PGPB (such as *Azospirillum*, *Bacillus*, *Pseudomonas* and *Streptomyces* spp.) have been largely studied and reported to have the potential to modulate plant secondary metabolite production (412). In many works, several species of these genera influenced phenolic compound production in different plants (548, 549). In Jain et al. (550), pea plants inoculated with *Pseudomonas aeruginosa* and *Bacillus subtilis* showed a significant higher content of gallic acid and other phenolics, and were more resistant to the same plant pathogen, if compared to control plants; furthermore, leaf flavonols (myricitin, quercetin, kaempferol) were subjected to an increased production only when pea plants were treated with both bacteria, and the same results were observed for salicylic acid (SA) content in the shoot. In other studies, the SA concentration significantly decreased in tomato plants inoculated with *Streptomyces lydicus* (551), highlighting that different bacterial strains can give rise to different plant

metabolic changes. Variations in plant phenolic compound composition and concentration could be related to specific plant-PGPB interactions, as reported in some studies on rice plants inoculated with *Rhizobium* (552) or *Azospirillum* (450), on maize plants inoculated with *Azospirillum* (548), and on grapevine colonized by endophytic bacteria (553). For instance, in rice plants inoculated with a specific *Azospirillum* strain, glycosylated flavone levels increased in the shoot of two different cultivars, whereas the same rice plants inoculated with another *Azospirillum* strain did not show any variations in the glycosylated flavone content (450). On the other side, only one rice cultivar showed a lowered content of feruloylquinic acid in the root when inoculated with the two different *Azospirillum* strains; this example highlights that changes in flavonoid content were related to the combinations of bacterial strain and plant cultivars (450). Modulations in the hydroxamic acids (benzoxazinoid contents) were also reported according to the combination of bacteria and plant host (458), as observed in experiments in which maize plants inoculated with one strain of *Azospirillum brasilense* had enriched in aglycone, while those inoculated with another *A. brasilense* strain had high content of glycosylated form of a typical benzoxazinoid of maize plant (548). Moreover, when plants were co-inoculated with different PGPB species, a marked increase in benzoxazinoid content has been noticed (554, 555). However, the exact components and plant target of microorganism elicitors are still unknown, and their identification could be crucial in order to trigger plant metabolism (412, 527).

Also alkaloid compound production can be modulated by PGPB: these substances are present in many families of plant (about 300; 556), but they are often related to a specific taxonomic group, as exemplified by steroidal glycoalkaloids in *Solanaceae* family (557). Instead in *Fabaceae* family, precisely in *Crotolaria* sp., plants are stimulated to increase pyrrolizidine alkaloids, a chemical defence against herbivores, when nodulated hereafter symbiotic

interaction with *Bradyrhizobium* and *Methylobacterium* genera, that belong to nitrogen-fixing bacteria (558).

Terpenes are another important and most diffused group of plant metabolites playing many roles in plant life, from defence to communication (556). In Banchio et al. (559) plants of oregano inoculated with *P. fluorescens* and *A. brasilense* showed an increased production of plant monoterpenes in the shoot, such as carvacrol, thymol, γ-terpinene and sabine hydrate. It has been later discovered that bacteria related to these two strains were capable to induce the expression of terpene plant synthase, driving to the accumulation of sesquiterpenes in the roots, and the same PGPB used these compounds as source of carbon metabolizing them into other ones (458). However, PGPB inoculation can have positive effects also on the fruit quality as a consequence of anthocyanin, chlorophyll, carotenoid, and vitamin enhancement, as reported in many studies on different crops (253, 328, 339, 352, 457); in general leading to an increase in antioxidant compounds as observed also in *Pisum sativum* (560), *Stevia rebaudiana* (561), *Glycine max* (545) and *Spinacia oleracea* (546). In a study on *Ocimum basilicum*, inoculation with *Bacillus subtilis* increased terpene accumulation (562), and many works showed that PGPB can increase secondary metabolite concentrations *in planta* determining a higher quality of plant material (563). In del Rosario Cappellari et al. (456), marigold (*Tagetes minuta*) plants inoculated with *A. brasilense* and/or *P. fluorescens* had different profiles of essential oil components, such as limonene, linalool, humulene, tagetone, ocimenone and β-ocimene, in the shoot. Banchio et al. (564) showed that plants of *Origanum majorana* inoculated with *P. fluorescens* or *Bradyrhizobium* had a higher total yield of essential oil compared to the control plants, probably due to an increased terpene biosynthesis. In another study, on *Anethum graveolens*, plants inoculated with *Pseudomonas putida* showed a higher content of carvone and a lower content of limonene, if compared with non-inoculated plants (565).

Regarding *A. annua* plants, a few studies have investigated the effects of PGPB on plant metabolism, particularly about artemisinin production (23, 25, 26, 459). In Awasthi et al. (23) inoculation with *Bacillus subtilis* and *Stenotrophomonas* spp. did not lead to an increase in artemisinin content in percentage, whereas significant differences were reported in artemisinin yield (grams per pot) in comparison to control plants. In other studies performed by Arora et al. (25, 26), a significant enhancement of artemisinin content in plants inoculated with *A. chroococcum* was reported, in comparison to control plants, also in presence of salinity stress (50-200 mM NaCl; 459). In some cases, the use of a consortium of bacteria is more effective to stimulate plant metabolism (566), as well as it is demonstrated in experiments on *Catharanthus roseus* inoculated with *Azospirillum brasilense* and *Pseudomonas fluorescens* (567), *Withania somnifera* inoculated with *Azospirillum*, *Azotobacter chroococcum*, *Pseudomonas fluorescens* and *Bacillus megaterium* (568); but also the combined use of PGPB and AMF is a good solution in order to trigger plant secondary metabolism (23, 418, 459, 569, 570).

4.4 - Mycorrhiza Helper Bacteria (MHB)

The belowground of plants is an environment where different organisms can interact with each other, so roots constantly interact with fungi and bacteria (571, 572). Mycorrhizosphere is defined as "the soil area influenced by the mycorrhizal roots and peripheral fungal mycelium" (573). However, some bacterial groups live in this area and they are able to stimulate mycorrhizal growth and development (29, 574), for this reason they are named Mycorrhiza Helper Bacteria (MHB; 575, 576). They belong to different phyla such as Actinobacteria (*Strepromyces* genus), Firmicutes (mostly *Bacilllus* and *Paenibacillus* genera) and Proteobacteria (*Azospirillum*, *Azotobacter*, *Bradyrhizobium, Burkholderia, Klebsiella*, *Pseudomonas*, and *Rhizobium* genera; 572, 577). Moreover, some

MHB are classified as PGPB, like several species of *Azospirillum*, *Bacillus* and *Pseudomonas* genera (578-580), but the great similarity between the species present in the same group makes it difficult to do a certain classification (581). Data on fossil records demonstrate that mycorrhizal symbiosis developed about 450 million years ago (582), but the discovery of bacteria involved in mycorrhizal symbiosis takes place from the observation that fumigation with methyl bromide improved or reduced infection of *Pinus radiata* by *Rhizopogon luteolus* according to different soil types (583). Later, several studies documented the capacity of bacteria to stimulate mycorrhiza formation in different plants, as in *Fagus sylvatica* in the presence of *Pisolithus tinctorius* (584) and *Hebeloma crustuliniforme* (585). Thus, it was consequently supposed that some helper bacteria adapted to live in a mutualistic relationship with fungi, so they probably were more abundant near the fungus, and they were isolated from *Pinus radiata* root system colonized by *Rhizopogon luteolus* (586).

MHB could stimulate mycorrhizal development during different stages of the root-fungus-bacteria interaction (576, 587, 588), always considered that mycorrhizal symbiosis is also influenced by fungus physiology, plant root susceptibility, biotic and abiotic factors (589). Several of these bacteria are able to influence mycorrhiza in many ways: influencing mycorrhizal symbiosis formation (590), modulating spore germination (591) and hyphal development (572), reducing negative environmental conditions (592), improving nutrient availability in the soil (576), and providing mycorrhizal nutrition (407). They can also be involved in the production of many compounds that stimulate root exudate release by plants, thus enhancing hyphal activity and root colonization by mycorrhizal fungi (593, 594). Secondary metabolites of MHB can trigger fungal spore germination leading to a fast mycelium elongation (595-597), as reported in a study in which *F. mosseae* hyphae development was faster in the presence of *P. fluorescens* (598). This growth effect has been attributed to many

metabolites like plant regulators or vitamins, for example IAA can promote AM symbiosis until a threshold concentration because this hormone inhibit hyphal growth at higher concentrations (599, 600). On the other hand, no effects of different IAA levels on *Rhizophagus intraradices* growth were observed, while *Paenibacillus* sp., that produces IAA, promoted hyphal growth of the same fungus (596), underlining that the interaction between the two partners may be not only due to the hormone production by bacteria (601, 602). AMF sporulation is a crucial phase in mycorrhizal plant development (603), and some bacteria could have a positive effect on this phenomenon, as observed in different indigenous AMF species in the presence of *Methylobacterium oryzae* on *Capsicum annuum* (604) or in *Glomus aggregatum* with *Bacillus polymyxa* in *Cymbogon martini* (605).

Other studies highlight the existence and the crucial role of compatibility and specificity of the interaction between bacteria and mycorrhizal fungi for having positive effects on fungal growth and sporulation (606-608). Also mycorrhizal colonization can be promoted (609, 610), as reported in Pivato et al. (598) in which *P. fluorescens* increased *F. mosseae* root colonization. In other experiments, it has been lighted the presence of molecules (flavonoids and Nod factors) interfering between mycorrhizal fungi and plant root communication, probably acting as fungal growth regulators, and hence leading to an improvement of mycorrhizal colonization in plant root (609, 611). Some of these compounds as reported to be gasses (612-614), one of them has been identified and named “auxofuran”, according to its auxin-like chemical structure (615). Moreover, it was identified a set of genes involved in the priming helper effect of bacteria on growth of its fungal partner (577, 616). Interestingly, some bacteria also have the capacity to inject effector proteins into eukaryotic cells of their partner by a syringe-like system named T3SS (617), but the types of bacterium effectors and the injection mode are still unknown. Nevertheless, mycorrhizal

fungi can produce toxic molecules to stop competitors, therefore bacteria are able to detoxify these fungal compounds (618, 619).

Another mechanism studied in MHB is their capacity to stimulate lateral root formation thanks to the production of auxin-like molecules, that can increase the number of sites where root-fungus interaction takes place (600, 614, 620), indirectly stimulating a higher mycorrhizal colonization. However, in some cases a helper bacterial strain can promote growth of either first or second order mycorrhizal roots, whereas another helper strain of the same fungus can only stimulate second order mycorrhizal root formation (600, 621), thus underlining that different helper strains could develop different traits even on the same fungus (618, 622, 623). Moreover, in an experiment with *Laccaria bicolor* co-cultivated with MHB strains, differences in the branching angles and hyphal density of apex were observed (616). In accordance with what has been before-mentioned, bacteria interact with plant roots earlier than fungi, thus MHB would release enzymes that digest root cell wall making infiltration points facilitating the extent of fungal hyphae inside the root tissue, as reported in a study with *Azospirillum brasilense* (572, 624). MHB also provide nutrient uptake to the fungus, such as nitrogen and solubilization of phosphate or iron (618). Furthermore, it has been hypothesised an increase in the mycobiont aggressiveness due to an enhancement of a phenolic fungal compound (hypaphorine) caused by MHB (625).

Physical contact with bacteria, for both roots and fungi, is crucial to carry on the stimulatory effect (587, 626). Metagenomic methods helped to find several bacteria associated with mycorrhizae (627), in some cases they highlight the presence of many unculturable bacteria, and that some culturable bacteria were modulators of plant symbiosis (628). Deveau et al. (629) sequenced the whole *Pseudomonas fluorescens* genome (strain BBc6R8) and data revealed that bacteria produce helper molecules in a constitutive manner; then, helper effect could be pleiotropic depending on trophic interactions (630, 631). Looking

among overexpressed genes of AMF toward MHB, tecnonin genes have sparked interest because they are well conserved, linked to organismic interactions, and associated to innate immunity (632). Tecnonins are a family of proteins (lectins), that could play a key role in the physical interaction, and hence in the recognition, between bacteria and fungus (629). Returning to the physical contact, in a work of Toljander et al. (633) it was noticed that different bacterial strains differed in their ability to attach on hyphae. It was proposed a mechanism made up of two steps: the first, consisting of a weak bind due to electrostatic attraction; the second is in relation to cellulose or bacterial polymer production (634).

In many studies, co-inoculation with bacteria and mycorrhizal fungi has been demonstrated very effective to modulate plant productivity and metabolism (25, 253, 339, 352, 609), as reported for instance in *Thymus daenensis* plants co-inoculated with *F. mosseae* and *Bacillus subtilis* (635) or in *A. annua* plants co-inoculated with three different AMF and two different bacteria (23), only to mention some of these studies. Usually, three major genera of bacteria are considered MHB: *Pseudomonas*, *Bacillus* and *Streptomyces*, as previously mentioned; some can have a positive effect, while other can have negative or neutral effect on the fungus (576, 592). Behind the bacterial genus, if bacterium has a positive, neutral or negative influence on mycorrhizal symbiosis, it is a consequence of its physiology and biochemistry (636). Co-inoculation with *Glomus deserticola* and *Azospirillum brasilense* in pepper plants, grown in poor soil conditions, resulted in a bigger plant size, in an improvement of nutrient uptake and in the production of more fruits, in comparison to plants non-inoculated or inoculated with one microorganism only (637). This fact demonstrates how the synergism between microorganisms can lead to a better plant growth. In several experiments performed on cucumber plants and *Gigaspora rosea* interactions, it has been registered that a specific strain of *Pseudomonas* (UW4) helped the root colonization of the fungus (638). Another

instance of synergism has been showed in tomato plants grown at reduced fertilization, in which plants restored plant productivity at levels comparable with those grown at optimal fertilization level, when inoculated with five different AM strains and one PGPB strain (639), also ameliorating sucrose fruit content and, hence, fruit quality (253). So, in addition to the improvement of crop productivity in mycorrhizal plants, MHB can also influence fruit quality through variations in secondary metabolites, as reported in studies on strawberry plants in which both AMF and MHB presence strongly increased the anthocyanin concentrations (328) and strawberry fruit quality (339).

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Chapter 2

Outline of the Thesis

The aim of this thesis has been to evaluate the effect of clonal variability and beneficial soil microorganisms on 5 different genotypes of *Artemisia annua* plant (var. Anamed), propagated *in vitro* by the micropropagation techniques, starting from seeds. The different clone plants were cultivated and inoculated with several beneficial soil microorganisms. The used microorganisms were PGPB: *Pseudomonas protegens* (strain Pf7) and *P. brassicearum* (strain SVB6R1) previously selected in our laboratory; and arbuscular mycorrhizal fungi (AMF): *Funneliformis mosseae* (BEG12, Biorize, Dijon, France), *Rhizophagus irregularis*, and a mix of AMF (*R. intraradices*, *R. aggregatus*, *Septoglomus viscosum*, *Claroideoglomus claroideum*, *C. etunicatum*, mixed in our laboratory). In a first phase different experiments were performed, in order to test clone stability *in vivo* conditions, and to evaluate the growth responses in the presence of microorganisms. The plants of each clone showed a low mycorrhizal colonization, and in some cases the co-inoculation with PGPB improved the root colonization by AMF, thus demonstrating their role as Mycorrhizal Helper Bacteria (MHB). Regarding the plant growth, different responses were observed, according to the used microorganisms and the plant clone. After this phase of clone selection, only the most stable one, both *in vitro* and *in vivo* condition, and another new clone were cultivated and inoculated with different combinations of the above-mentioned microorganisms. Since the low mycorrhizal colonization observed in the previous experiments, two samplings at different times (30 and 60 days) were performed, in order to monitor the mycorrhizal symbiosis trend over the time besides the plant growth responses. In this last experiment, the artemisinin production was also evaluated, using a HPLC method, for

investigating the differences in its concentration related to the different plant genotypes and the combination of the used microbes, also over the time. Furthermore, during the cultivation of plants, different smells among each plant treatment were noticed, mostly in one plant clone; therefore, a leaf volatile characterization by GC-MS analysis was performed with the purpose to investigate whether the leaf volatile profile varied in the presence of beneficial soil microorganisms.

Chapter 3

Materials and Methods

1 - Inocula propagation

1.1 - Arbuscular mycorrhizal fungi (AMF)

Sorghum bicolor L. seeds were washed three times (5 minutes each) in sterile deionized water, then they were sterilized for 5 minutes in a 20% sodium hypochlorite solution and washed three times (5 minutes each) with sterile deionized water, then imbibed in sterile water for 40 minutes. Seeds were pre-germinated in Petri dishes at 25°C for 72 h in the dark. Sprouted seeds were transplanted in a plastics pots (700 mL), which were previously sterilized in a 20% sodium hypochlorite solution. On the bottom of the pots a layer of quartz sand (size 4/5 mm) was used to have optimal drainage. The culture substrate consisted of a mixture of sand (50%) and inoculum (50%), finally the upper part of the pot was covered with an anti-algae layer of quartz sand (size 2/3 mm). The inoculum was formed by pieces of sorghum mycorrhized roots with the arbuscular mycorrhizal fungi: *Funneliformis mosseae* (BEG12, Biorize, Dijon, France) or *Rhizophagus irregularis*. The sands were sterilized in an oven at 180°C for 4 h. The plants were cultivated in a climatic chamber with a light/dark cycle of 16/8 h, a temperature of 24°C at morning and 21°C at night and irrigated every other day with Long Ashton nutrient solution at 32 µM of phosphorous (1). After about 70 days plants were irrigated until complete desiccation of the aboveground part. Subsequently, the roots were chopped and mixed with the sand substrate, placed into plastic bags and stored at 4°C.

AMF mix were composed by different fungus species (*Rhizophagus intraradices*, *R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C.*

etunicatum), and it was propagated in the same manner as the before mentioned fungus, using *S. bicolor* L. and *Trifolium pratense* L. as plants, but in a different substrate composed by zeolite, blond peat, pumice and vermiculite (5:3:2.5:2; v/v/v/v) under a greenhouse tunnel. Subsequently, the roots were chopped and mixed with the substrate of cultivation, placed into plastic bags and stored at 4°C.

1.2 - Plant Growth Promoting Bacteria (PGPB)

The used PGPB were *Pseudomonas protegens* strain Pf7 (briefly Pf7) and/or *P. brassicacearum* strain SVB6R1 (briefly SVB6R1), previously isolated and characterized in our Microbiology Lab (2), having the characteristics reported in the Table 2. They were cultivated on Tryptic Soy Agar (TSA) medium for three days at 28°C and then resuspended in magnesium sulphate heptahydrate ($MgSO_4$ x $7H_2O$; 0.1 M) to obtain an inoculum OD_{600} = 0.5 corresponding to 10^8 CFU mL^{-1}.

Table 2 | Biochemical characteristics of the used PGPB.

	Bacterial strain	
Bacterial characterization	*P. protegens* strain Pf7	*P. brassicacearum* strain SVB6R1
IAA production	+	+
Siderophore production	+	+
P-solubilization (DCP)	-	+
P-solubilization (TCP)	+	-
ACC deaminase activity	Not tested	+

The sign "+" indicates that the bacterial strain possesses the specified biochemical characteristic. DCP: dicalcium phosphate; TCP: tricalcium phosphate.

2 - Plant micropropagation protocol

In order to start *in vitro* culture 50 seeds of *A. annua* plants were placed in a paper bag and sterilized in NaClO 1% for 5 minutes, rinsed in deionized sterile water (2 washes 5 minutes each, and 2 washes 20 minutes each). Afterwards, sterilized

seeds were put inside glass tubes containing 10 mL of semi-solid substrate with MS salts, MS vitamins and sucrose 3% (pH= 5.8; 3). The glass tubes were grown in a climatic chamber at 23 ± 1°C under white LED light (Philips T8 LED Tube Light 20W-200 ± 5 $\mu Em^{-2}s^{-1}$). The obtained plants were numbered and micropropagated according to Sharafi et al. (4) method. Shoot explants were put in a semi-solid substrate with MS salts, MS vitamins, 3% sucrose, 1 mg/mL 6-benzyl-amino purine (BA) and 0.05 mg/L α-naphtalenic-acetic acid (NAA) at pH=5.8 in glass jars (10 cm height and Ø 6 cm; Figure 10 A) Then later, cultures of the most vigorous clones were maintained alive for the next clonal selection *in vivo* step.

3 - Experimental design

In collaboration with the "Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria" (CREA) of Sanremo, different clones of *A. annua* plant were propagated through the micropropagation technique, starting from seeds (var. Anamed), to rule out the genetic variability of plant seed population. The plant clones were named clone 26 (CL26), clone 10 (CL10), clone 24 (CL24), and clone 6 (CL6), maintained *in vitro* and gradually supplied by CREA; then they were cultivated *in vivo* to verify clone stability and plant growth responses in the presence of different beneficial soil microorganisms. After this first phase of cultivation, CREA has informed us that the CL26 was the only clone still alive *in vitro* culture, and also the more stable one in all the different stages of micropropagation and in *vivo* condition. Therefore, it was decided to test other fungal and bacterial inocula on this plant clone; furthermore, at the same time, CREA provided us an additional clone named clone 7 (CL7), that was grown in a single experiment with the CL26, in which two samplings (after 30 and 60 days) were carried out. These different times of sampling were chosen in order to evaluate the mycorrhizal symbiosis trend over the time. In addition, during the

cultivation, we noticed different smells among the different plant treatments, mostly in the CL26 plants, so we also proceed to analyse the leaf volatile profile composition besides the artemisinin concentration, in order to assess changes related to the microorganism presence on the plants. Furthermore, the analysis on the leaf volatile profile composition was also performed in the CL26 plants of the fourth experiment, in order to compare the effect of other different microbes. During these three years of PhD project 337 plants were cultivated in controlled conditions, two clones per time due to the climatic chamber dimension limits; therefore different experiments (6 in total) were set up and carried out, all in the same conditions.

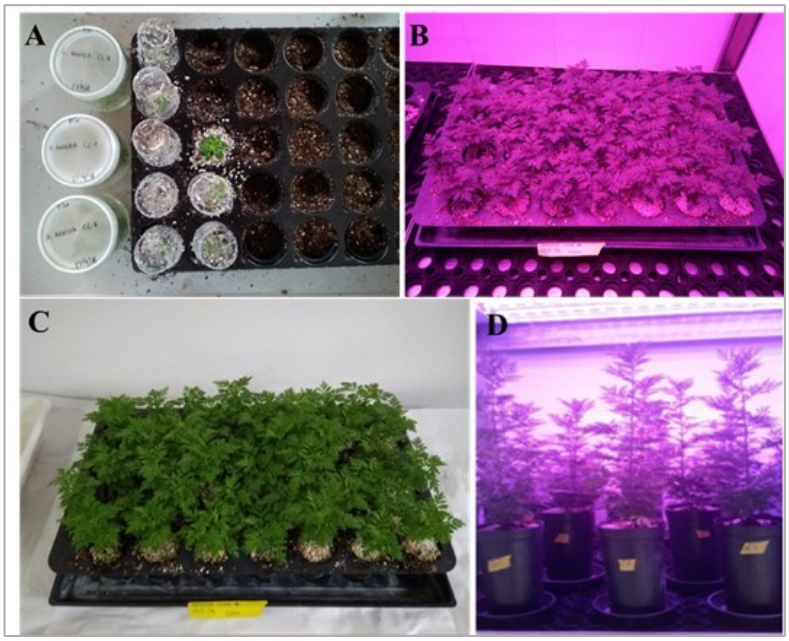

Figure 10. The figure shows *A. annua* micropropagated plants during the transplanting from vitro to alveolar boxes covered with plastic drilled containers (**A**), during the acclimatization period in the climatic chamber under blue/red led lights (**B**), after the acclimatization period (**C**), and plants after repotting into bigger pots during their growth in the climatic chamber (**D**).

In all the experiments, *A. annua* micropropagated plants were transplanted from *vitro* to alveolar boxes (80 mL of capacity) in a sterile substrate formed by quartz

sand (size 4/5 mm) as a draining bottom (10 mL), a mixture of peat and quartz sand of different granulometry (2:1; v/v) and covered with an anti-algae layer of quartz sand (size 2/3 mm). In order to maintain a constant humidity and to limit plant transpiration, plantlets were covered with plastic drilled containers and constantly nebulized with sterile water (Figure 10 A, B). This phase of acclimation lasted for 10 days (Figure 10 C). Then, plants were transplanted into pots of a bigger volume (700 mL) filled with a growth substrate composed by a mixture of sterile quartz sands of different granulometry and peat (as showed in Figure 10 D and 11).

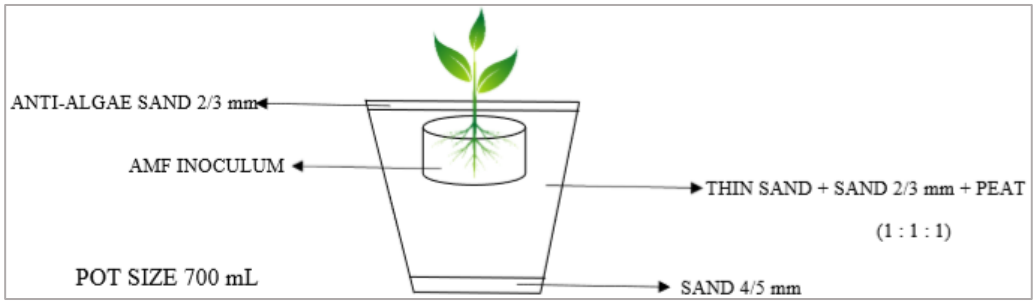

Figure 11. Substrate composition of the pots.

After transplanting, each plant was watered with 150 mL of deionized sterile water. During this step, plants were inoculated or not with different beneficial soil microorganisms: AM fungi were *F. mosseae* BEG12, a mix of different AMF species and *R. irregularis*; PGPB included two species of *Pseudomonas* genus: *P. protegens* (strain Pf7) and *P. brassicacearum* (strain SVB6R1). AMF inoculum (150 mL) was added near the plant roots (Figure 11), PGPB inoculum (10 mL) was added on the substrate surface near the plant roots, while plants which were not inoculated with PGPB received magnesium sulphate solution only (10 mL; $MgSO_4$ x $7H_2O$; 0.1 M). After 30 days a reinforcement inoculum of PGPB (10 mL) was added. Details on the used microorganisms-plant clones

will be reported later, in each single experiment results. Plants were tested with each microorganisms according to the clone plant availability, for this reason some clones did not inoculate with all the available microbes. The plastic materials used for the experiments were previously sterilized in sodium hypochlorite solution (20% v/v). The peat (Vigor Plant's Complete Peat; composed by 21% baltic peat, 20% humified peat, 26%, irish peat, 18% calibrated peat, 13% pumice; pH 6.5; containing a granular slow release mineral fertilizer NPK 15-9-15 all in a soluble form and not subjected to leach) was previously sterilized with flowing steam at 104°C for 1 hour, and after the vessels containing the peat were put in an oven at 40°C for 24 hours to reduce humidity in order to avoid mould formation. All the quartz sands were sterilized in an oven at 180°C for 4 hours. Plants were grown in controlled conditions for 60 days (except for the last experiment where also a first sampling at 30 days was done), with a photoperiod of 16/8 h light/dark at a light intensity of 140 µmol m^{-2} s^{-1} under reduced spectrum led light (red and blue light wavelengths). The temperature was 25°C in the light and 21°C in the dark (Figure 10 D). All the plants were soaked with Long Ashton nutrient solution, with phosphate concentration of 32 µM, three times a week. The Long Ashton solution consisted of 5 macronutrient solutions [$Ca(NO_3)_2$ x $4H_2O$ (2 mM); $MgSO_4$ x $7H_2O$ (0.75 mM); KNO_3 (2 mM); FeNa EDTA (50 µM; NaH_2PO_4 (32 µM)] and a micronutrient solution [$MnSO_4$ x H_2O (10 µM), $CuSO_4$ x $5H_2O$ (1 µM), H_3BO_3 (40 µM), $ZnSO_4$ x $7H_2O$ (2 µM), NaCl (100 µM), Na_2MoO_4 x $2H_2O$ (0.5 µM)]. All solutions were autoclaved at 121°C for 15 minutes. The amount of nutrient solution used ranged from 20 mL to 100 mL according to the plant growth.

4 - Analysed parameters

After 60 days, in each experiment, and also after 30 days in the last experiment, plants were harvested and different parameters were recorded (Figure 12 A, B, C

D): mycorrhizal colonization in the root system, fresh and dry weight (60°C in oven for 1 week) of different plant organs, and photosynthetic pigment concentrations. Instead, artemisinin concentration was evaluated in all the plants cultivated in the last experiment, both at 30 and 60 days; while, the leaf volatile composition was recorded only in the CL26 plants (at 30 and 60 days) in the sixth experiment and in the same clone plants but in the fourth experiment.

Figure 12. The figure shows representative images of CL26 plants (from right to left: Control, bacterial, mycorrhizal plants and those co-inoculated with both microorganisms) during the last experiment samplings at 30 days (**A**) and 60 days (**B**) and during processing in laboratory (**B** and **D**).

5 - Mycorrhizal colonization

The mycorrhizal colonization was calculated according to Trouvelot et al. (5). Sixty pieces of root randomly chosen, they were clarified in KOH 10% in a water bath at 60°C for 20 min. Then, samples were rinsed with deionized water, dried and stained with 1% lactic blue (methyl blue 1% in lactic acid). The excess dye

was removed with a series of lactic acid washes. Finally, the samples were stored at 4°C for 24 hours in lactic acid. The following day the obtained samples were mounted on slide and observed under light microscope: two slides for each plant (30 root pieces each) were prepared. The samples were included into classes on the base of mycorrhizal degree, arbuscule and vesicles abundance, as reported in the following table:

Classe	mycorrhization %
0	lack of colonization
1	traces of colonization
2	less than 10%
3	from 11 to 50%
4	from 51 to 90%
5	more than 90%

Classe	% of arbuscule abundance
X	lack of arbuscules
A	low abundance (10%)
A	medium abundance (50%)
A	high abundance (100%)

In order to obtain the mycorrhization percentage (M%) the following formula was used:

$$M\% = (95n5 + 70n4 + 30n3 + 5n2 + n1) / N$$

in which "n5, n4….n1" represent the number of fragments classified in each respective class, whereas N represents the total number of the analysed root fragments. For evaluating the frequency of mycorrhization (F%) and the arbuscule abundance (A%), both in the mycorrhizal part (a%) and root apparatus (A%), the following formulae were applied:

$$F\% = 100 \cdot (N - n_0) / N$$

in which F% is the percentage frequency of mycorrhization and N is the total number of analysed root fragments, n_0 is the number of fragments into class 0, where the fungus was not present.

$$a\% = (100mA + 50mA + 10mA) / 100$$

mA…mA calculated as showed:

$$mA = (95n5A + 70n4A + 30n3A + 5n2A + n1A) \cdot F / M \cdot (N - n_0)$$

in which "n5A…n1A" represent the number of fragments indicated with 5A, 4A…1A, respectively. According to this latter, it is obtained:

$$A\% = a \cdot M / 100$$

6 - Morphometric and weight parameters

For each plant, stem height, root length, and fresh weights of root, shoot and leaves were registered; furthermore, samples were dried in an oven at 60°C for one week in order to evaluate the dry weights of the aforementioned plant organs.

7 - Analysis of leaf photosynthetic pigments

Chlorophyll *a*, *b* and carotenoid concentrations were determined according to Porra et al. (6). Briefly, 0.02 g of fresh leaves were taken from each plant and 1.5 mL of N, N-dimethyl formamide was added. The samples were kept in the dark at 4°C for a week, until the complete pigment extraction. The concentration of chlorophylls and carotenoids was evaluated spectrophotometrically using the following formula.

$[Chl_a]\ \mu g/mL = 12A_{663,8} - 3.11\ A_{646.8}$

$[Chl_b]\ \mu g/mL = 20.78\ A_{646.8} - 4.88\ A_{663.8}$

[Car] µg/mL = (1000 A_{480} – 1.12 [Chl $_a$] – 34.7 [Chl $_b$] / 245)

8 - Artemisinin extraction and HPLC analysis

Dried leaves were finely crushed in a mortar to obtain a homogenous compound that was used for artemisinin extraction according with Lapkin et al. (7), with some modifications. For each extraction, 12.5 mL of acetone 100% were added to 0.5 g of leaf dry material (of each plant), in a centrifuge tube. The sample was stirred at 250 rpm for 30 minutes at room temperature, and centrifuged at 13000 rpm for 60 minutes at 22°C. The pellet was removed, and the supernatant filtered with 0.20 µm filter and aliquoted into eppendorfs, each containing 800 µL of extract. Later, samples were concentrated in speedvac for 30 minutes, resuspended in 900 µL of mobile phase (Acetonitrile 50%, HPLC Water 30%, Methanol 20%) and left to settle for one hour at room temperature. Then, they were centrifuged at 13000 rpm for 1 minute and 20 µL of each sample were diluted 1:10 in the mobile phase, filtered with a 0.20 µm filter, loaded into vials and analyzed with HPLC. The used HPLC (Agilent 1260 Infinity Series Quaternary LC) composed by a solvent cabinet, a quaternary pump, an autosampler with thermostat, and a diode array or variable wavelength detector (UVD-DAD). Artemisinin detection was performed at 280 nm, using an injection volume of 5 µL. The artemisinin calibration curve was constructed using different concentrations of an analytical standard (artemisinin No. 69532 - 10 mg, Sigma-Aldrich). The chromatographic run was performed in isocratic mode with mobile phase consisting of Acetonitrile (5%), HPLC Water (65%), Methanol (30%) at a flow rate of 1.2 mL/min and a column temperature of 45°C. In addition, the artemisinin peak was identified in comparison to the retention time of artemisinin standard and through the injection of the sample containing a "spike" of the analytical standard. The artemisinin peak was also detected with mass spectrometry.

9 - Volatile profile analysis

Plant leaves (about 2 g for each plant) were picked during the sampling, frozen in liquid nitrogen and stored at -80°C until their using time. Shoot volatiles were analysed by Gas Chromatography-Mass Spectrometry (GC-MS) according to Vidic et al. (8), 1 g of frozen leaves were crushed in liquid nitrogen into a mortar and put into a glass vial filled with 10 mL of dichloromethane (CH_2Cl_2 Sigma-Aldrich 95%). It was hermetically closed and put in stirring at 200 rpm for 24 h at room temperature. Then the samples were analysed with Thermo Scientific Trace 1300 Gas Chromatography-Single Quadrupole Mass Spectometer (ISQ-LT) instrument; GC separation was done with a Phenomenez DB5-5ms capillary column, at an injection temperature of 250°C in spitless mode, utilizing helium as a gas carrier at flow rate of 1.0 mL min^{-1}. MS signal was acquired through El+ mode with 70.0 eV ionization energy, source temperature of 290°C. The detection was done both in full-scan mode (range 35-500 m/z) and Single Ion Monitoring (SIM). The peak integration and identification were done with Chromeleon Chromatography Studio, using different database: nist_msms, nist_msms2, nist_ri, NISTDEMO, and WileyFragrans.

10 - Statistical analysis

The mean value and the relative standard error of the considered parameters were calculated. The data obtained were compared by means of the ANOVA test. The significance of the differences ($p < 0.05$) were established by the Fisher's post hoc comparison test. For $p < 0.05$ the differences among the parameters were considered significant; for values of $p < 0.001$ they were considered highly significant; for values above 0.05 they were considered not significant. Furthermore, data were analysed by the two-way ANOVA using "Fungus" (F)

and “Bacterium” (B), and “Treatment” and “Time” as factors. The processing was carried out using Statview 4.5 software (Abacus Concepts).

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Chapter 4

Results

1 - First experiment | Clone 26 (CL26)

The number of plants and the used microorganisms are reported in the following table:

Treatment	Label	Number of plants
No microorganisms	Control	8
P. protegens strain Pf7	Pf7	10
F. mosseae BEG12	BEG12	10
F. mosseae BEG12 and *P. protegens* strain Pf7	Pf7 + BEG12	10

1.1 - Mycorrhizal colonization

The CL26 uninoculated plants and those inoculated with *P. protegens* (Pf7) showed no fungus presence (Figure 13). The plants inoculated with *F. mosseae* (BEG12) showed low mycorrhization frequency and percentage (F%= 1%, M%= 0,2%) and the arbuscule abundance was negligible (A% next to zero). In plants co-inoculated with *F. mosseae* (BEG12) and *P. protegens* (Pf7), the F%, M% and A% had higher values than the other treatments. This last treatment exhibited significant differences compared to all the others. Regarding the F%, as showed by the two-way ANOVA, both factors ("Fungus" and "Bacterium") and their interaction were highly significant; instead, concerning the M%, the "Fungus" (F) factors was highly significant, and the "Bacterium" (B) factor and the interaction between both factors (F*B) were significant (two-way ANOVA).

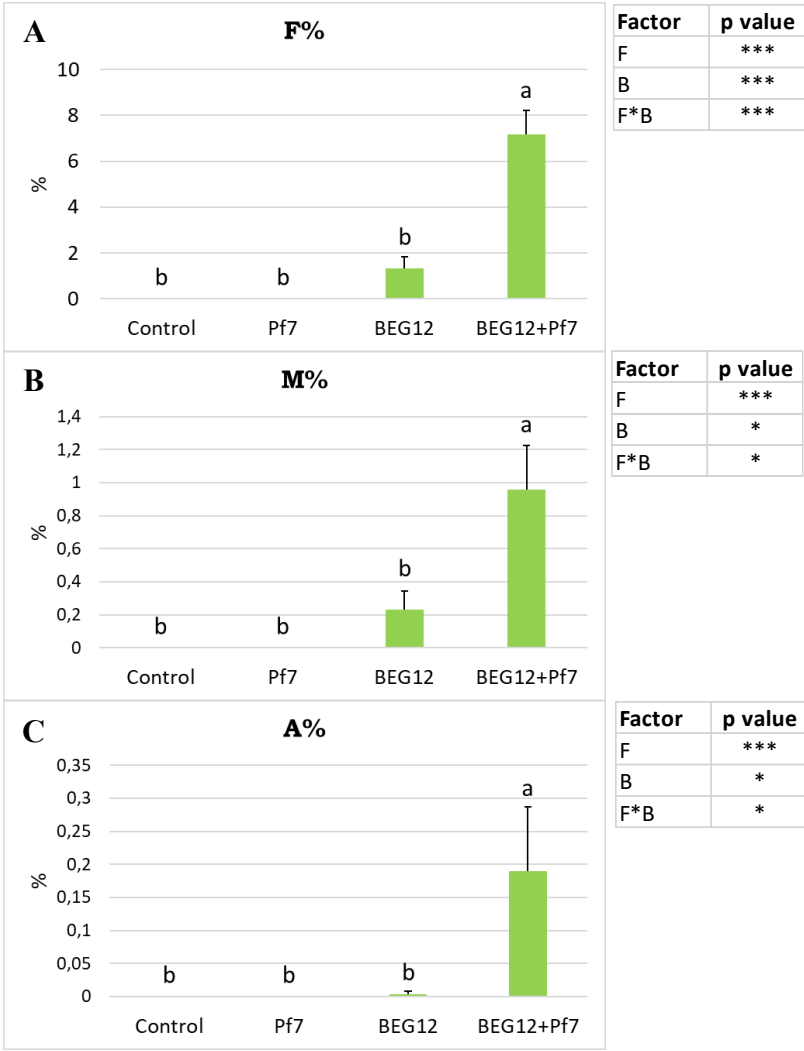


Figure 13. The figure shows the mean values and the relative standard error of the mycorrhization frequency: F% (**A**), of the mycorrhization percentage: M% (**B**), of the arbuscule abundance: A% (**C**) of *A. annua* plants (clone 26). Control: control plants, Pf7: plants inoculated with *P. protegens* Pf7, BEG12: plants inoculated with *F. mosseae* and BEG12+Pf7: plants co-inoculated with *F. mosseae* and *P. protegens* Pf7. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA. F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

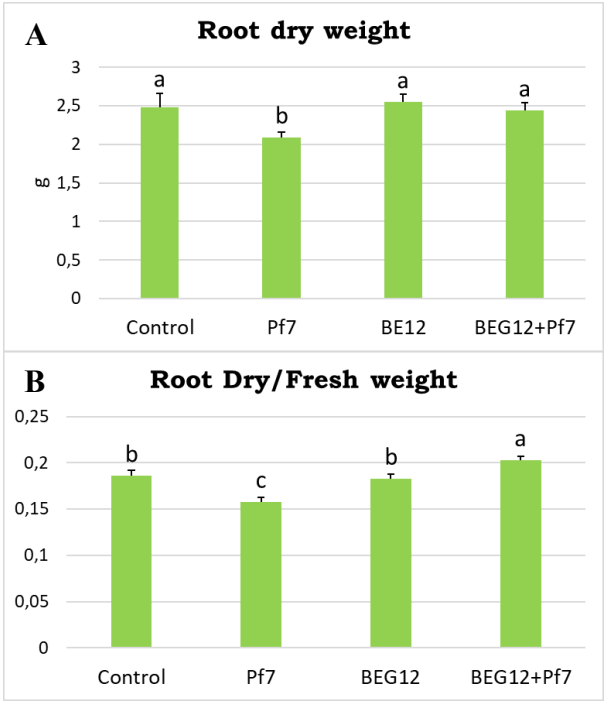
1.2 - Morphometric and weight parameters

1.2.1 - Shoot fresh and dry weight, leaf fresh and dry weight, and ratio of shoot dry/fresh weight

The shoot and leaf biomass, both in fresh and dry weight, and the ratio of shoot dry/fresh weight did not show significant differences among the plant treatments (data are reported in Annex A, table 1).

1.2.2 - Root fresh and dry weight, and ratio of root dry/fresh weight

Concerning the root system, no differences in the root fresh weight among the plant treatments were reported (data are reported in Annex A, table 1); instead, the root dry weight (Figure 14 A) had the lowest values in the plants inoculated with the bacterium alone (Pf7), and these plants were significantly different from all other plant treatments. Control plants, plants inoculated with the fungus alone (BEG12) and those co-inoculated with both microorganisms (BEG12+Pf7) were similar to each other. According to the two-way ANOVA, only the "Bacterium" factor (B) was significant. Instead, the root ratio of dry on fresh weight (Figure 14 B) had the highest values in the co-inoculated plants (BEG12+Pf7) and these plants were significantly different from all other plant treatments. The plants inoculated with Pf7 alone showed the lowest values, and they were significantly different from all other plant treatments; whereas the plants inoculated with the fungus alone (BEG12) and those uninoculated were similar to each other. They showed intermediate ratio values between the two aforementioned treatments. Both the "Fungus" factor (F) and the interaction between F and B were highly significant (two-way ANOVA).



Factor	p value
F	ns
B	*
F*B	ns

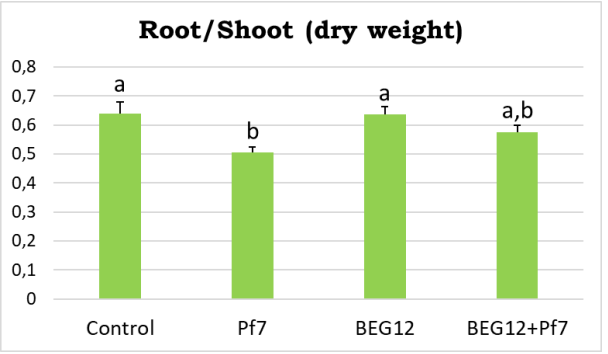
Factor	p value
F	***
B	ns
F*B	***

Figure 14. The figure shows the mean values and the relative standard error of the dry weight (**A**), and root dry to fresh weight (**B**) in *A. annua* plants (clone 26). Control: control plants, Pf7: plants inoculated with *P. protegens* Pf7, BEG12: plants inoculated with *F. mosseae* and BEG12+Pf7: plants co-inoculated with *F. mosseae* and *P. protegens* Pf7. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

1.2.3 - Root/shoot ratio of fresh weight and root/shoot ratio of dry weight

The values of root/shoot ratio of fresh weight, did not show significant differences between all the plant treatments (data are reported in Annex A, table 1). On the contrary, the root/shoot ratio of dry weight had the lowest values in the plants inoculated with Pf7 alone, these plants were significantly different from the uninoculated plants and the plants inoculated with BEG12 alone (Figure 15). Uninoculated, co-inoculated (BEG12+Pf7) plants and those inoculated with BEG12 alone were similar to each other, and they had higher ratio values

comparing with plants inoculated with Pf7 alone. Only the "Bacterium" factor (B) was significant (two-way ANOVA).



Factor	p value
F	ns
B	**
F*B	ns

Figure 15. The figure shows the mean values and the relative standard error of the root to shoot of fresh dry weight in *A. annua* plants (clone 26). Control: control plants, Pf7: plants inoculated with *P. protegens* Pf7, BEG12: plants inoculated with *F. mosseae* and BEG12+Pf7: plants co-inoculated with *F. mosseae* and *P. protegens* Pf7. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

1.2.4 - Stem height, root length and their ratio

Regarding the stem height, the root length, and the ratio between these two last parameters, no significant differences between the different plant treatments were observed (data are reported in Annex A, table 1).

1.3 - Leaf photosynthetic pigment concentrations

Concerning the photosynthetic pigments, the chlorophyll *a* and *b* concentrations showed the same trend (Figure 16 A, B). The highest concentrations were registered in the co-inoculated plants (BEG12+Pf7) and they were significantly different if compared to all other plant treatments. The plants inoculated with Pf7 alone and those inoculated with BEG12 alone showed lower concentrations of chlorophylls compared to the previous one; they were similar to each other, but significantly different to the other plant treatments. Control plants had the lowest

concentrations of chlorophylls in the leaves, and these plants were significantly different from all other plant treatments except for the chlorophyll *a* concentration in which they were similar to the plants inoculated with BEG12 alone (Figure 16 A). According to the two-way ANOVA, the "Fungus" factor (F) was significant, and the "Bacterium" factor was highly significant.

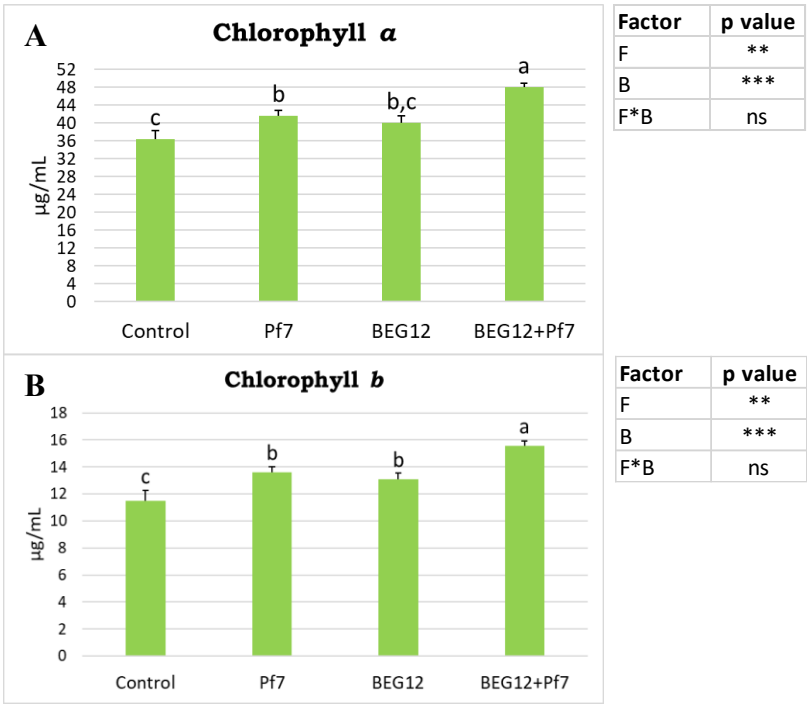
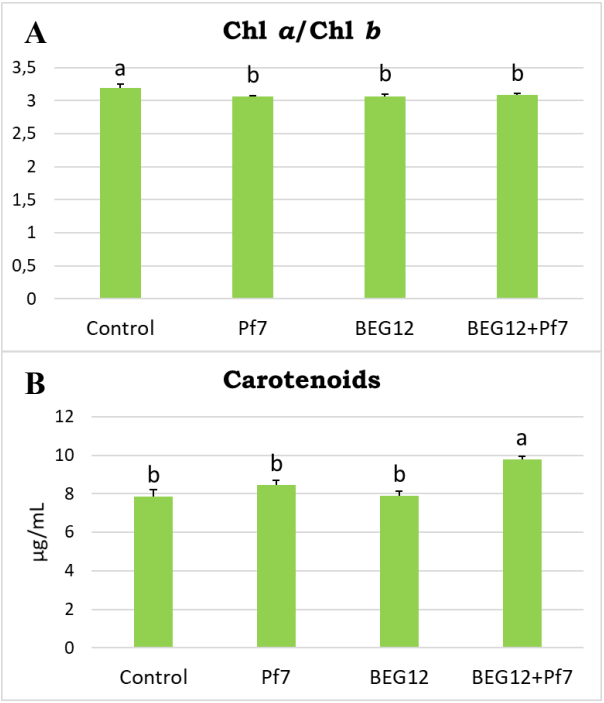


Figure 16. The figure shows the mean values and the relative standard error of the chlorophyll *a* (**A**) and chlorophyll *b* (**B**) concentrations in *A. annua* plants (clone 26). Control: control plants, Pf7: plants inoculated with *P. protegens* Pf7, BEG12: plants inoculated with *F. mosseae*; BEG12+Pf7: plants co-inoculated with *F. mosseae* and *P. protegens* Pf7. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

The chlorophyll a/b ratio (Chl *a*/Chl *b*, Figure 17 A) had the highest values in the control plants: these plants were significantly different from all other plant treatments. Whereas plants inoculated with microorganisms alone (Pf7 and BEG12) and those co-inoculated (BEG12+Pf7) showed lower values compared

to controls, and they were similar to each other. According to the two-way ANOVA, the interaction between “Fungus” and “Bacterium” factors (F*B) only was significant.



Factor	p value
F	ns
B	ns
F*B	*

Factor	p value
F	**
B	***
F*B	*

Figure 17. The figure shows the mean values and the relative standard error of the chlorophyll *a* to chlorophyll *b* ratio (**A**), and the carotenoid concentration (**B**) in *A. annua* plants (clone 26). Control: control plants, Pf7: plants inoculated with *P. protegens* Pf7, BEG12: plants inoculated with *F. mosseae* and BEG12+Pf7: plants co-inoculated with *F. mosseae* and *P. protegens* Pf7. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

Carotenoids (Figure 17 B) had the highest concentrations in the leaves of the co-inoculated plants (BEG12+Pf7), and these plants were significantly different from all other plant treatments. The control plants, plants inoculated with Pf7 alone and those inoculated with BEG12 alone had lower carotenoid concentrations compared to the co-inoculated plants. In this case, the “Bacterium” factor (B) was highly significant and the “Fungus” factor (F) and

the interaction between the two factors (F*B) were significant (two-way ANOVA).

2 - Second experiment – Clone 10 (CL10) and Clone 24 (CL24)

In this experiment two clones (CL10 and CL24) were inoculated with different soil microorganisms. The used materials and methods are the same reported in the section "Materials and Methods".

2.1 | Clone 10 (CL10)

The number of plants and the soil microorganisms used in each treatment are reported in the following table:

Treatment	Label	Number of plants
No microorganisms	Control	5
P. protegens strain Pf7	Pf7	5
P. brassicacearum strain SVB6R1	SVB6R1	6
P. protegens strain Pf7 and *P. brassicacearum* strain SVB6R1	Pf7 + SVB6R1	7
F. mosseae BEG12	BEG12	6
F. mosseae BEG12 and *P. protegens* strain Pf7	BEG12 + Pf7	7
F. mosseae BEG12 and *P. brassicacearum* strain SVB6R1	BEG12 + SVB6R1	7

2.1.1 - Mycorrhizal colonization

In the Figure 18 are represented the mycorrhizal parameters of CL10 plants: the control plants, plants inoculated with Pf7 or SVB6R1, and those co-inoculated with both bacteria (Pf7+SVB6R1) did not show any traces of fungal colonization in the root.

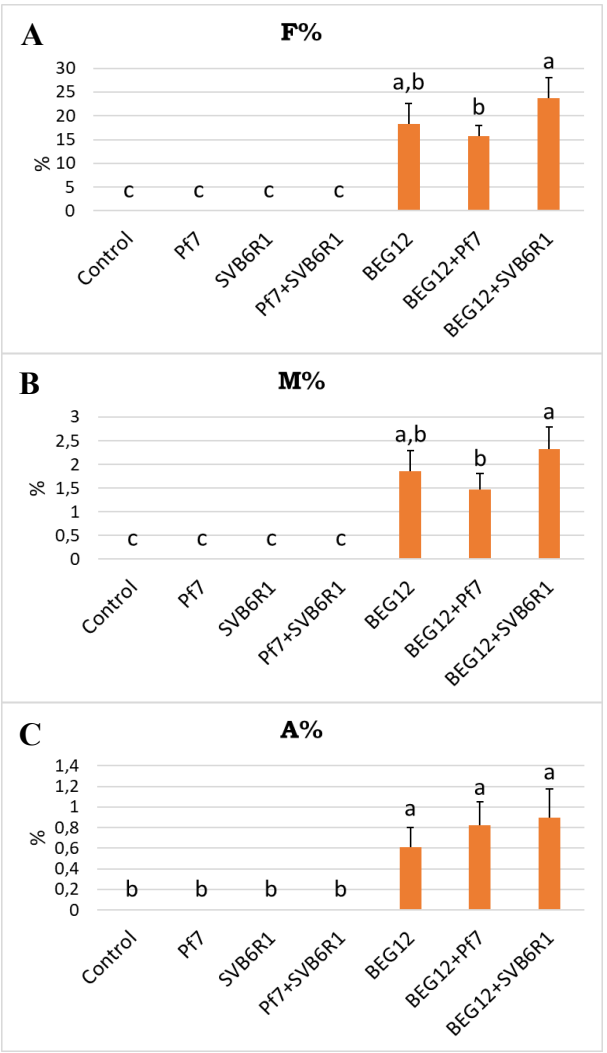


Figure 18. The figure shows the mean values and the relative standard error of the mycorrhization frequency: F% (**A**), of the mycorrhization percentage: M% (**B**), of the arbuscule abundance A% (**C**) of *A. annua* plants (clone 10). Control: control plants, Pf7: plants inoculated with *P. protegens*; SVB6R1: plants inoculated with *P. brassicacearum*; Pf7+SVB6R1: plants co-inoculated with *P. protegens* and *P. brassicacearum*; BEG12: plants inoculated with *F. mosseae*; BEG12+Pf7: plants co-inoculated with *F. mosseae* and *P. protegens*; BEG12+SVB6R1: pants co-inoculated with *F. mosseae* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

On the other hand, all the inoculated plants showed low values of colonization; the plants co-inoculated with BEG12 and SVB6R1 (Figure 18 A, B) had the

highest frequency of mycorrhization (F%) and the mycorrhizal percentage (M%), about 25% and 2.5% respectively, followed by plants inoculated with BEG12 alone, and finally by those co-inoculated with BEG12 and Pf7. Significant differences were reported between plants co-inoculated with the same fungus but with the two different bacteria. Concerning the arbuscule abundance (A%; Figure 18 C), no significant differences were reported between plants inoculated (BEG12) and co-inoculated (BEG12+Pf7; BEG12+SVB6R1); instead, these plants were significantly different from the control and bacterial plants. For all the considered parameters only the "Fungus" factor was highly significant (two-way ANOVA). Moreover, no traces of vesicles in the root system were registered.

2.1.2 - Morphometric and weight parameters

2.1.2.1 - Shoot fresh and dry weight, leaf fresh and dry weight, and ratio of shoot dry/fresh weight

The shoot biomass showed significant differences between the plant treatments only for the dry biomass (leaf and shoot), whereas no significant differences in the fresh biomass (leaf and shoot; data are reported in Annex A, table 2.1) were recorded. Regarding the shoot and leaf dry weights (Figure 19 A, B), plants co-inoculated with BEG12 and Pf7 or SVB6R1 had the highest values, and these plants were significantly different from control plants, plants inoculated with Pf7 or BEG12 alone. Instead, these latter plant treatments had the lowest weights, and in this case only the plants inoculated with Pf7 alone were significantly different from plants inoculated with SVB6R1. The two-way ANOVA showed that only the "Bacterium" factor influenced the analysis.

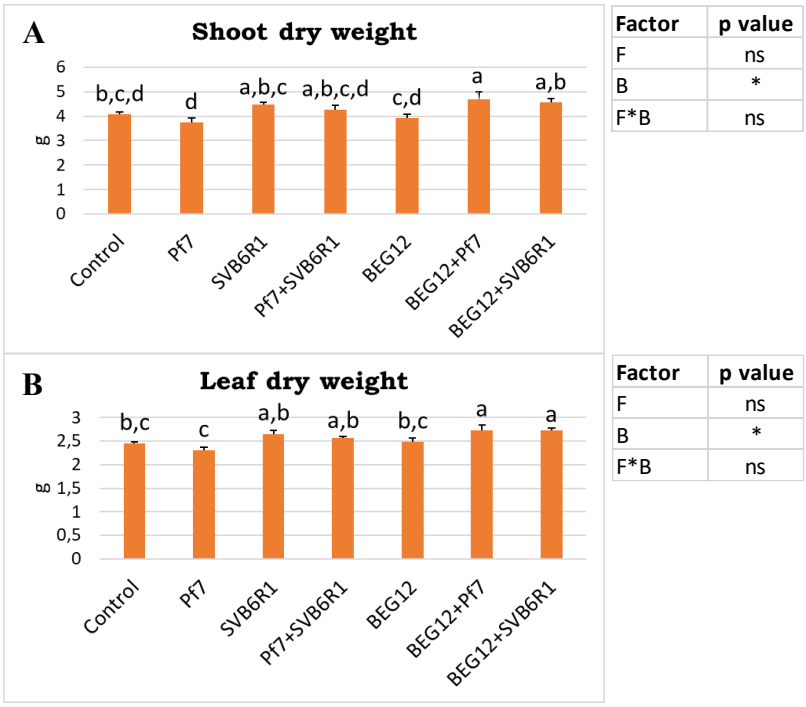
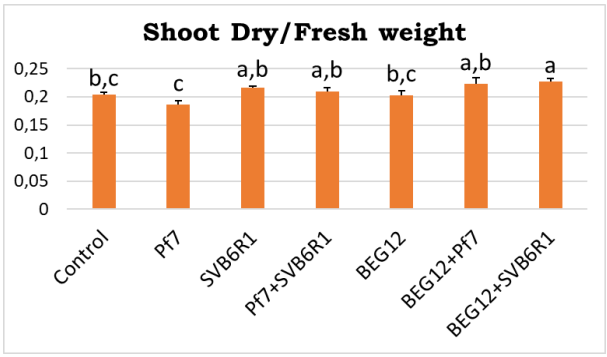


Figure 19. The figure shows the mean values and the relative standard error of the shoot (**A**) and leaf (**B**) dry weight of *A. annua* plants (clone 10). Control: control plants, Pf7: plants inoculated with *P. protegens*; SVB6R1: plants inoculated with *P. brassicacearum*; Pf7+SVB6R1: plants co-inoculated with *P. protegens* and *P. brassicacearum*; BEG12: plants inoculated with *F. mosseae*; BEG12+Pf7: plants co-inoculated with *F. mosseae* and *P. protegens*; BEG12+SVB6R1: pants co-inoculated with *F. mosseae* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

Looking at the shoot ratio of dry on fresh weight (Figure 20), it was registered the same trend observed in shoot and leaf dry weights, with a difference: plants co-inoculated with BEG12 and Pf7 were similar to control plants. In regard to these two latter parameters, the differences between the various treatments were due to the bacterial inoculation (see the two-way ANOVA).

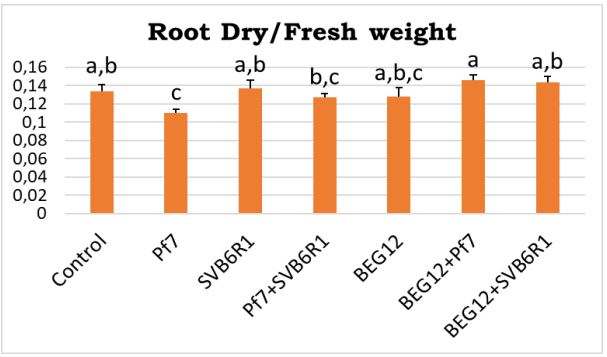


Factor	p value
F	ns
B	*
F*B	ns

Figure 20. The figure shows the mean values and the relative standard error of the shoot dry weight to fresh weight of *A. annua* plants (clone 10). Control: control plants, Pf7: plants inoculated with *P. protegens*; SVB6R1: plants inoculated with *P. brassicacearum*; Pf7+SVB6R1: plants co-inoculated with *P. protegens* and *P. brassicacearum*; BEG12: plants inoculated with *F. mosseae*; BEG12+Pf7: plants co-inoculated with *F. mosseae* and *P. protegens*; BEG12+SVB6R1: pants co-inoculated with *F. mosseae* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

2.1.2.2 - Root fresh and dry weight, and ratio of root dry/fresh weight

Concerning the fresh and dry weights of the root system, no significant differences between all the plant treatments were recorded (data are reported in Annex A, table 2.1). A different trend was registered for the ratio of root dry/fresh weight (Figure 21), in fact plants co-inoculated with BEG12 and Pf7 or SVB6R1 had the higher values if compared to all the other plant treatments. Furthermore, plants co-inoculated with BEG12 and Pf7 were significantly different from those inoculated with Pf7 alone and those co-inoculated with both bacteria (Pf7+SVB6R1), whereas plants co-inoculated with BEG12 and SVB6R1 were significantly different only from those inoculated with Pf7 alone. The interaction between the two factors ("Fungus" and "Bacterium") was significant (two-way ANOVA).

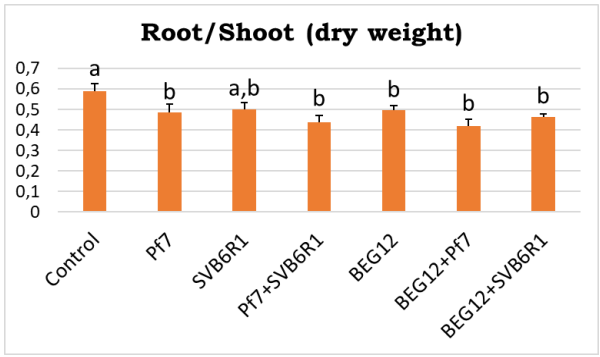


Factor	p value
F	ns
B	ns
F*B	*

Figure 21. The figure shows the mean values and the relative standard error of the root dry to fresh weight of *A. annua* plants (clone 10). Control: control plants, Pf7: plants inoculated with *P. protegens*; SVB6R1: plants inoculated with *P. brassicacearum*; Pf7+SVB6R1: plants co-inoculated with *P. protegens* and *P. brassicacearum*; BEG12: plants inoculated with *F. mosseae*; BEG12+Pf7: plants co-inoculated with *F. mosseae* and *P. protegens*; BEG12+SVB6R1: pants co-inoculated with *F. mosseae* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

2.1.2.3 - Root/shoot ratio of fresh weight and root/shoot ratio of dry weight

In regard to the values of fresh weight ratio, no significant differences were registered between all the plant treatments (data are reported in Annex A, table 2.1). Instead, in the case of the dry weight ratio (Figure 22), the control plants were significantly different from all the other plant treatments, except for the plants inoculated with SVB6R1 alone. The two-way ANOVA showed that both the “Fungus” and the “Bacterium” factors influenced this parameter.



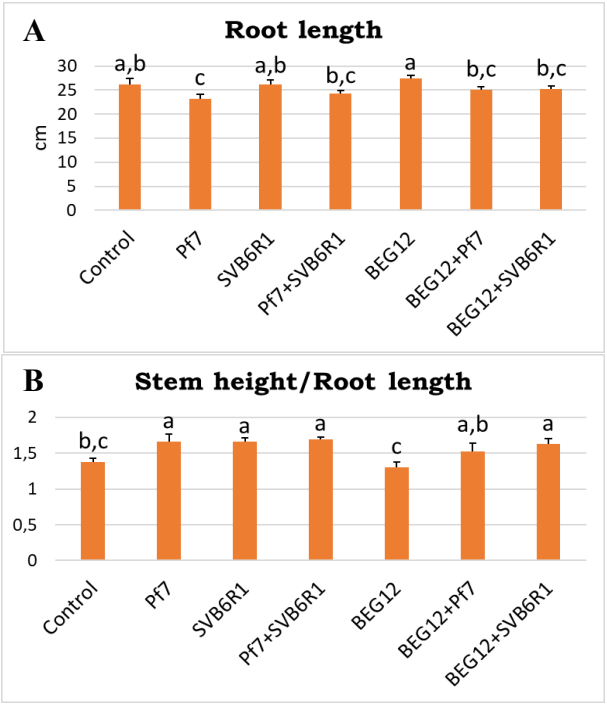
Factor	p value
F	*
B	**
F*B	ns

Figure 22. The figure shows the mean values and the relative standard error of the root to shoot ratio of dry weight of *A. annua* plants (clone 10). Control: control plants, Pf7: plants inoculated with *P. protegens*; SVB6R1: plants inoculated with *P. brassicacearum*; Pf7+SVB6R1: plants co-inoculated with *P. protegens* and *P. brassicacearum*; BEG12: plants inoculated with *F. mosseae*; BEG12+Pf7: plants co-inoculated with *F. mosseae* and *P. protegens*; BEG12+SVB6R1: pants co-inoculated with *F. mosseae* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

2.1.2.4 - Stem height, root length and their ratio

In stem height no significant differences between all the plant treatments were reported (data are reported in Annex A, table 2.1). Instead, the control plants, the plants inoculated with SVB6R1 alone, and those inoculated with BEG12 alone had the highest root length (Figure 23 A). However, plants inoculated with BEG12 alone were significantly different from those inoculated with Pf7 alone, co-inoculated plants with both bacteria (Pf7+SVB6R1), and co-inoculated with BEG12 and Pf7 or SVB6R1; while, control plants and plants inoculated with SVB6R1 alone were different only from those inoculated with Pf7 alone. These last plants had the lowest length of root, and they were similar to all the co-inoculated plants (Pf7+SVB6R1; BEG12+Pf7; BEG12+SVB6R1). The ratio of stem height on root length (Figure 23 B) showed a different trend: control plants and those inoculated with BEG12 alone had the lowest values, and these plants were similar to each other. However, plants inoculated with BEG12 alone were significantly different from all the other plant treatments, whereas control plants

were not different from plants co-inoculated with BEG12 and Pf7. The "Bacterium" factor significantly affected the considered parameters, as confirmed by the two-way ANOVA.



Factor	p value
F	ns
B	**
F*B	ns

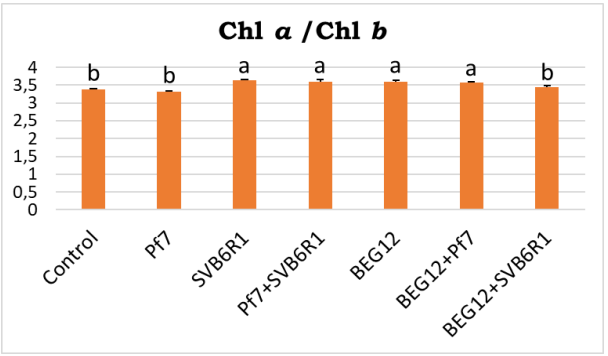
Factor	p value
F	ns
B	***
F*B	ns

Figure 23. The figure shows the mean values and the relative standard error of the root length (**A**), and stem height to root length ratio (**B**) of *A. annua* plants (clone 10). Control: control plants, Pf7: plants inoculated with *P. protegens*; SVB6R1: plants inoculated with *P. brassicacearum*; Pf7+SVB6R1: plants co-inoculated with *P. protegens* and *P. brassicacearum*; BEG12: plants inoculated with *F. mosseae*; BEG12+Pf7: plants co-inoculated with *F. mosseae* and *P. protegens*; BEG12+SVB6R1: pants co-inoculated with *F. mosseae* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

2.1.3 - Leaf photosynthetic pigment concentrations

No significant differences in the concentrations of chlorophyll *a*, *b*, and carotenoids between all the plant treatments were recorded (data are reported in Annex A, table 2.1). The chlorophyll *a*/*b* ratio (Figure 24) was higher in plants inoculated with SVB6R1 alone, BEG12 alone, and in those co-inoculated with

both bacteria (Pf7+SVB6R1) or with BEG12+Pf7, if compared to all the other plant treatments. These four plant treatments were significantly different from control plants, plants inoculated with Pf7 alone, and those co-inoculated with BEG12+SVB6R1, that showed a decrease in the ratio. The co-inoculation (F*B) was responsible for these differences, as showed by the two-way ANOVA.



Factor	p value
F	ns
B	ns
F*B	*

Figure 24. The figure shows the mean values and the relative standard error of the chlorophyll *a* / *b* ratio in the leaves of *A. annua* plants (clone 10). Control: control plants, Pf7: plants inoculated with *P. protegens*; SVB6R1: plants inoculated with *P. brassicacearum*; Pf7+SVB6R1: plants co-inoculated with *P. protegens* and *P. brassicacearum*; BEG12: plants inoculated with *F. mosseae*; BEG12+Pf7: plants co-inoculated with *F. mosseae* and *P. protegens*; BEG12+SVB6R1: pants co-inoculated with *F. mosseae* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

2.2 | Clone 24 (CL24)

The number of plants and the soil microorganisms used in each treatment are reported in the following table:

Treatment	Label	Number of plants
No microorganisms	Control	6
P. protegens strain Pf7	Pf7	6
P. brassicacearum strain SVB6R1	SVB6R1	7
P. protegens strain Pf7 and *P. brassicacearum* strain SVB6R1	Pf7 + SVB6R1	7
Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum;	AMF mix	6
Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum and *P. protegens* strain Pf7	AMF mix + Pf7	7
Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum and *P. brassicacearum* strain SVB6R1	AMF mix + SVB6R1	5

2.2.1 - Mycorrhizal colonization

Control plants and plants inoculated or co-inoculated with both bacteria did not show any trace of mycorrhizal colonization, as we expected. Plants inoculated with AMF mix had the highest frequency of mycorrhization (F% about 22%; Figure 25 A), followed by AMF mix+SVB6R1 and AMF mix+Pf7, respectively. The first two plant treatments were similar to each other but significantly different from this latter. The two-way ANOVA showed that the “Bacterium” factor and the interaction between the two factors (“Bacterium” and “Fungus”) were significant, while the “Fungus” factor was highly significant.

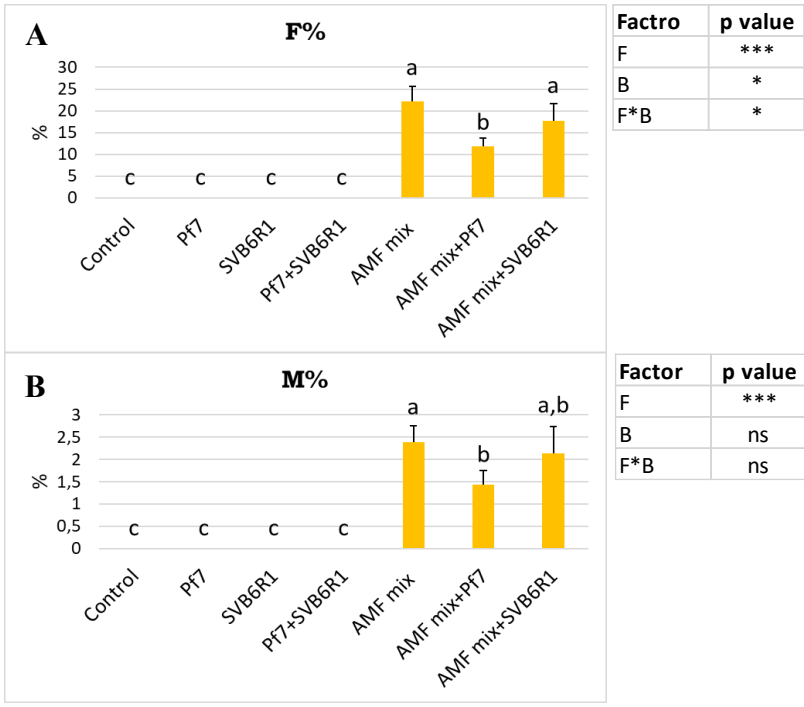
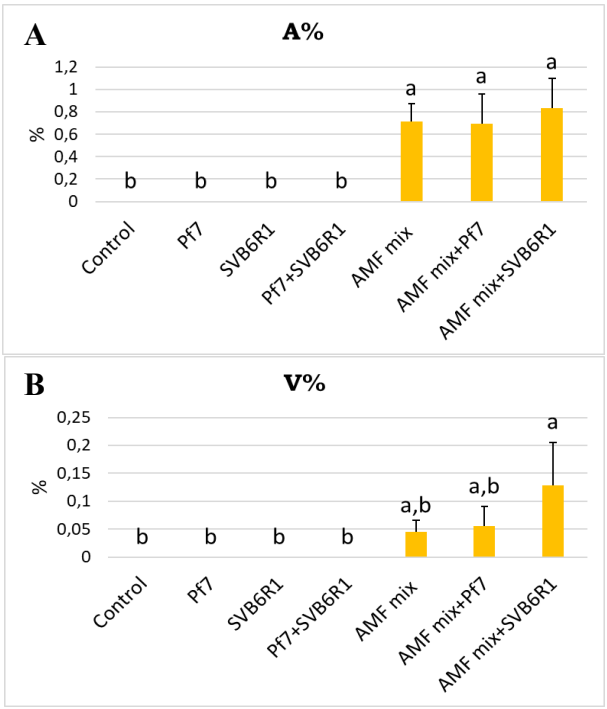


Figure 25. The figure shows the mean values and the relative standard error of the mycorrhization frequency: F% (**A**) and of the mycorrhization percentage: M% (**B**) of *A. annua* plants (clone 24). Control: control plants, Pf7: plants inoculated with *P. protegens*; SVB6R1: plants inoculated with *P. brassicacearum*; Pf7+SVB6R1: plants co-inoculated with *P. protegens* and *P. brassicacearum*; AMF mix: plants inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum*; AMF mix+Pf7: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. protegens*; AMF mix+SVB6R1: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

A similar trend was recorded for the mycorrhizal percentage (M%; Figure 25 B), it was about 2.5% in plants inoculated with AMF mix alone, 2% in plants co-inoculated with AMF mix and SVB6R1, and less than 1.5% in plants co-inoculated with AMF mix+Pf7. However, plants co-inoculated with AMF mix and SVB6R1 were not different from those inoculated with fungal mix and Pf7. In this case, the "Fungus" factor was highly significant (Two-way ANOVA).



Factor	p value
F	***
B	ns
F*B	ns

Factor	p value
F	**
B	ns
F*B	ns

Figure 26. The figure shows the mean values and the relative standard error of the arbuscule abundance: A% (**A**) and of the vesicle abundance: V% (**B**) of *A. annua* plants (clone 24). Control: control plants, Pf7: plants inoculated with *P. protegens*; SVB6R1: plants inoculated with *P. brassicacearum*; Pf7+SVB6R1: plants co-inoculated with *P. protegens* and *P. brassicacearum*; AMF mix: plants inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum*; AMF mix+Pf7: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. protegens*; AMF mix+SVB6R1: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

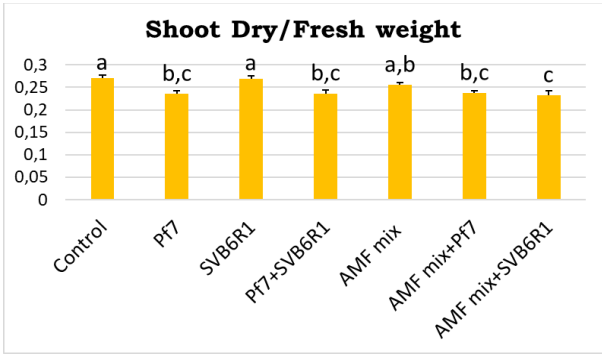
The arbuscule abundance (A%; Figure 26 A) did not show significant differences between plants inoculated with AMF mix alone and those co-inoculated with AMF mix and Pf7; nevertheless, these three plant treatments were significantly different from control plants and plants inoculated or co-inoculated with Pf7 and SVB6R1. The percentage of vesicles (V%; Figure 26 B) had the same trend above described, with values of about 0.15%, but in this case only the plant co-inoculated with AMF mix and SVB6R1 were significantly different from control

and bacterial plants. In both cases, as confirmed by the two-way ANOVA, the "Fungus" factor only was responsible for the detected differences.

2.2.2 - Morphometric and weight parameters

2.2.2.1 - Shoot fresh and dry weight, leaf fresh and dry weight, and ratio of shoot dry/fresh weight

The shoot and leaf biomass, both fresh and dry, did not show significant differences between the different plant treatments (data are reported in Annex A table 2.2).



Factor	p value
F	ns
B	**
F*B	ns

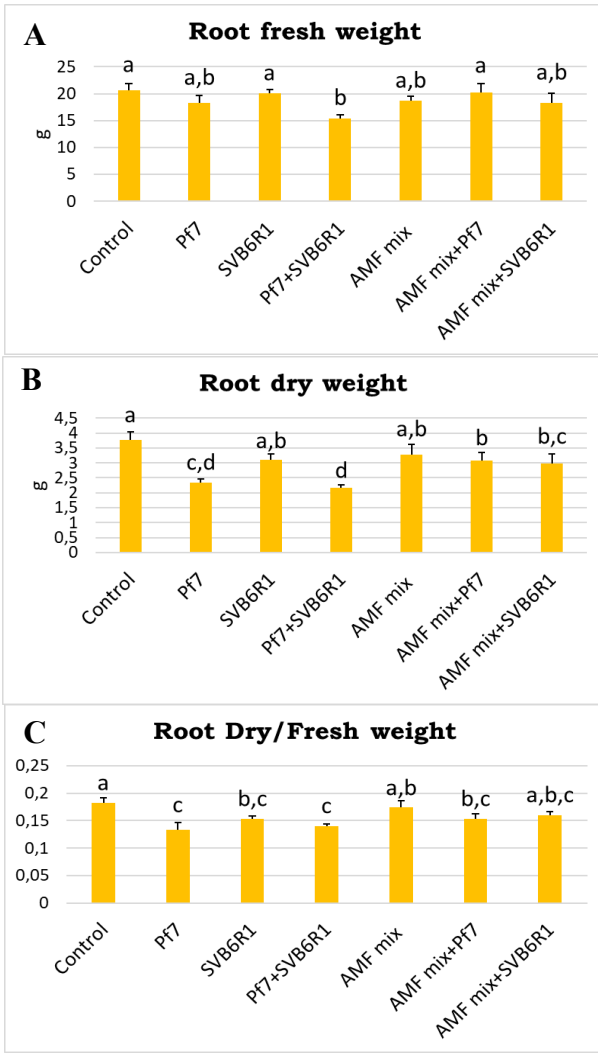
Figure 27. The figure shows the mean values and the relative standard error of the shoot dry to fresh weight of *A. annua* plants (clone 24). Control: control plants, Pf7: plants inoculated with *P. protegens*; SVB6R1: plants inoculated with *P. brassicacearum*; Pf7+SVB6R1: plants co-inoculated with *P. protegens* and *P. brassicacearum*; AMF mix: plants inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum*; AMF mix+Pf7: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. protegens*; AMF mix+SVB6R1: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

Concerning the shoot dry/fresh weight ratio (Figure 27), the highest values were observed in control plants, plants inoculated with Pf7 alone and in those inoculated with AMF mix alone, and these plants were similar to each other.

However, the first two groups of plants were significantly different from all the other plant treatments, whereas the latter group (AMF mix) was significantly different only from AMF mix+SVB6R1 co-inoculated plants. All the other plant treatments had significantly lower values of the ratio comparing to the previous ones, and they were similar to each other. According to the two-way ANOVA, the "Bacterium" factor only was significant.

2.2.2.2 - Root fresh and dry weight, and ratio of root dry/fresh weight

Control plants, plants inoculated with SVB6R1 alone and those co-inoculated with AMF mix+Pf7 showed higher values of fresh root biomass (Figure 28 A), if compared to all the other plant treatments; they were similar to each other and significantly different only from plants co-inoculated with both bacteria (Pf7+SVB6R1). This latter group of plants had the lowest values of fresh biomass, nevertheless it was similar to plants inoculated with Pf7 alone, or AMF mix alone and those co-inoculated with fungal mix and SVB6R1. The two-way ANOVA did not show any significance for the considered factors. The root dry weight had a different trend (Figure 28 B): the highest weight was recorded in control plants, and these plants were significantly different from all the other plant treatments, except for the plants inoculated with SVB6R1 alone and with AMF mix alone. These two last groups of plants had lower root dry weights, comparable to the plants co-inoculated with AMF mix and Pf7 or SVB6R1, and they were different only from plants inoculated with Pf7 alone and co-inoculated with both bacteria.



Factor	p value
F	ns
B	ns
F*B	ns

Factor	p value
F	ns
B	**
F*B	*

Factor	p value
F	ns
B	***
F*B	ns

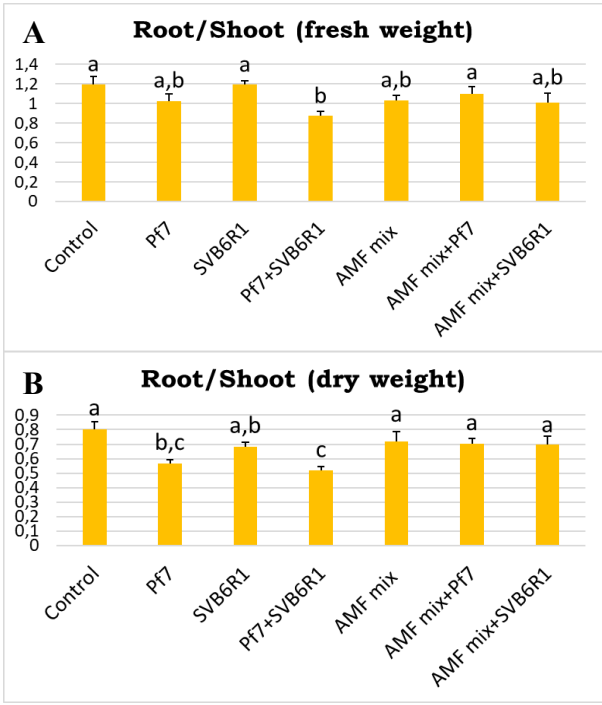
Figure 28. The figure shows the mean values and the relative standard error of the root fresh (**A**) and dry (**B**) weight, and root ratio of dry to fresh (**C**) of *A. annua* plants (clone 24). Control: control plants, Pf7: plants inoculated with *P. protegens*; SVB6R1: plants inoculated with *P. brassicacearum*; Pf7+SVB6R1: plants co-inoculated with *P. protegens* and *P. brassicacearum*; AMF mix: plants inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum*; AMF mix+Pf7: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. protegens*; AMF mix+SVB6R1: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

Finally, plants co-inoculated with both bacteria and those inoculated with Pf7 alone had the lowest values of root dry biomass, thus showing similar to one another; however, the plants inoculated with both bacteria (Pf7+SVB6R1) were also similar to those co-inoculated with the fungal mix and SVB6R1. Either the "Bacterium" factor or its interaction with the "Fungus" factor significantly influenced this parameter, as underlined by the two-way ANOVA. Looking at the ratio of root dry/fresh weight (Figure 28 C), control plants and those inoculated with AMF mix alone had higher values in comparison with all the other plant treatments, and they were similar to each other and to the plants co-inoculated with AMF mix and SVB6R1. Nevertheless, only the control plants were significantly different from all the other plant treatments. Instead, plants inoculated with Pf7 alone and those co-inoculated with both bacteria (Pf7+SVB6R1) showed lower values of the ratio, in comparison to the previous ones, and they were similar to each other and to all the other plant treatments, with the exception of plants inoculated with AMF mix and control plants. According to the two-way ANOVA, only the "Bacterium" factor was significant.

2.2.2.3 - Root/shoot ratio of fresh weight and root/shoot ratio of dry weight

Controls and plants inoculated with SVB6R1 alone had the higher values of root/shoot ratio (fresh weight; Figure 29 A), and they were similar to one another but significantly different only from the plants co-inoculated with Pf7+SVB6R1. This latter group of plants showed the lowest values and differed even from AMF mix+Pf7 plants. The ratio between root/shoot dry biomass (Figure 29 B) reported a slightly different trend: control plants had the highest values, but they were different only from the plants inoculated with Pf7 and from those co-inoculated with both bacteria (Pf7+SVB6R1). These two latter groups of plants, on the contrary, showed the lowest values. The two-way ANOVA underlined that the

"Bacterium" factor and its interaction with the "Fungus" factor (F*B) were significant.



Factor	p value
F	ns
B	ns
F*B	ns

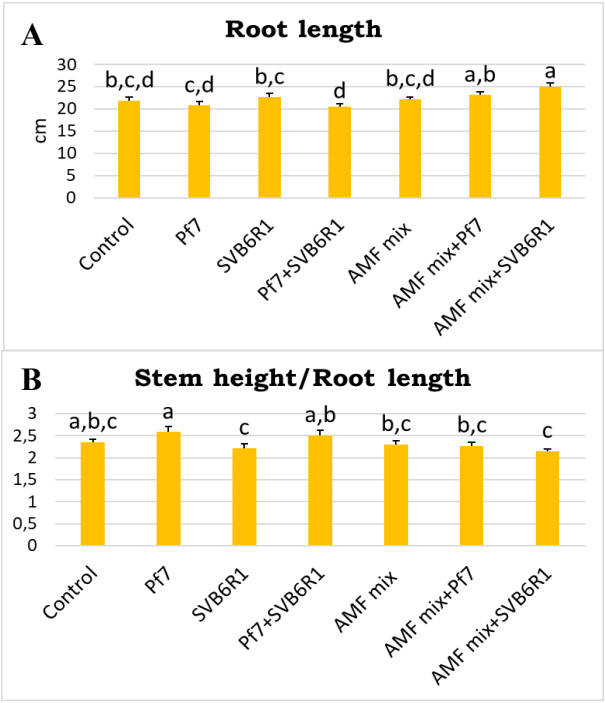
Factor	p value
F	ns
B	**
F*B	*

Figure 29. The figure shows the mean values and the relative standard error of the root to shoot ratio of fresh weight (**A**), and root to shoot ratio of dry weight (**B**) of *A. annua* plants (clone 24). Control: control plants, Pf7: plants inoculated with *P. protegens*; SVB6R1: plants inoculated with *P. brassicacearum*; Pf7+SVB6R1: plants co-inoculated with *P. protegens* and *P. brassicacearum*; AMF mix: plants inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum*; AMF mix+Pf7: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. protegens*; AMF mix+SVB6R1: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

2.2.2.4 - Stem height, root length and their ratio

All the plants had similar heights of the stem, and no significant differences between the various treatments were recorded (data are reported in Annex A, table 2.2). Instead, plants co-inoculated with AMF mix and SVB6R1 had a higher

root length (30 A), if compared to all the other plant treatments; furthermore, they were different from all the other plant treatments, except for the plants co-inoculated with AMF mix+Pf7.



Factor	p value
F	*
B	ns
F*B	ns

Factor	p value
F	ns
B	**
F*B	*

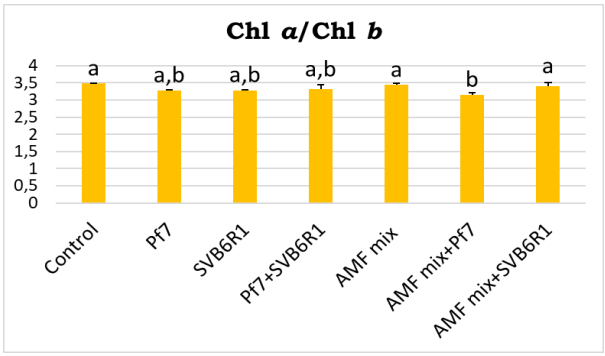
Figure 30. The figure shows the mean values and the relative standard error of the root length (**A**), and stem height to root length (**B**) of *A. annua* plants (clone 24). Control: control plants, Pf7: plants inoculated with *P. protegens*; SVB6R1: plants inoculated with *P. brassicacearum*; Pf7+SVB6R1: plants co-inoculated with *P. protegens* and *P. brassicacearum*; AMF mix: plants inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum*; AMF mix+Pf7: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. protegens*; AMF mix+SVB6R1: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

On the contrary, the lowest length was registered in plants co-inoculated with both bacteria (Pf7+SVB6R1): these plants were also significantly different from those inoculated with SVB6R1 alone and co-inoculated with AMF mix and Pf7. According to the two-way ANOVA, only the “Fungus” factor was significant.

The ratio between stem height and root length (Figure 30 B) was the highest in plants inoculated with Pf7 alone and in those co-inoculated with Pf7+SVB6R1, these plants were similar to one another and to the control plants. Whereas lower values of the ratio were reported in plants inoculated with SVB6R1 alone and co-inoculated with AMF mix and SVB6R1; however, they were significantly different only from plants inoculated with Pf7 and those co-inoculated with both bacteria. In this case, the two-way ANOVA showed that both "Bacterium" factor (B) and its interaction with the "Fungus" factor (F*B) were responsible for this result.

2.2.3 - Leaf photosynthetic pigment concentrations

Concerning the chlorophyll *a, b*, and carotenoid concentrations, no significant differences between the different plant treatments were recorded (data are reported in Annex A, table 2.2). The chlorophyll *a*/*b* ratio (Figure 39 A) decreased in AMF mix+Pf7 co-inoculated plants. These plants were significantly different from control plants, plants inoculated with AMF mix alone, and from those co-inoculated with AMF mix+SVB6R1. According to the two-way ANOVA, only the "Bacterium" factor significantly affected this parameter.



Factor	p value
F	ns
B	**
F*B	ns

Figure 31. The figure shows the mean values and the relative standard error of the chlorophyll *a* / *b* ratio in the leaves of *A. annua* plants (clone 24). Control: control plants, Pf7: plants inoculated with *P. protegens*; SVB6R1: plants inoculated with *P. brassicacearum*; Pf7+SVB6R1: plants co-inoculated with *P. protegens* and *P. brassicacearum*; AMF mix: plants inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum*; AMF mix+Pf7: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. protegens*; AMF mix+SVB6R1: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

3 - Third experiment – Clone 10 (CL10) and Clone 6 (CL6)

In this experiment two clones (CL10 and CL6) were inoculated with different soil microorganisms. The used materials and methods are the same reported in the section "Materials and Methods".

3.1 | Clone 10 (CL10)

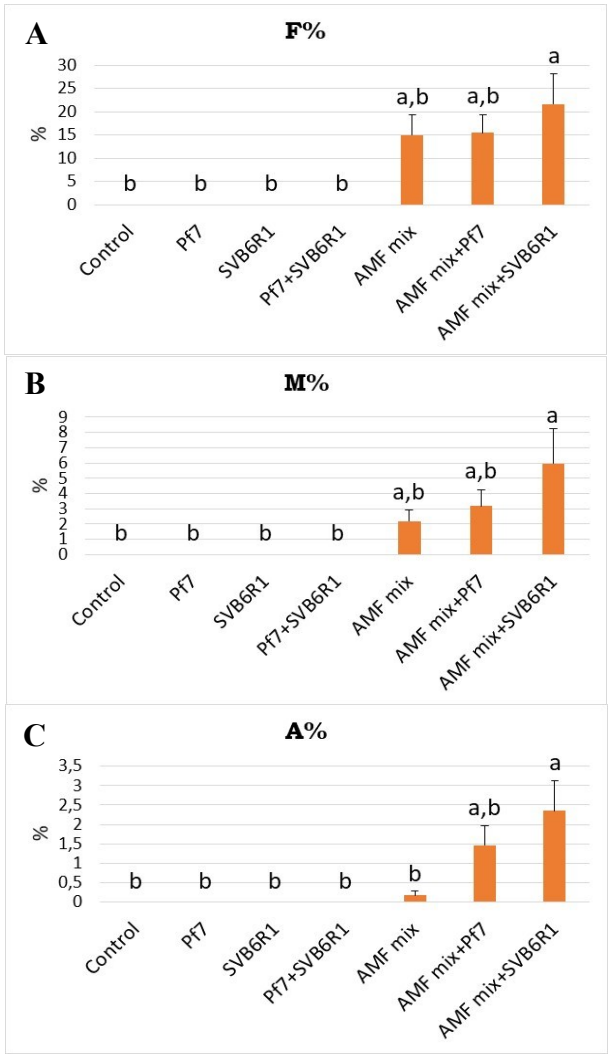
The number of plants and the soil microorganisms used in each treatment were reported in the following table:

Treatment	Label	Number of plants
No microorganisms	Control	3
P. protegens strain Pf7	Pf7	3
P. brassicacearum strain SVB6R1	SVB6R1	3
P. protegens strain Pf7 and *P. brassicacearum* strain SVB6R1	Pf7 + SVB6R1	3
Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum	AMF mix	6
Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum and *P. protegens* strain Pf7	AMF mix + Pf7	7
Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum and *P. brassicacearum* strain SVB6R1	AMF mix + SVB6R1	7

3.1.1 - Mycorrhizal colonization

The control plants and plants inoculated and co-inoculated with bacteria did not show any trace of root mycorrhization (Figure 32). Plants co-inoculated with AMF mix+SVB6R1 had the highest frequency of mycorrhization (F% about

20%; Figure 32 A) and root colonization (M% about 6%; Figure 32 B), while plants inoculated with AMF mix alone and those co-inoculated with AMF mix+Pf7 showed lower values compared to the previous ones. Nevertheless, no significant differences were registered between the before mentioned groups of plants. Only the AMF mix+SVB6R1 plants were significantly different from controls and plants inoculated or co-inoculated with both bacteria. The two-way ANOVA put on light that the "Fungus" factor was highly significant. The arbuscule abundance (A%, Figure 32 C) was higher in plants co-inoculated with AMF mix+SVB6R1, about 2.5%; while plants co-inoculated with AMF mix+Pf7 had slightly lower percentage of arbuscules (about 1.5%, followed by plants inoculate with AMF mix alone (less than 0.5%). The last two groups of plants were similar to the control plants and to the plants inoculated and co-inoculated with both bacteria whom did not show any arbuscule presence. Instead, the plants co-inoculated with AMF mix+SVB6R1 were significantly different from all the other plant treatments, except for the plants co-inoculated with AMF mix+Pf7. In this case the "Fungus" factor was significant (Two-way ANOVA). Vesicles (V%) were present in all the plants inoculated with AM fungi, but the values were low and no significant differences were detected between the various treatments (data are reported in Annex A, table 3.1).



Factor	p value
F	***
B	ns
F*B	ns

Factor	p value
F	***
B	ns
F*B	ns

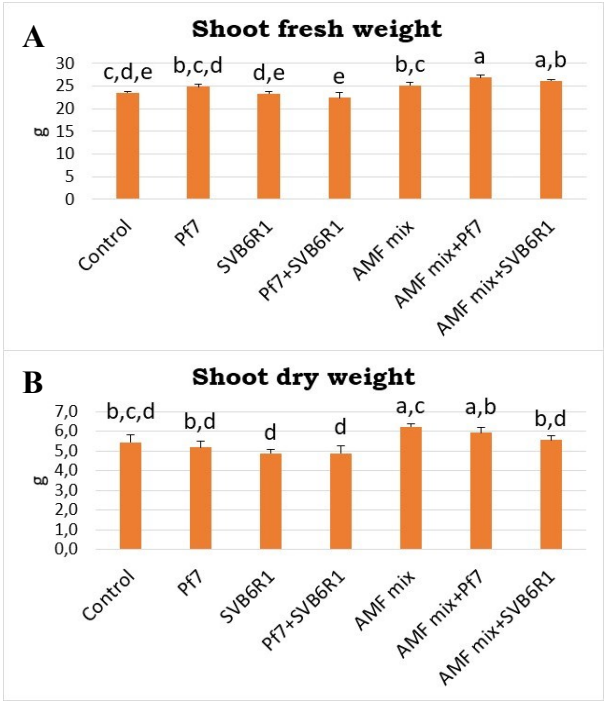
Factor	p value
F	*
B	ns
F*B	ns

Figure 32. The figure shows the mean values and the relative standard error of the mycorrhization frequency: F% (**A**), of the mycorrhization percentage: M% (**B**), and of the arbuscule abundance: A% (**C**) of *A. annua* plants (clone 10). Control: control plants, Pf7: plants inoculated with *P. protegens*; SVB6R1: plants inoculated with *P. brassicacearum*; Pf7+SVB6R1: plants co-inoculated with *P. protegens* and *P. brassicacearum*; AMF mix: plants inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum*; AMF mix+Pf7: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. protegens*; AMF mix+SVB6R1: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

3.1.2 - Morphometric and weight parameters

3.1.2.1 - Shoot fresh and dry weight, leaf fresh and dry weight, and ratio of shoot dry/fresh weight

The shoot fresh biomass (Figure 33 A) had the highest values in plants co-inoculated with AMF mix+Pf7 or SVB6R1, and these plants were similar to one another but only the plants co-inoculated with AMF mix and Pf7 were significant different from all the other plant treatments. Instead, those co-inoculated with AMF mix+SVB6R1 were different only from control plants, those inoculated with SVB6R1 and co-inoculated with both bacteria (Pf7+SVB6R1). In this latter group of plants, the lowest fresh weight of the epigeous biomass was observed, and it was significantly different from all the other plant treatments, with the exception of the control plants and plants inoculated with Pf7 alone. According to the two-way ANOVA, the "Fungus" factor was highly significant. On the other side, the dry weight of shoot biomass showed a different trend (Figure 33 B): plants inoculated with AMF mix alone had the highest dry weight, and they were significantly different from all the other plant treatments, except for the control plants and plants co-inoculated with AMF mix and Pf7. While the lowest values of dry biomass were registered in plants inoculated with SVB6R1 alone and in those co-inoculated with both bacteria, they were similar to each other but significantly different from plants inoculated with fungal mix alone and from those co-inoculated with AMF mix and Pf7. Both the "Fungus" and "Bacterium" factors were significant, as underlined by the two-way ANOVA.



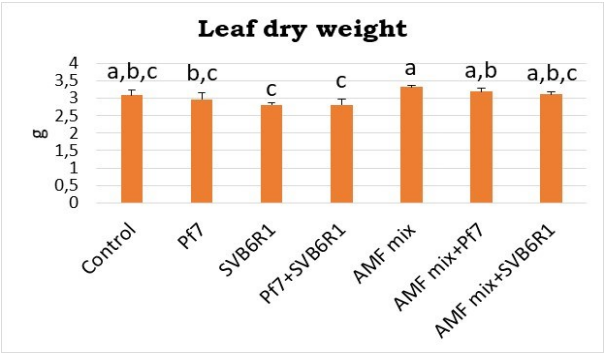
Factor	p value
F	***
B	ns
F*B	ns

Factor	p value
F	**
B	*
F*B	ns

Figure 33. The figure shows the mean values and the relative standard error of the shoot fresh (**A**) and dry (**B**) weight of *A. annua* plants (clone 10). Control: control plants, Pf7: plants inoculated with *P. protegens*; SVB6R1: plants inoculated with *P. brassicacearum*; Pf7+SVB6R1: plants co-inoculated with *P. protegens* and *P. brassicacearum*; AMF mix: plants inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum*; AMF mix+Pf7: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. protegens*; AMF mix+SVB6R1: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

The leaf fresh weight did not vary in a significant manner between the various treatments (data are reported in Annex A, table 3.1). Whereas, the leaf dry weight (Figure 34) had the same trend observed for the epigeous biomass: plants inoculated with Pf7 alone and those co-inoculated with both bacteria showed the lowest weights, and they were significantly different only from the plants inoculated with AMF mix alone and co-inoculated with AMF mix+Pf7. The plants inoculated with AMF mix alone had higher values of leaf dry biomass and

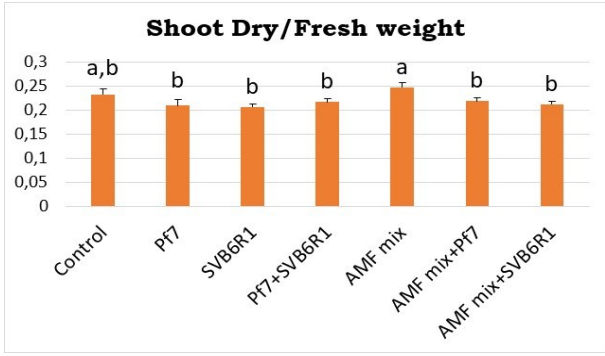
were also significantly different from those inoculated with Pf7 alone. According to the two-way ANOVA, both factors ("Fungus" and "Bacterium") influenced this parameter.



Factor	p value
F	**
B	*
F*B	ns

Figure 34. The figure shows the mean values and the relative standard error of the leaf dry weight of *A. annua* plants (clone 10). Control: control plants, Pf7: plants inoculated with *P. protegens*; SVB6R1: plants inoculated with *P. brassicacearum*; Pf7+SVB6R1: plants co-inoculated with *P. protegens* and *P. brassicacearum*; AMF mix: plants inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum*; AMF mix+Pf7: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. protegens*; AMF mix+SVB6R1: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

The ratio of shoot dry/fresh weight (Figure 35) was the highest in plants inoculated with AMF mix alone and in control plants, these two groups were similar to one another. However, only those inoculated with AMF mix were significantly different from all the other plant treatments. While, all the other treatments had lower and similar values of the ratio, showing similar to each other. In this case, only the "Bacterium" factor influenced this parameter, as showed by the two-way ANOVA.



Factor	p value
F	ns
B	**
F*B	ns

Figure 35. The figure shows the mean values and the relative standard error of the shoot dry to fresh weight of *A. annua* plants (clone 10). Control: control plants, Pf7: plants inoculated with *P. protegens*; SVB6R1: plants inoculated with *P. brassicacearum*; Pf7+SVB6R1: plants co-inoculated with *P. protegens* and *P. brassicacearum*; AMF mix: plants inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum*; AMF mix+Pf7: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. protegens*; AMF mix+SVB6R1: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

3.1.2.2 - Root fresh and dry weight, and ratio of root dry/fresh weight

Root biomass and root dry/fresh weight ratio (Figure 36) values changed in response to the inoculation of different microorganisms. In particular, plants inoculated with both bacteria and control plants showed an increase of root fresh biomass (A) comparing to all the other treatments, and were similar to each other and to the plants inoculated with Pf7 alone and co-inoculated with AMF mix and Pf7. The lowest values of the fresh weight were observed in plants inoculated with fungal mix alone, and these plants were significantly different from those co-inoculated with both bacteria or with AMF mix+Pf7, and control plants. The "Fungus" factor and its interaction with "Bacterium" factor were significant (two-way ANOVA). Instead, no significant differences in the root dry weight

between all the plant treatments were recorded (data are reported in Annex A, table 3.1).

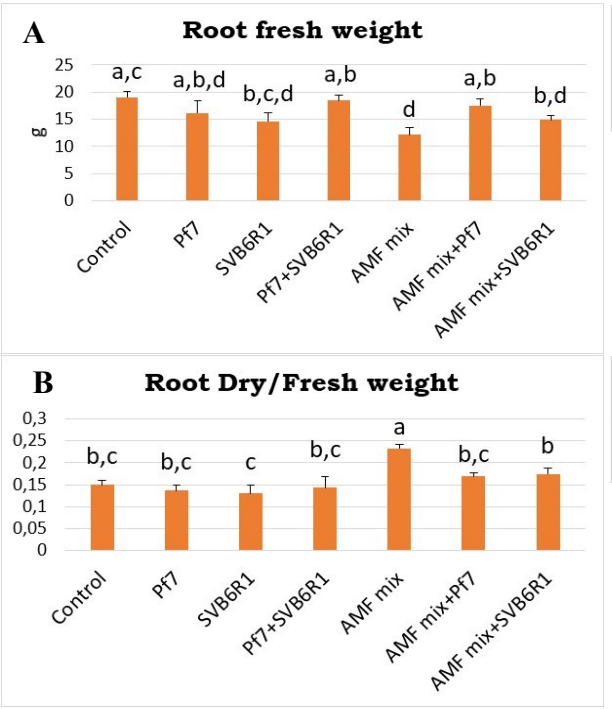


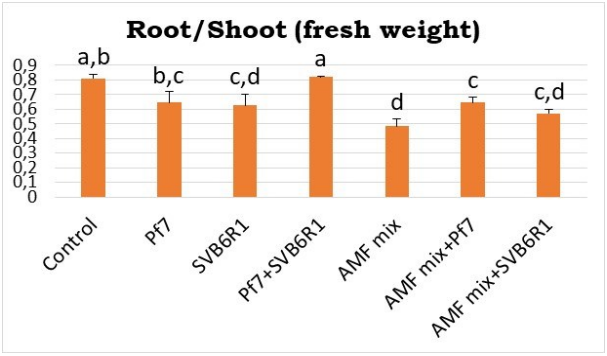
Figure 36. The figure shows the mean values and the relative standard error of the root fresh (**A**), and of the root dry to fresh weight ratio (**B**) of *A. annua* plants (clone 10). Control: control plants, Pf7: plants inoculated with *P. protegens*; SVB6R1: plants inoculated with *P. brassicacearum*; Pf7+SVB6R1: plants co-inoculated with *P. protegens* and *P. brassicacearum*; AMF mix: plants inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum*; AMF mix+Pf7: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. protegens*; AMF mix+SVB6R1: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

Different speech for the ratio of root dry/fresh weight (Figure 36 B): plants inoculated with AMF mix alone had the highest values, which were significantly different from all the other plant treatments. All the other plant treatments showed lower values of the ratio, and they were similar to each other except for the plants

inoculated with SVB6R1 alone and those co-inoculated with AMF mix+SVB6R1. According to the two-way ANOVA, the "Fungus" factor was highly significant, and the "Bacterium" factor and the interaction between the two factors (F*B) were also significant.

3.1.2.3 - Root/shoot ratio of fresh weight and root/shoot ratio of dry weight

Looking at the ratio of root/shoot fresh weight (Figure 37), it was lower in plants inoculated with Pf7 or SVB6R1 alone, in plant inoculated with AMF mix alone, and in those co-inoculated with AMF mix and Pf7 or SVB6R1, if compared to the control plants and plants co-inoculated withPf7+SVB6R1. These two latter groups of plants were similar to each other, but significantly different from all the other plant treatments, with the exception of control plants and plants inoculated with Pf7 alone.



Factor	p value
F	***
B	ns
F*B	*

Figure 37. The figure shows the mean values and the relative standard error of the root to shoot ratio of fresh weight of *A. annua* plants (clone 10). Control: control plants, Pf7: plants inoculated with *P. protegens*; SVB6R1: plants inoculated with *P. brassicacearum*; Pf7+SVB6R1: plants co-inoculated with *P. protegens* and *P. brassicacearum*; AMF mix: plants inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum*; AMF mix+Pf7: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. protegens*; AMF mix+SVB6R1: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

While plants inoculated with AMF mix were also different from those inoculated with Pf7 alone and from those co-inoculated with AMF mix+Pf7. The two-way ANOVA put on light that the "Fungus" factor was highly significant, and its interaction with the "Bacterium" factor was significant. Instead, in regard to the ratio of root/shoot dry weight, no significant differences among all the plant treatments were recorded (data are reported in Annex A, table 3.1).

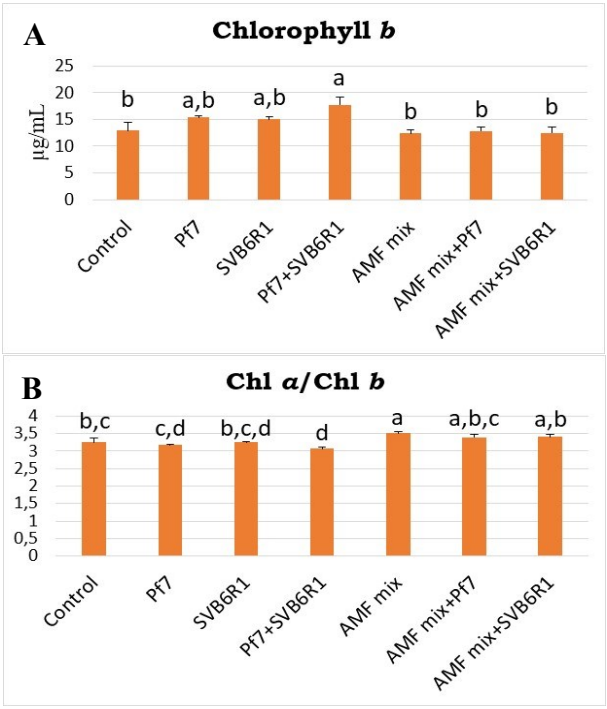
3.1.2.4 - Stem height

The stem height did not show significant differences among all the plant treatments (data are reported in Annex A, table 3.1).

3.1.3 - Leaf photosynthetic pigment concentrations

The concentration of chlorophyll *a* and carotenoids did not show significant differences between the plant treatments (data are reported in Annex A, table 3.1). Instead, the chlorophyll *b* concentration (Figure 38 A) had the highest value in plants co-inoculated with both bacteria, and these plants were significantly different from all the other plant treatments, with the exception of the plants inoculated with Pf7 or SVB6R1 alone. All the other remained plant treatments had lower concentrations and were similar to each other. In this case, only the "Fungus" factor was responsible for these differences (two-way ANOVA). Regarding the ratio between chlorophyll *a* and *b* (Figure 38 B), all the plants inoculated or co-inoculated with AMF mix had higher values of the ratio, comparing to all the other plant treatments. However, only the plants inoculated with fungal mix alone were different from the remained treatments. Whereas the lowest value of the ratio was observed in plants co-inoculated with both bacteria, and these plants were significantly different from control plants, plants inoculated with AMF mix, and from those co-inoculated with AMF mix and Pf7 or

SVB6R1. In this case, the two-way ANOVA underlined that the “Fungus” factor was highly significant.



Factor	p value
F	*
B	ns
F*B	ns

Factor	p value
F	***
B	ns
F*B	ns

Figure 38. The figure shows the mean values and the relative standard error of the chlorophyll *b* (**A**) concentrations, and chl *a* / *b* ratio (**B**) in the leaves of *A. annua* plants (clone 10). Control: control plants, Pf7: plants inoculated with *P. protegens*; SVB6R1: plants inoculated with *P. brassicacearum*; Pf7+SVB6R1: plants co-inoculated with *P. protegens* and *P. brassicacearum*; AMF mix: plants inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum*; AMF mix+Pf7: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. protegens*; AMF mix+SVB6R1: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

3.2 | Clone 6 (CL6)

The number of plants and the soil microorganisms used in each treatment are reported in the following table:

Treatment	Label	Number of plants
No microorganisms	Control	6
P. protegens strain Pf7	Pf7	6
P. brassicacearum strain SVB6R1	SVB6R1	7
P. protegens strain Pf7 and *P. brassicacearum* strain SVB6R1	Pf7 + SVB6R1	7
F. mosseae BEG12	BEG12	6
F. mosseae BEG12 and *P. protegens* strain Pf7	BEG12 + Pf7	7
F. mosseae BEG12 and *P. brassicacearum* strain SVB6R1	BEG12 + SVB6R1	7

3.2.1 - Mycorrhizal colonization

In control plants and in plants inoculated or co-inoculated with both bacteria, traces of fungal colonization in the root were not detected (Figure 39). Plants co-inoculated with BEG12+SVB6R1 showed higher frequency of mycorrhization (F% less than 5%; Figure 39 A), compared to the plants inoculated with BEG12 alone and those co-inoculated with BEG12+Pf7, which showed slightly lower values (less than 3% and about 3%, respectively). However, only the plants co-inoculated with BEG12 and Pf7 or SVB6R1 were significantly different from plants not inoculated with the fungus. The two-way ANOVA showed that the "Fungus" factor was highly significant. Looking at the percentage of colonization (M%; Figure 39 B), it followed the same trend before described for the F%, with values about of 1.2% in plants co-inoculated with BEG12+SVB6R1, 0.7% in plants co-inoculated with BEG12+Pf7, and slightly over 0.4% in plants

inoculated with BEG12 alone. Moreover, only the plants co-inoculated with fungus and SVB6R1 were significantly different from plants not inoculated with the fungus. Also in this case the "Fungus" factor was significant, as showed by the two-way ANOVA.

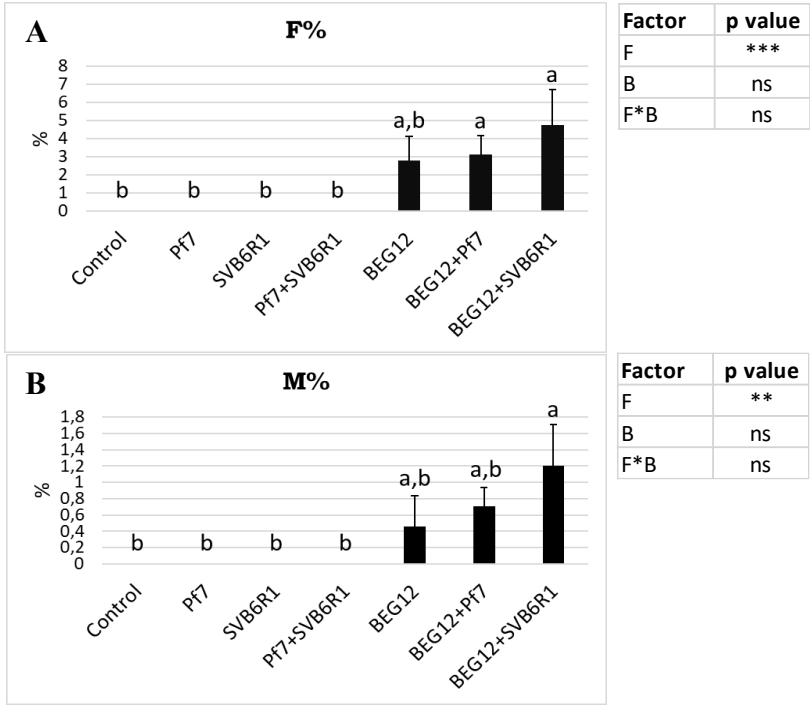


Figure 39. The figure shows the mean values and the relative standard error of the frequency of mycorrhization: F% (**A**), and of the mycorrhization percentage: M% (**B**) in the root of *A. annua* plants (clone 6). Control: control plants, Pf7: plants inoculated with *P. protegens*; SVB6R1: plants inoculated with *P. brassicacearum*; Pf7+SVB6R1: plants co-inoculated with *P. protegens* and *P. brassicacearum*; BEG12: plants inoculated with *F. mosseae*; BEG12+Pf7: plants co-inoculated with *F. mosseae* and *P. protegens*; BEG12+SVB6R1: plants co-inoculated with *F. mosseae* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

The arbuscules (A%) were present only in plants inoculated or co-inoculated with the fungus, with a low percentage of arbuscules, about and less than 0.2%, in all the three mycorrhizal groups of plants (BEG12, BEG12+Pf7, BEG12+SVB6R1),

and no differences between all the plant treatments were detected (data are reported in Annex A, table 3.2).

3.2.2 - Morphometric and weight parameters

3.2.2.1 - Shoot fresh and dry weight, leaf fresh and dry weight, and ratio of shoot dry/fresh weight

Concerning the epigeous biomass (shoot and leaves), plants did not show significant differences among all the plant treatments, both in fresh and dry weight (data are reported in Annex A, table 3.2). Also for the dry on fresh weight ratio of the shoot, plants were all similar to each other and no significantly differences between the plant treatments were recorded (data are reported in Annex A, table 3.2).

3.2.2.2 - Root fresh and dry weight, and ratio of root dry/fresh weight

The root biomass (fresh and dry) and the ratio of root dry/fresh weight did not show significant differences among all the plant treatments (data are reported in Annex A, table 3.2).

3.2.2.3 - Root/shoot ratio of fresh weight and root/shoot ratio of dry weight

No significant differences in the ratio of root/shoot fresh and dry weight between the various treatments were observed (data are reported in Annex A, table 3.2).

3.2.2.4 - Stem height

Concerning the stem height, plants were similar to each other and no significant differences among all the treatments were registered (data are reported in Annex A, table 3.2).

3.2.3 - Leaf photosynthetic pigment concentrations

Regarding the concentrations of chlorophylls (Chl *a* and *b*), their ratio, and carotenoid concentration, no significant differences between all the plant treatments were detected (data are reported in Annex A, table 3.2).

4 - Fourth experiment | Clone 26 (CL26)

In this experiment one plant clone (CL26) was inoculated with different soil microorganisms. The used materials and methods are the same reported in the section "Materials and Methods".

The number of plants and the soil microorganisms used in each treatment are reported in the following table:

Treatment	Label	Number of plants
No microorganisms	Control	5
P. brassicacearum strain SVB6R1	SVB6R1	5
F. mosseae BEG12 and *P. brassicacearum* strain SVB6R1	BEG12 + SVB6R1	5
Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum and *P. brassicacearum* strain SVB6R1	AMF mix + SVB6R1	5

4.1 - Mycorrhizal colonization

In regard to the frequency of mycorrhization (F%; Figure 40 A) and the percentage of fungal colonization (M%; Figure 40 B) in the root system, plants co-inoculated with AMF mix+SVB6R1 had higher values (less than 20% and about 4%, respectively), if compared with plants co-inoculated with BEG12 and SVB6R1. However, these differences were not significant. Whereas significant differences were reported in comparison to control plants and those inoculated with the bacterium alone (SVB6R1) whom did not show any trace of mycorrhizal colonization in the root.

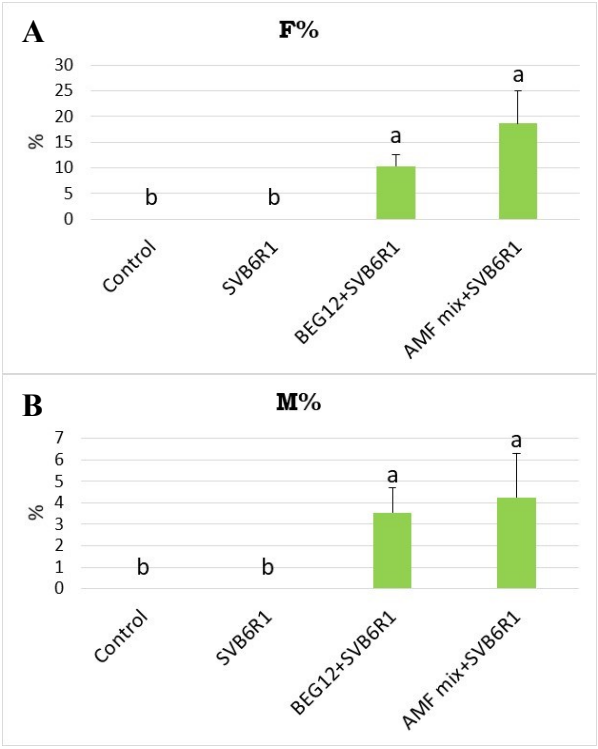


Figure 40. The figure shows the mean values and the relative standard error of the frequency of mycorrhization: F% (**A**) and of the mycorrhization percentage: M% (**B**) in the root of *A. annua* plants (clone 26). Control: control plants, SVB6R1: plants inoculated with *P. brassicacearum*; BEG12+SVB6R1: plants co-inoculated with *F. mosseae* and *P. brassicacearum*, AMF mix+SVB6R1: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). In table 4.2, Annex A is reported the one-way ANOVA, in which ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

The arbuscule abundance (A%; Figure 41 A) was the highest ever in plants co-inoculated with AMF mix and SVB6R1 (over than 1.5%), while lower percentage (about 1%) was observed in plants co-inoculated with BEG12+SVB6R1; however, only the plants co-inoculated with AMF mix+SVB6R1 were significantly different from control plants and those inoculated with SVB6R1 alone. The vesicles (V%; Figure 41 B), instead, were detected only in plants co-inoculated with AMF mix+SVB6R (about of 0.1%), and these plants were significantly different from all the other plant treatments.

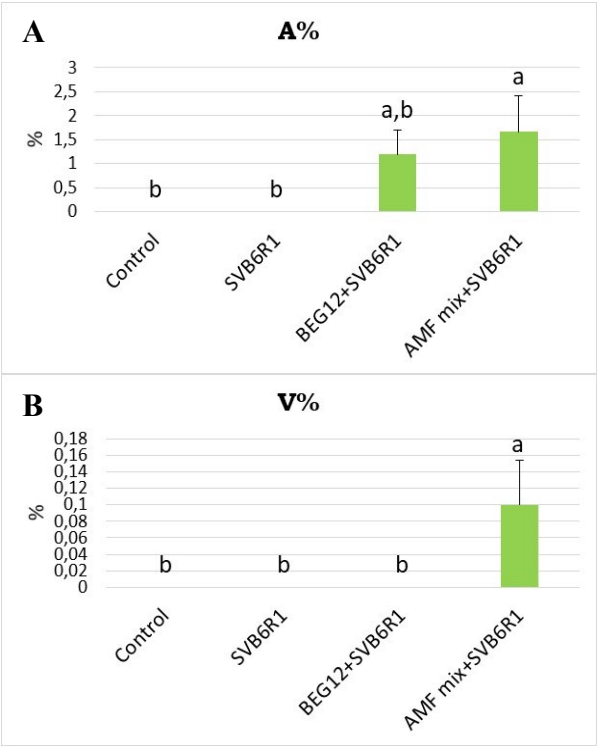


Figure 41. The figure shows the mean values and the relative standard error of the arbuscule abundance: A% (**A**) and of the vesicle abundance: M% (**B**) in the root of *A. annua* plants (clone 26). Control: control plants, SVB6R1: plants inoculated with *P. brassicacearum*; BEG12+SVB6R1: plants co-inoculated with *F. mosseae* and *P. brassicacearum*, AMF mix+SVB6R1: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). In table 4.2, Annex A is reported the one-way ANOVA, in which ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

4.2 - Morphometric and weight parameters

4.2.1 - Shoot fresh and dry weight, leaf fresh and dry weight, and ratio of shoot dry/fresh weight

Regarding the shoot fresh biomass (Figure 42), plants co-inoculated with AMF mix+SVB6R1 showed the highest weight, while the control plants, plants inoculated with the bacterium only and those co-inoculated with

BEG12+SVB6R1 had lower values. Moreover, the first group of plants were significantly different from all the other plant treatments.

Figure 42. The figure shows the mean values and the relative standard error of the shoot fresh weight of *A. annua* plants (clone 26). Control: control plants, SVB6R1: plants inoculated with *P. brassicacearum*; BEG12+SVB6R1: plants co-inoculated with *F. mosseae* and *P. brassicacearum*, AMF mix+SVB6R1: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). In Annex A, table 4.2 is reported the one-way ANOVA, in which ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

Instead, no significant differences between all the plant treatments were observed in the shoot dry weight, in the leaf biomass (fresh and dry), and in the ratio of shoot dry/fresh weight (data are reported in Annex A, table 4.1).

4.2.2 - Root fresh and dry weight, and ratio of root dry/fresh weight

Root fresh and dry weight, and their ratio did not show significant differences between all the plant treatments (data are reported in Annex A, table 4.1).

4.2.3 - Root/shoot ratio of fresh weight and root/shoot ratio of dry weight

Concerning the ratio of root/shoot fresh weight and the ratio of root/shoot dry weight, the differences between the plant treatments were not statistically significant (data are reported in Annex A, table 4.1).

4.2.4 - Stem height, root length and their ratio

The stem height had the same value in all the plants, and all the plants were similar to each other (data are reported in Annex A, table 4.1).

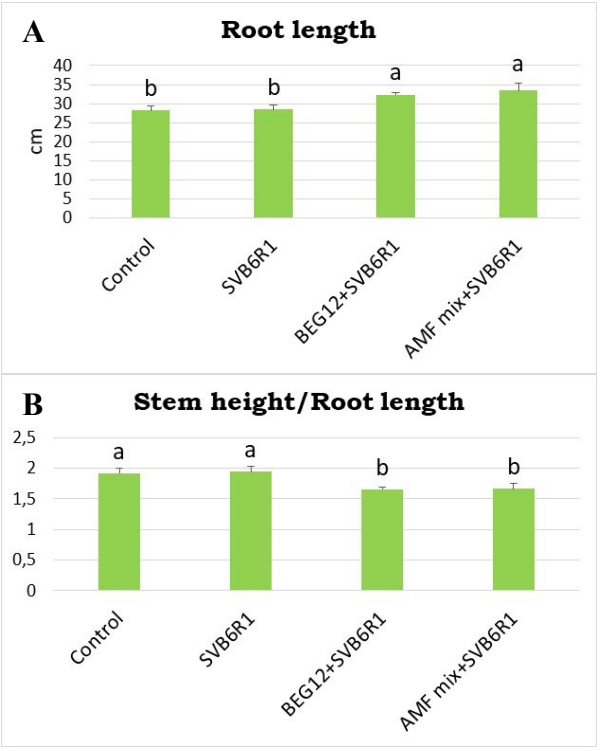


Figure 43. The figure shows the mean values and the relative standard error of the root length (**A**), and of the stem height to root length ratio (**B**) of *A. annua* plants (clone 26). Control: control plants, SVB6R1: plants inoculated with *P. brassicacearum*; BEG12+SVB6R1: plants co-inoculated with *F. mosseae* and *P. brassicacearum*, AMF mix+SVB6R1: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). In Annex A, table 4.2 is reported the one-way ANOVA, in which ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

Instead, plants co-inoculated with SVB6R1 and BEG12 or AMF mix showed the highest values of root length (Figure 43 A), and these plants were similar to one another, but significantly different from control plants and from those inoculated with SVB6R1 alone. The stem height/root length ratio (Figure 43 B) had an opposite trend: control plants and plants inoculated with the SVB6R1 alone had

the highest values, and were significantly different from plants co-inoculated with SVB6R1 and BEG12 or AMF mix whom showed lower values of the ratio.

4.3 - Leaf photosynthetic pigment concentrations

The chlorophyll *a* concentration (Figure 44 A) was the highest in plants co-inoculated with BEG12+SVB6R1 (about 50 µg/mL), but these plants were different only from those inoculated with SVB6R1 alone and from those co-inoculated with AMF mix+SVB6R1.

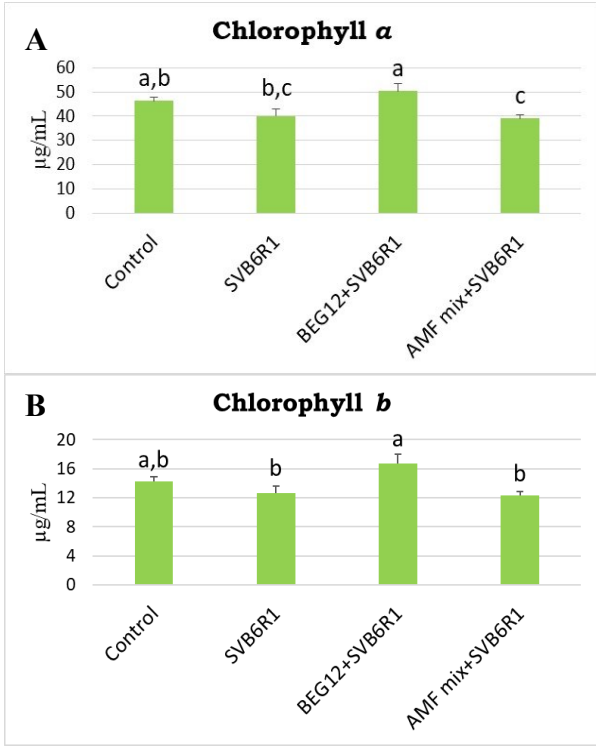


Figure 44. The figure shows the mean values and the relative standard error of the chlorophyll *a* (**A**), of the chlorophyll *b* (**B**) concentration in the leaves of *A. annua* plants (clone 26). Control: control plants, SVB6R1: plants inoculated with *P. brassicacearum*; BEG12+SVB6R1: plants co-inoculated with *F. mosseae* and *P. brassicacearum*, AMF mix+SVB6R1: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). In Annex A, table 4.2 is reported the one-way ANOVA, in which ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

This latter group of plants showed the lowest concentration of chlorophyll *a* (less than 40 µg/mL), and they were also different from control plants. The concentration of chlorophyll *b* (Figure 44 B) had the same trend before observed in the chlorophyll *a*; however, in this case, plants co-inoculated with AMF mix+SVB6R1 were not different from control plants.

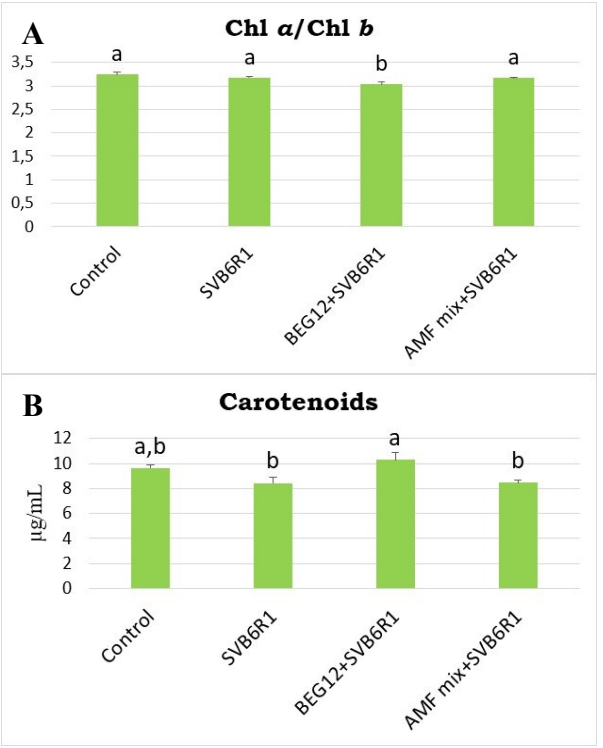


Figure 45. The figure shows the mean values and the relative standard error of the chlorophyll *a* / *b* ratio (**A**), and of the carotenoid concentration (**B**) in the leaves of *A. annua* plants (clone 26). Control: control plants, SVB6R1: plants inoculated with *P. brassicacearum*; BEG12+SVB6R1: plants co-inoculated with *F. mosseae* and *P. brassicacearum*, AMF mix+SVB6R1: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). In Annex A, table 4.2 is reported the one-way ANOVA, in which ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

Concerning the ratio between chlorophyll *a* and *b* (Figure 45 A), the plants co-inoculated with BEG12+SVB6R1 had the lowest values of this parameter, about of 3, while the other plant treatments showed values over than 3. Moreover,

plants co-inoculated with BEG12+SVB6R1 were significant different to all the other treatments. The carotenoid concentration in the leaf (Figure 45 B) had the same trend and significance level above described in chlorophyll *b* concentration.

5 - Fifth experiment | Clone 26 (CL26)

In this experiment one plant clone (CL26) was inoculated with different soil microorganisms. The used materials and methods are the same reported in the section "Materials and Methods".

The number of plants and the soil microorganisms used in each treatment are reported in the following table:

Treatment	Tag	Number of plants
No microorganisms	Control	6
Rhizophagus irregularis	Ri	6
Rhizophagus irregularis and *P. protegens* strain Pf7	Ri + Pf7	7
Rhizophagus irregularis and *P. brassicacearum* strain SVB6R1	Ri + SVB6R1	7

5.1 - Mycorrhizal colonization

Control plants did not show any trace of fungal colonization, as we expect; instead, plants co-inoculated with Ri+Pf7 had the highest frequency of mycorrhization (F% about 40%; Figure 46 A), while plants inoculated with Ri alone and those co-inoculated with Ri+SVB6R1 showed lower values compared to the previous ones, about 26%. However, these three groups of plants were similar to each other, but significantly different from control plants. The percentage of mycorrhizal colonization (M%; Figure 46 B) had the same trend abovementioned for F%: plants co-inoculated with Ri+Pf7 showed the highest M%, slightly less than 16%, and these plants were significantly different from all the other plant treatments, with the exception of the plants co-inoculated with Ri+SVB6R1. These latter showed a M% about of 11%, but they were significantly different only from control plants. Finally, plants inoculated with Ri

alone, that have a M% of about 9%, were significantly different from control plants and plants co-inoculated with Ri+Pf7.

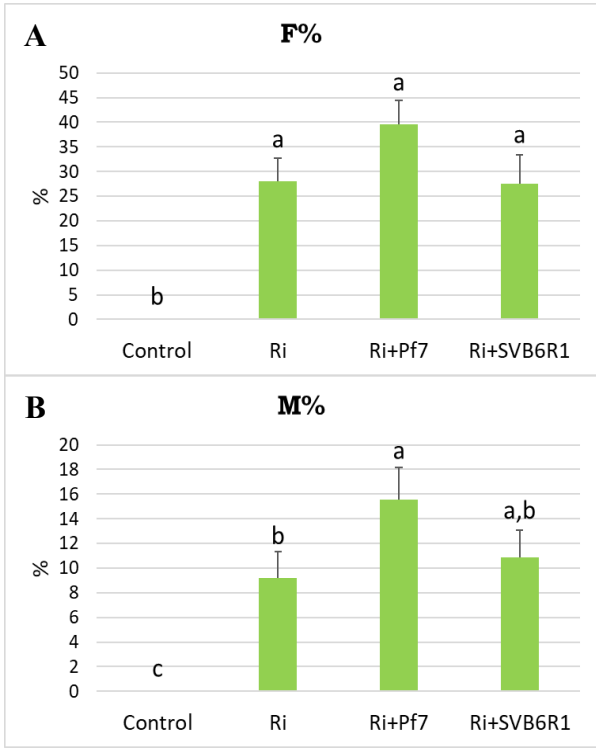


Figure 46. The figure shows the mean values and the relative standard error of the frequency of mycorrhization: F% (**A**) and of the mycorrhization percentage: M% (**B**) in the root of *A. annua* plants (clone 26). Control: control plants, Ri: plants inoculated with *R. irregularis*, Ri+Pf7: plants co-inoculated with *R. irregularis* and *P. protegens*, Ri+SVB6R1: plants co-inoculated with *R. irregularis* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). In Annex A, table 5.2 is reported the one-way ANOVA, in which ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

The arbuscule percentage (A%; Figure 47 A) also followed the same trend described for the M%. Instead, no significant differences in the percentage of vesicles in the root (V%; Figure 47 B) were observed in all the plants inoculated or co-inoculated with the fungus, but they were significantly different from control plants.

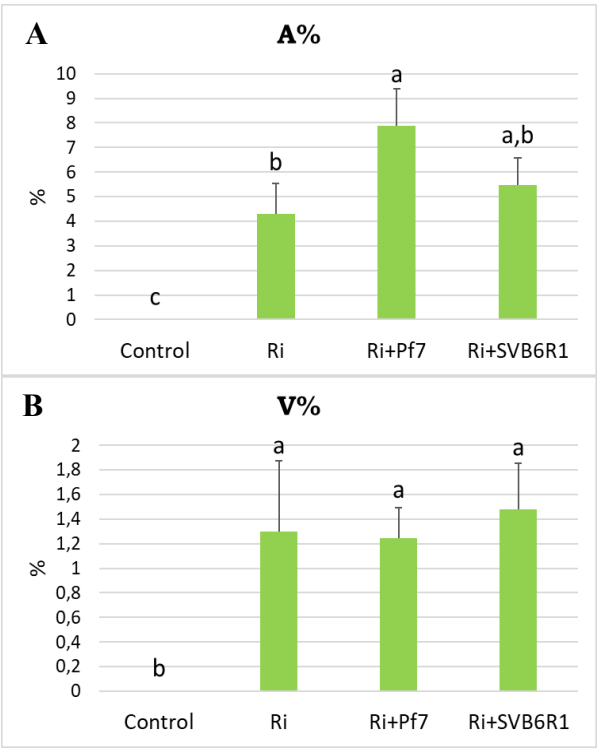


Figure 47. The figure shows the mean values and the relative standard error of the arbuscule abundance: A% (**A**) and of the vesicle abundance: V% (**B**) in the root of *A. annua* plants (clone 26). Control: control plants, Ri: plants inoculated with *R. irregularis*, Ri+Pf7: plants co-inoculated with *R. irregularis* and *P. protegens*, Ri+SVB6R1: plants co-inoculated with *R. irregularis* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). In table 5.2, Annex A is reported the one-way ANOVA, in which ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

5.2 - Morphometric and weight parameters

5.2.1 - Shoot fresh and dry weight, leaf fresh and dry weight, and ratio of shoot dry/fresh weight

Regarding the shoot biomass (fresh and dry weight, and their ratio), and the leaf fresh weight, all the plants were rather homogeneous, and no significant differences between the various treatments were reported (data are reported in Annex A, table 5.1).

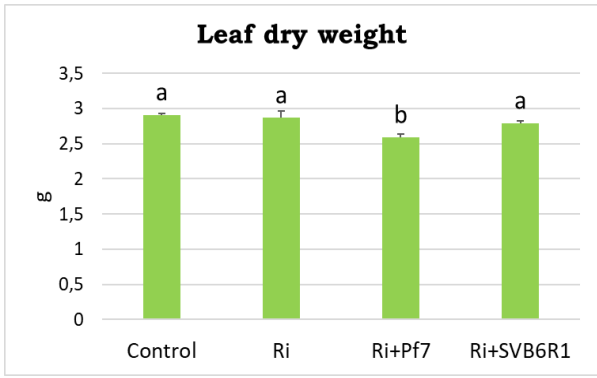


Figure 48. The figure shows the mean values and the relative standard error of the leaf dry weight of *A. annua* plants (clone 26). Control: control plants, Ri: plants inoculated with *R. irregularis*, Ri+Pf7: plants co-inoculated with *R. irregularis* and *P. protegens*, Ri+SVB6R1: plants co-inoculated with *R. irregularis* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). In Annex A, table 5.2 is reported the one-way ANOVA, in which ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

Looking at the leaf dry weight (Figure 48), plants co-inoculated with Ri+Pf7 showed a significant lower leaf dry weight, if compared to all the other plant treatments.

5.2.2 - Root fresh and dry weight, and ratio of root dry/fresh weight

Control plants had the highest root fresh weight (Figure 49 A), and they were significantly different from plants co-inoculated with Ri and Pf7 or SVB6R1. Slightly lower fresh weight was observed in plants inoculated with Ri alone, however these plants were significantly different only from those co-inoculated with Ri+Pf7. Instead, these latter group of plants showed the lowest fresh weight of the root and they were significantly different from all the other plant treatments, except for the plants co-inoculated with Ri+SVB6R1. Instead, the differences between all the plant treatments in regard to the root dry weight and the ratio of root dry/fresh weight were not statistically significant (data are reported in Annex A, table 5.1).

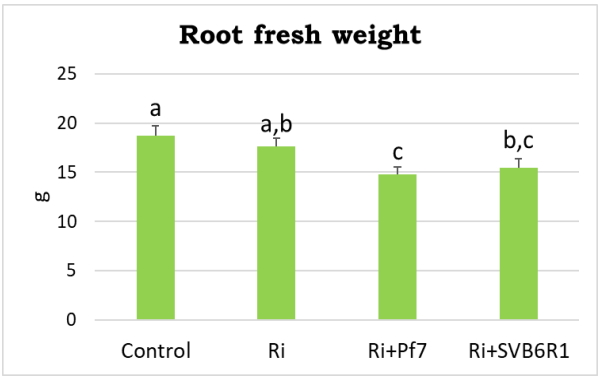


Figure 49. The figure shows the mean values and the relative standard error of the root fresh weight of *A. annua* plants (clone 26). Control: control plants, Ri: plants inoculated with *R. irregularis*, Ri+Pf7: plants co-inoculated with *R. irregularis* and *P. protegens*, Ri+SVB6R1: plants co-inoculated with *R. irregularis* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). In Annex A, table 5.2 is reported the one-way ANOVA, in which ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

5.2.3 - Root/shoot ratio of fresh weight and root/shoot ratio of dry weight

The ratio between root and shoot of fresh weight and dry weight did not show significant differences between all the plant treatments (data are reported in Annex A, table 5.1).

5.2.4 - Stem height, root length and stem/root ratio of height

Concerning the stem height, all the plants were similar to each other (data are reported in Annex A, table 5.1); whereas the plants co-inoculated with Ri and SVB6R1 showed a significantly lower length of the root (Figure 50 A) in comparison to all the other plant treatments, except for the plants co-inoculated with Ri+Pf7. These latter plants showed slightly higher values of root length, and they were significantly different only from control plants. Control plants and plants inoculated with Ri alone showed the highest root length and were similar to each other. The ratio of stem height/root length (Figure 50 B) showed an opposite trend compared to that before observed in root length: control plants and

plants inoculated with Ri alone had the lowest values compared to both co-inoculated plants. These last two groups of plants (Ri+Pf7 and Ri+SVB6R1) had higher values of the ratio and were similar to one another, but significantly different from all the other plant treatments, except for Ri+Pf7 plants.

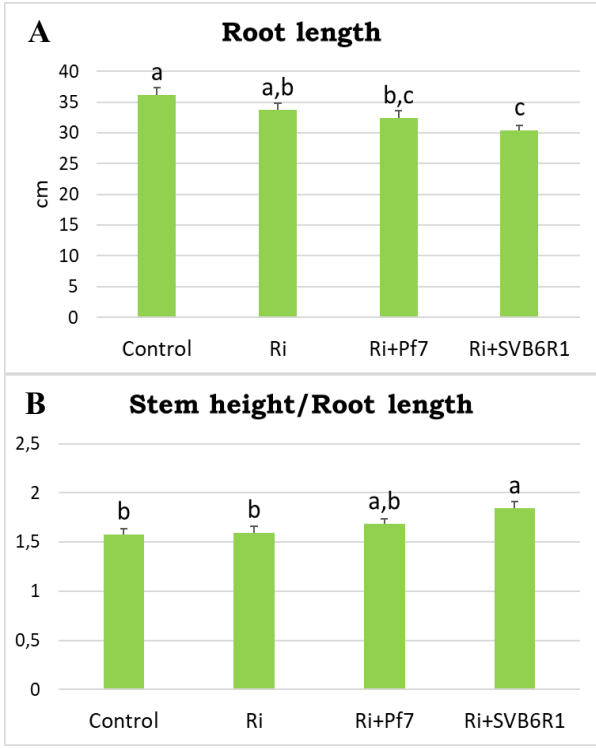


Figure 50. The figure shows the mean values and the relative standard error of the root length (**A**), and of the stem height to root length ratio (**B**) of *A. annua* plants (clone 26). Control: control plants, Ri: plants inoculated with *R. irregularis*, Ri+Pf7: plants co-inoculated with *R. irregularis* and *P. protegens*, Ri+SVB6R1: plants co-inoculated with *R. irregularis* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). In Annex A, table 5.2 is reported the one-way ANOVA, in which ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

5.3 - Leaf photosynthetic pigment concentrations

Concerning the photosynthetic system, the chlorophyll *a*, *b*, and carotenoid concentrations did not show significant differences between all the plant treatments (data are reported in Annex A, table 5.1). On the contrary, all the plants inoculated and co-inoculated showed lower values of the chlorophyll ratio

(Figure 51) comparing to the control plants, which had the highest value of this parameter.

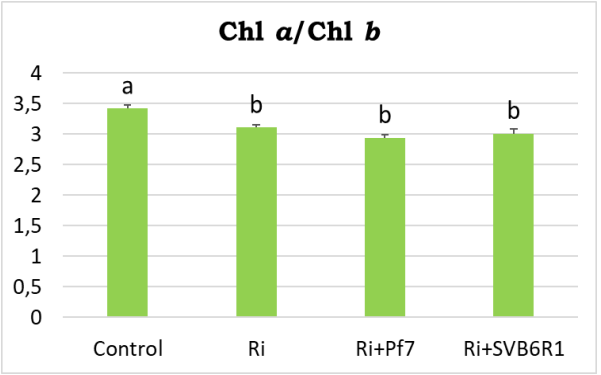


Figure 51. The figure shows the mean values and the relative standard error of the chlorophyll *a* / *b* ratio in the leaves of *A. annua* plants (clone 26). Control: control plants, Ri: plants inoculated with *R. irregularis*, Ri+Pf7: plants co-inoculated with *R. irregularis* and *P. protegens*, Ri+SVB6R1: plants co-inoculated with *R. irregularis* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$). In Annex A, table 5.2 is reported the one-way ANOVA, in which ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

6 - Sixth experiment – Clone 26 (CL26) and Clone 7 (CL7)

In this experiment two clones (CL26 and CL7) were inoculated with different soil microorganisms, and two samplings were done, at 30 and 60 days respectively. The used materials and methods are the same reported in the section "Materials and Methods".

6.1 - I° sampling | Clone 26 (CL26)

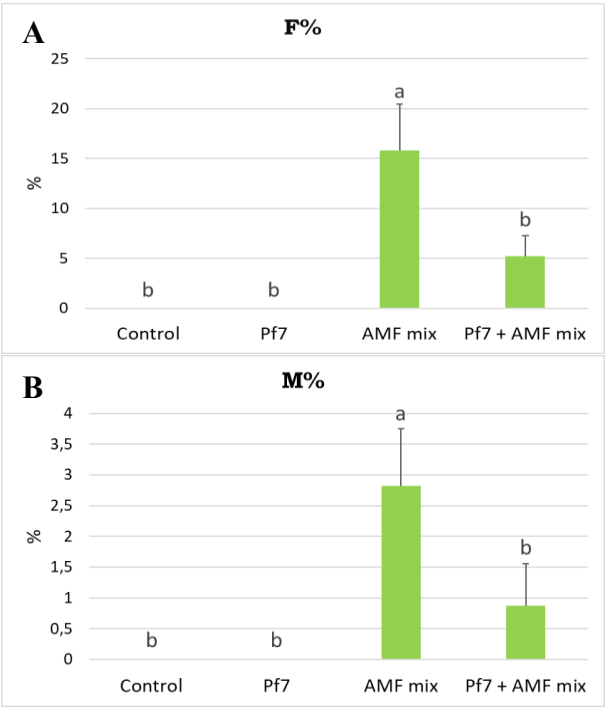
The number of plants and the soil microorganisms used in each treatment are reported in the following table:

Treatment	Tag	Number of plants
No microorganisms	Control	6
P. protegens strain Pf7	Pf7	6
Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum	AMF mix	7
Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum and *P. protegens* strain Pf7	Pf7 + AMF mix	6

6.1.1 - Mycorrhizal colonization

The fungus was not present inside the root system of control and bacterial (Pf7) plants, as we expected. The frequency of mycorrhization (F%; Figure 52 A) and the percentage of mycorrhizal colonization (M%; Figure 52 B) had similar trends: the highest values of these parameters were observed in plants inoculated with AMF mix alone, about 15% and less than 3% respectively, and these plants were significantly different from those co-inoculated with Pf7 and AMF mix, which had lower values (M% about 0.8% and F% about 5%). Furthermore, this latter

group of plants were similar to control plants. Both for F% and M%, the "Fungus" factor had a significant influence, as underlined by the two-way ANOVA.



Factor	p value
F	**
B	ns
F*B	ns

Factor	p value
F	**
B	ns
F*B	ns

Figure 52. The figure shows the mean values and the relative standard error of the mycorrhization frequency: F% (**A**) and of the mycorrhization percentage: M% (**B**) in the root of *A. annua* plants (clone 26). Control: control plants, Pf7: plants inoculated with *P. protegens*; AMF mix: plants inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum*; Pf7+AMF mix: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. protegens*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

Arbuscules were present only in plants inoculated and co-inoculated with the fungus, its percentage (A%) was higher in plants inoculated with the fungal mix alone, if compared with plants co-inoculated with both microorganisms; however, no significant differences between all the plant treatments were observed. Instead, vesicles (V%) were observed only in the root of plants

inoculated with AMF mix, even if the differences were not significant (data are reported in Annex A, table 6.1)

6.1.2 - Morphometric and weight parameters

6.1.2.1 - Shoot fresh and dry weight, leaf fresh and dry weight, and ratio of shoot dry/fresh weight

Looking at the shoot and leaf biomass, either in the fresh or dry weight, and in the ratio of shoot dry weight on fresh weight no significant differences between all the plant treatments were recorded (data are reported in Annex A, table 6.1).

6.1.2.2 - Root fresh and dry weight, and ratio of root dry/fresh weight

The root fresh and dry weight, and their ratio did not show significant differences between all the plant treatments (data are reported in Annex A, table 6.1).

6.1.2.3 - Root/shoot ratio of fresh weight and root/shoot ratio of dry weight

Regarding the ratio of root/shoot fresh weight and the root/shoot ratio of dry weight (Figure 85 B), the differences between all the plant treatments were not significant (data are reported in Annex A, table 6.1).

6.1.2.4 - Stem height, root length and their ratio

Concerning the stem height, the root length, and their ratio, the differences between all the plant treatments were statistically not significant (data are reported in Annex A, table 6.1).

6.1.3 - Leaf photosynthetic pigment concentrations

Concerning the photosynthetic pigments, the concentrations of chlorophyll *a*, *b* and carotenoids, and the ratio of chlorophyll *a* on chlorophyll *b* the observed

differences were statistically not significant (data are reported in Annex A, table 6.1).

6.1.4 – Artemisinin concentration in the leaves

Concerning the artemisinin content (data are reported in Annex A, table 6.1), the plants co-inoculated with AMF mix+Pf7 had the lowest concentration, about of 4 mg/mL, comparing with all the other plant treatments. These latter three groups of plants showed a higher concentration of artemisinin in the leaves, and were similar concentration to each other. However, the observed differences were not statistically significant.

6.2 - I° sampling | Clone 7 (CL7)

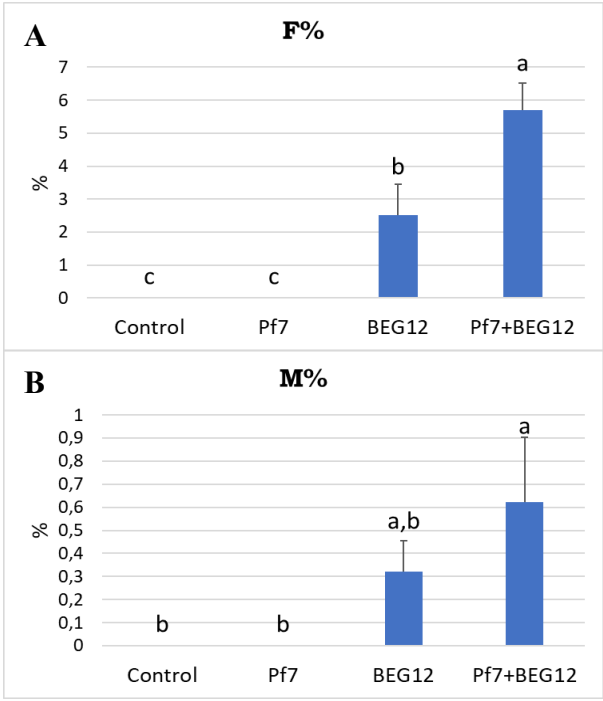
The number of plants and the soil microorganisms used in each treatment are reported in the following table:

Treatment	Label	Number of plants
No microorganisms	Control	6
P. protegens strain Pf7	Pf7	5
F. mosseae BEG12	BEG12	6
F. mosseae BEG12 and *P. protegens* strain Pf7	Pf7 + BEG12	5

6.2.1 - Mycorrhizal colonization

The mycorrhizal colonization in the root system was absent in control and bacterial plants, as we expected. The frequency of mycorrhization (F%; Figure 53 A) had the highest values in co-inoculated plants (Pf7+BEG12), about of 6%, and these plants were significantly different from all the other plant treatments. Plants inoculated with BEG12 alone showed significantly lower values of F% (less than 3%) compared to the previous one, and they were significant different to all the other plant treatments. According to the two-way ANOVA, the

"Bacterium" factor (B) and the interaction with the "Fungus" factor (F*B) were significant, and the "Fungus" factor (F) was highly significant. The percentage of mycorrhizal colonization (M%; Figure 53 B) was the highest in plants co-inoculated with Pf7+BEG12 (about of 0.6%), instead plants inoculated with BEG12 alone showed a M% about of 0.3%. The differences between these two last groups of plant were not significant, whereas both groups were significantly different from control and bacterial plants. The two-way ANOVA underlined that only the "Fungus" factor had a significant influence.



Factor	p value
F	***
B	*
F*B	*

Factor	p value
F	**
B	ns
F*B	ns

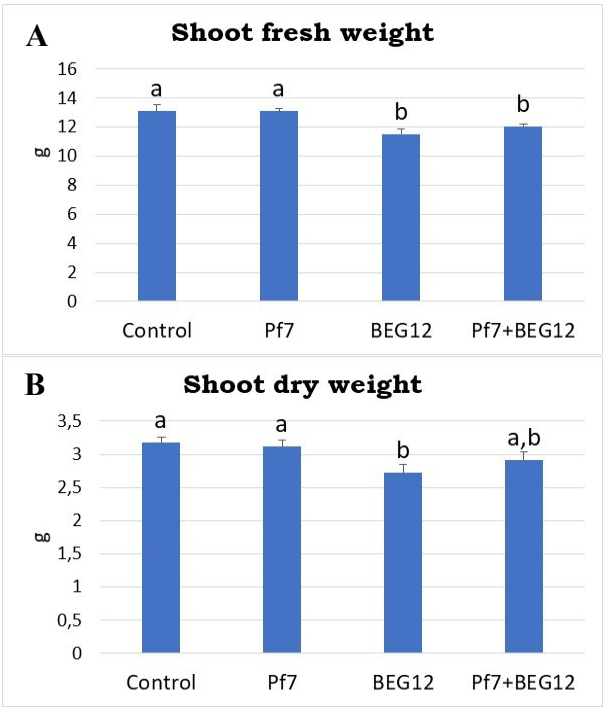
Figure 53. The figure shows the mean values and the relative standard error of the mycorrhization frequency: F% (**A**) and of the mycorrhization percentage: M% (**B**) in the root of *A. annua* plants (clone 7). Control: control plants, Pf7: plants inoculated with *P. protegens*; BEG12: plants inoculated with *F. mosseae,* Pf7+BEG12: plants co-inoculated with *F. mosseae* and *P. protegens*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

Arbuscules (A%) were present in all the plants inoculated with the fungus, but no significant differences between inoculated and co-inoculated plants were registered. Only in plants co-inoculated with both microorganisms were detected vesicles (V%), but the differences were not significant (data are reported in Annex A, table 6.2)

6.2.2 - Morphometric and weight parameters

6.2.2.1 - Shoot fresh and dry weight, leaf fresh and dry weight, and ratio of shoot dry/fresh weight

The plants inoculated with BEG12 alone and those co-inoculated with Pf7+BEG12 showed the lowest values of fresh biomass (Figure 54 A), and these two groups of plants were similar to each other, but significantly different from control and bacterial plants. As underlined by the two-way ANOVA, the "Fungus" factor was highly significant. The same trend was observed for the shoot dry weight (Figure 54 B), but the plants co-inoculated with both microorganisms were also similar to control and bacterial plants. Even for this parameter the only the "Fungus" factor was significant (two-way ANOVA). In regard to the leaf fresh and dry weight, and the ratio of shoot dry/fresh weight, no significant differences were reported between all the plant treatments (data are reported in Annex A, table 6.2).



Factor	p value
F	***
B	ns
F*B	ns

Factor	p value
F	**
B	ns
F*B	ns

Figure 54. The figure shows the mean values and the relative standard error of the shoot fresh (**A**) and of the dry (**B**) weight of *A. annua* plants (clone 7). Control: control plants, Pf7: plants inoculated with *P. protegens,* BEG12: plants inoculated with *F. mosseae*, Pf7+BEG12: plants co-inoculated with *F. mosseae* and *P. protegens*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

6.2.2.2 - Root fresh and dry weight, and ratio of root dry/fresh weight

Looking at the root biomass, both fresh and dry, and the ratio of root dry/fresh weight, the differences between all the plant treatments were not significant (data are reported in Annex A, table 6.2).

6.2.2.3 - Root/shoot ratio of fresh weight and root/shoot ratio of dry weight

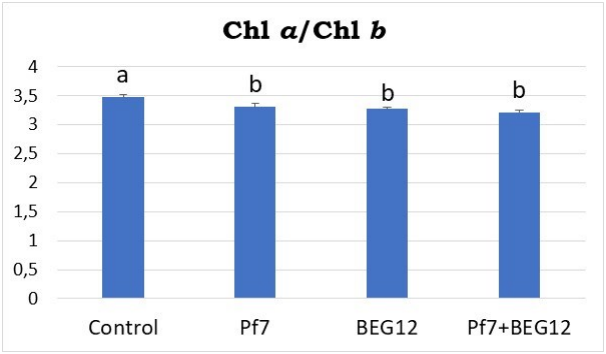
Regarding the ratio of root/shoot weight (fresh and dry), the observed differences were not statistically significant (data are reported in Annex A, table 6.2).

6.2.2.4 - Stem height, root length and their ratio

The stem height, the root length, and their ratio did not show significant differences between all the plant treatments (data are reported in Annex A, table 6.2).

6.2.3 - Leaf photosynthetic pigment concentrations

The chlorophyll (*a* and *b*) concentrations, and the concentration of carotenoids (Figure 99 B) did not show significant differences between all the plant treatments (data are reported in Annex A, table 6.2). On the other side, the chlorophyll *a*/*b* ratio (Figure 55) was significantly lower in plants inoculated and co-inoculated with microorganisms, compared to the control plants. The two-way ANOVA underlined that the "Fungus" factor and the "Bacterium" factor was significant.



Factor	p value
F	**
B	*
F*B	ns

Figure 55. The figure shows the mean values and the relative standard error of the chlorophyll *a* (**A**) and of the chlorophyll *b* (**B**) in the leaves of *A. annua* plants (clone 7). Control: control plants, Pf7: plants inoculated with *P. protegens,* BEG12: plants inoculated with *F. mosseae*, Pf7+BEG12: plants co-inoculated with *F. mosseae* and *P. protegens*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

6.2.4 - Artemisinin concentration in the leaves

Concerning the artemisinin concentration after 30 days of cultivation, no significant differences between all the plant treatments were detected (data are reported in Annex A, table 6.2).

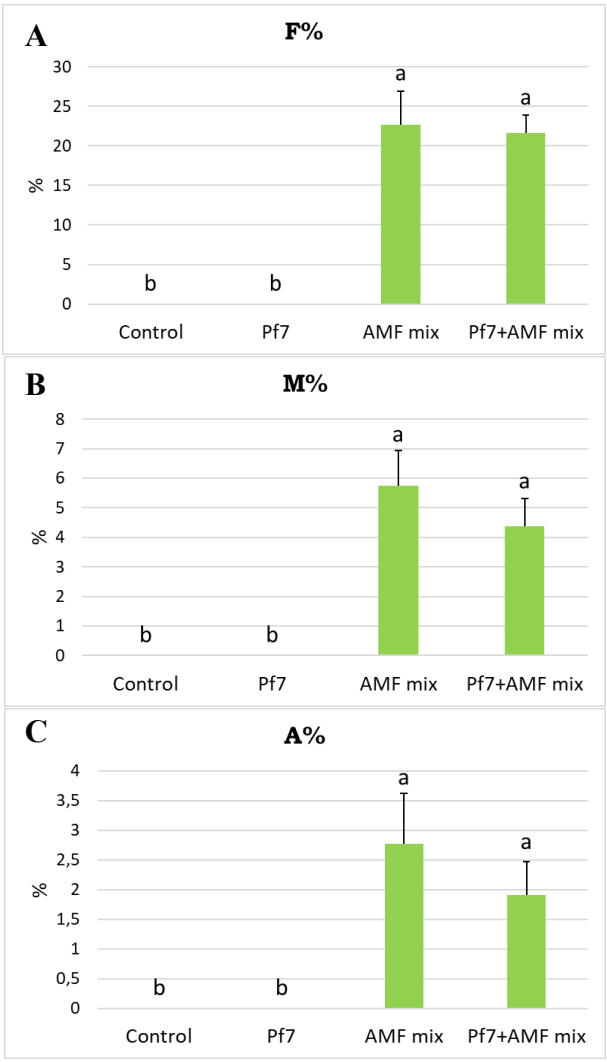
6.3 - II° sampling | Clone 26 (CL26).

The number of plants and the soil microorganisms used in each treatment are reported in the following table:

Treatment	Label	Number of plants
No microorganisms	Control	4
P. protegens strain Pf7	Pf7	5
Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum	AMF mix	5
Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum and *P. protegens* strain Pf7	Pf7 + AMF mix	6

6.3.1 - Mycorrhizal colonization

In the Figure 56 are represented the frequency of mycorrhjzation (F%; A), mycorrhizal percentage (M%; B), arbuscule abundance (A%; C). In the control plants and plants inoculated with the bacterium alone (Pf7), the fungi were not present, as we expected. Plants inoculated with different species of AMF (AMF mix) and those co-inoculated with both microorganisms (Pf7+AMF mix) had the same values of the beforementioned parameters, and these plants were similar to each other, but significantly different from control plants and those inoculated with Pf7 alone. According to the two-way ANOVA, the “Fungus” factor only was highly significant.



Factor	p value
F	***
B	ns
F*B	ns

Factor	p value
F	***
B	ns
F*B	ns

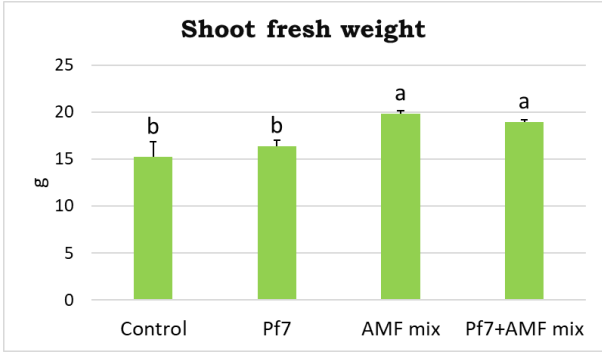
Factor	p value
F	***
B	ns
F*B	ns

Figure 56. The figure shows the mean values and the relative standard error of the mycorrhization frequency: F% (**A**), of the mycorrhization percentage: M% (**B**), and of the arbuscule abundance: A% (**C**) in the root of *A. annua* plants (clone 26). Control: control plants, Pf7: plants inoculated with *P. protegens*; AMF mix: plants inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum*; Pf7+AMF mix: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. protegens*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

6.3.2 - Morphometric and weight parameters

6.3.2.1 - Shoot fresh and dry weight, leaf fresh and dry weight, and ratio of shoot dry/fresh weight

The shoot fresh biomass (Figure 57) had the highest values in the plants inoculated with AMF mix alone and in those co-inoculated with Pf7+AMF mix, and these plant were similar to each other but significantly different from control plants and those inoculated with Pf7. These two latter plant groups had lower values compared to the previous ones and they were similar to each other. The "Fungus" factor was highly significant.

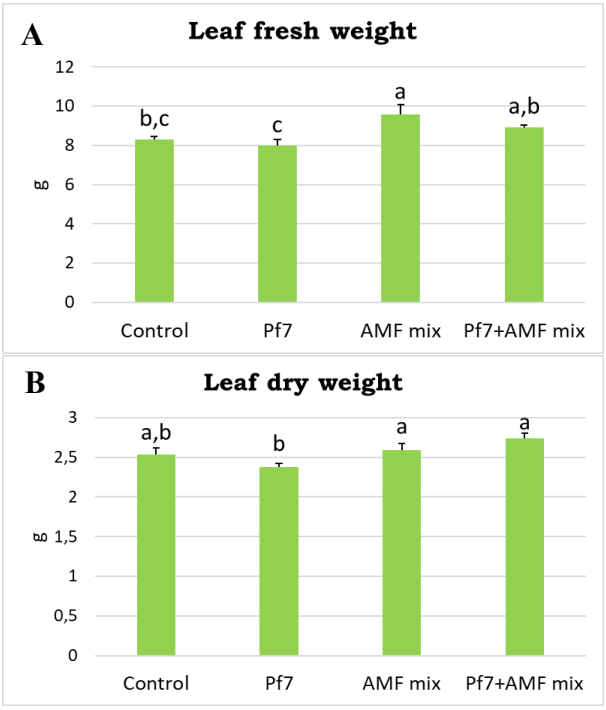


Factor	p value
F	***
B	ns
F*B	ns

Figure 57. The figure shows the mean values and the relative standard error of the shoot fresh weight of *A. annua* plants (clone 26). Control: control plants, Pf7: plants inoculated with *P. protegens*; AMF mix: plants inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum*; Pf7+AMF mix: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. protegens*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

On the other hand, no differences in the shoot dry weight between all the plant treatments were observed (data are reported in Annex A, table 6.3) even if the trend was similar to the shoot fresh weight. Regarding the leaf weights (Figure 58), the highest leaf fresh weight (A) was registered in the plants inoculated with AMF mix alone, and these plants were significantly different if compared to all

other plant treatments, except for the co-inoculated plants (Pf7+AMF mix). Instead the lowest values of leaf fresh weight was observed in the plants inoculated with Pf7 alone, and these plants were different from all other plant treatments, except for the control plants. According to the two-way ANOVA, the "Fungus" factor only was highly significant.



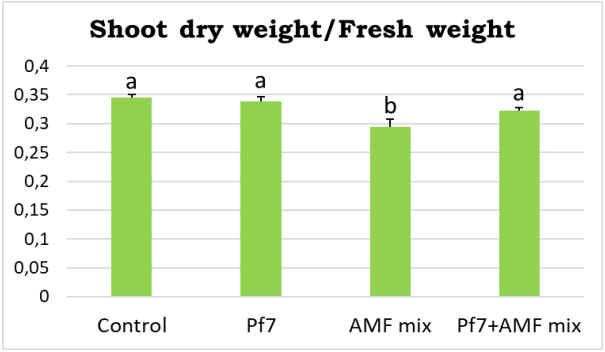
Factor	p value
F	**
B	ns
F*B	ns

Factor	p value
F	**
B	ns
F*B	*

Figure 58. The figure shows the mean values and the relative standard error of the leaf fresh (**A**) and dry (**B**) weight of *A. annua* plants (clone 26). Control: control plants, Pf7: plants inoculated with *P. protegens*; AMF mix: plants inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum*; Pf7+AMF mix: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. protegens*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

The leaf dry weight (Figure 58 B) had the same trend of the fresh one, however, in this case, the control plants, the plants inoculated with AMF mix alone and those co-inoculated (AMF+Pf7) had the highest values compared to the plants

inoculated with Pf7 alone. These three plant treatments were similar to each other, whereas the plants inoculated with Pf7 were significantly different to all other plant treatments, except for the control plants. The "Fungus" factor and the interaction between the two factors (F*B) were significant. The shoot ratio of dry on fresh weight (Figure 59) showed the lowest values in the plants inoculated with AMF mix alone, and these plants were significantly different compared to all the other plant treatments. Instead, the control plants, the plants inoculated with Pf7 alone and those co-inoculated (Pf7+AMF mix) had higher values comparing to the previous one and they were similar to each other. According to the two-way ANOVA, the "Fungus" factor (F) only was significant.

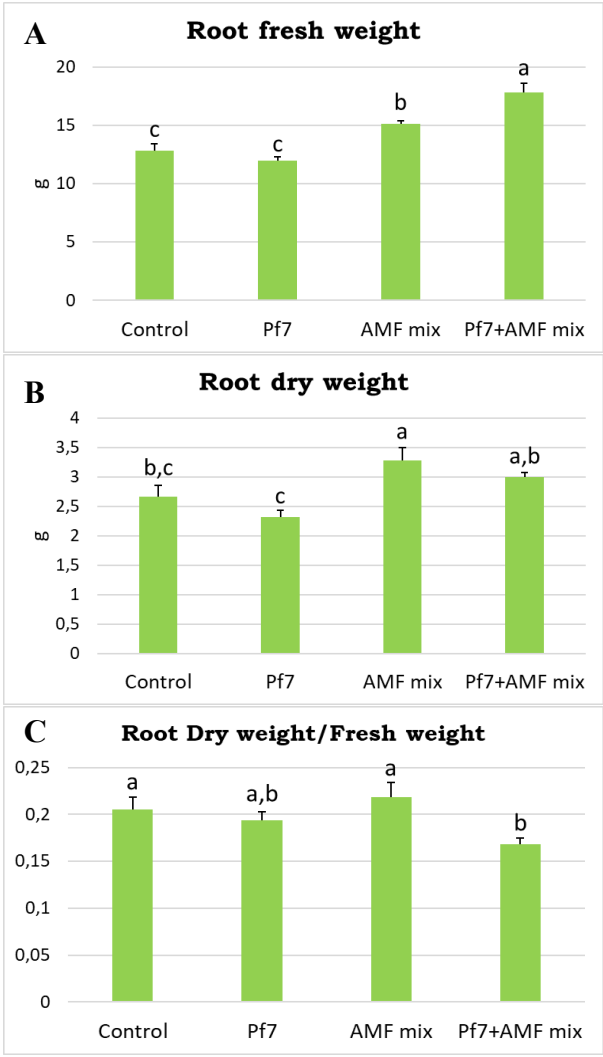


Factor	p value
F	**
B	ns
F*B	ns

Figure 59. The figure shows the mean values and the relative standard error of the shoot dry to fresh weight of *A. annua* plants (clone 26). Control: control plants, Pf7: plants inoculated with *P. protegens*; AMF mix: plants inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum*; Pf7+AMF mix: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. protegens*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

6.3.2.2 - Root fresh and dry weight, and ratio of root dry/fresh weight

The root fresh weight (Figure 60 A) had the highest values in the co-inoculated plants (Pf7+AMF mix), and these plants were significantly different from all the other plant treatments. Plants inoculated with AMF mix alone showed lower values in comparison to the previous one, and these plants were also significantly different from all other plant treatments. Control plants and those inoculated with Pf7 alone had the lowest root fresh weight, and they were similar to each other. The "Fungus" factor (F) was highly significant, and the interaction between the two factors (F*B) was significant (two-way ANOVA). The root dry weight (Figure 60 B) had higher values in the plants inoculated with fungal AMF mix alone and in those co-inoculated (Pf7+AMF mix), if compared to all other plant treatments; they were significantly different from control plants and plants inoculated with Pf7 alone, except for the co-inoculated plants that were similar to the control plants. Also in this case, control plants and plants inoculated with Pf7 alone had lower values, and they were similar to each other. According to the two-way ANOVA, the "Fungus" factor was highly significant. The ratio of root dry/fresh weight (Figure 60 C) decreased in the co-inoculated plants (Pf7+AMF mix) comparing to all other plant treatments, and these plants were significantly different to all other plant treatments, except for those inoculated with Pf7 alone. Instead, control plants, plants inoculated with Pf7 alone and co-inoculated plants (Pf7+AMF mix) had higher values and they were similar to each other. In this case the "Bacterium" factor (B) only was significant.



Factor	p value
F	***
B	ns
F*B	**

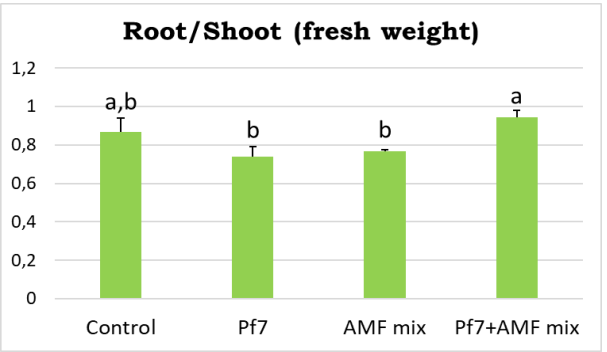
Factor	p value
F	***
B	ns
F*B	ns

Factor	p value
F	ns
B	*
F*B	ns

Figure 60. The figure shows the mean values and the relative standard error of the root to shoot ratio of fresh weight (**A**) and of the root to shoot ratio of dry weight (**B**) of *A. annua* plants (clone 26). Control: control plants, Pf7: plants inoculated with *P. protegens*; AMF mix: plants inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum*; Pf7+AMF mix: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. protegens*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

6.3.2.3 - Root/shoot ratio of fresh weight and root/shoot ratio of dry weight

Looking at the root/shoot ratio of fresh weight (Figure 61), it had the highest values in the co-inoculated plants (Pf7+AMF mix), and these plants were significantly different from those inoculated with Pf7 alone and from those inoculated with AMF mix alone. Instead, these latter two plant treatments showed lower values and they were similar to each other and to the control plants. According to the two-way ANOVA, the interaction between the two factors (F*B) only was significant.



Factor	p value
F	ns
B	ns
F*B	**

Figure 61. The figure shows the mean values and the relative standard error of the root to shoot ratio of fresh weight of *A. annua* plants (clone 26). Control: control plants, Pf7: plants inoculated with *P. protegens*; AMF mix: plants inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum*; Pf7+AMF mix: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. protegens*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

No significant differences in the root/shoot ratio of dry weight between all the plant tratments were observed (data are reported in Annex A, table 6.3).

6.3.2.4 - Stem height, root length and their ratio

The stem height, the root length, and the stem height/root length ratio did not show significant differences between all the plant treatments (data are reported in Annex A, table 6.3).

6.3.3 - Leaf photosynthetic pigment concentrations

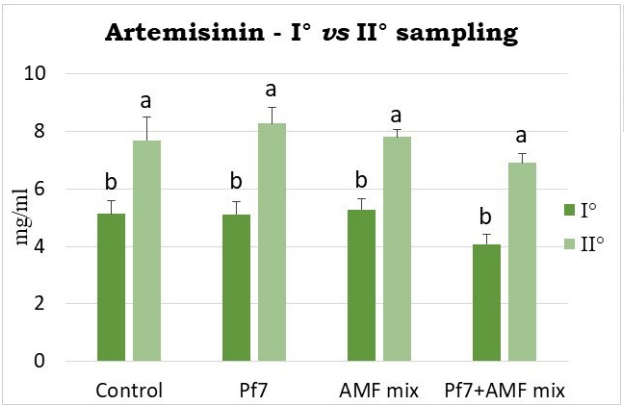
The concentrations of chlorophylls (*a* and *b*) and carotenoids, and the chlorophyll *a*/*b* ratio did not show significant differences between all the plant treatments (data are reported in Annex A, table 6.3).

6.3.4 -Artemisinin concentration in the leaves

The concentrations of artemisinin did not show significant differences between all the plant treatments (data are reported in Annex A, table 6.3). Nevertheless, the plants inoculated with Pf7 had slightly higher concentration of artemisinin in comparison with all the other plant treatments; instead, plants co-inoculated with both microorganisms (Pf7+AMF mix) showed a lower artemisinin concentration (about 7 mg/mL), if compared to the previous ones.

6.3.5 - Artemisinin concentration in the leaves: first versus second sampling

The comparison of the leaf artemisinin concentration between the first and the second sampling (Figure 62) showed that it significantly increased over the time, in each single treatment. The “Time” factor was the only responsible for this result, as underlined by the two-way ANOVA.



Factor	p value
Tr	ns
Ti	***
Tr*Ti	ns

Figure 62. The figure shows the mean values and the relative standard error of the artemisinin concentration in the leaves in the I° sampling (dark green) and in the II° sampling (light green) of A. annua plants (clone 26). Control: control plants, Pf7: plants inoculated with *P. protegens*; AMF mix: plants inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum*, Pf7+AMF mix: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. protegens*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which Tr: Treatment factor; Ti: Time factor; Tr*Ti: interaction between Treatment and Time factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

6.4 - II° sampling | Clone 7 (CL7)

The number of plants and the soil microorganisms used in each treatment are reported in the following table:

Treatment	Label	Number of plants
No microorganisms	Control	5
P. protegens strain Pf7	Pf7	5
F. mosseae BEG12	BEG12	6
F. mosseae BEG12 and *P. protegens* strain Pf7	Pf7 + BEG12	5

6.4.1 - Mycorrhizal colonization

Control plants and plants inoculated with Pf7 alone did not show any traces of mycorrhizal colonization, as we expect. The frequency of mycorrhization (F%;

Figure 63 A) was about of 15% in the co-inoculated plants (Pf7+BEG12) while lower values were observed in plants inoculated with BEG12 alone; however, these two plant treatments were not significantly different to each other.

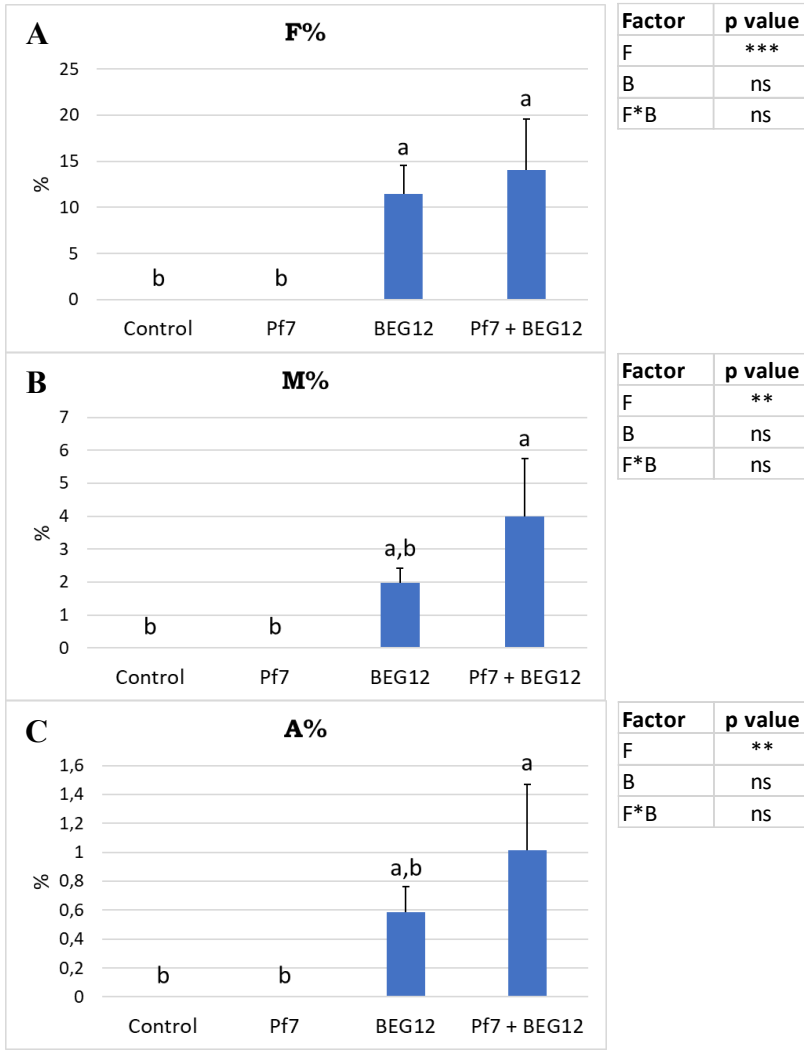


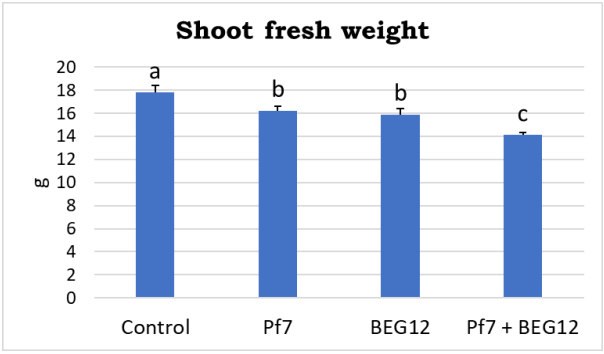
Figure 63. The figure shows the mean values and the relative standard error of the mycorrhization frequency: F% (**A**), of the mycorrhization percentage: M% (**B**), and of the arbuscule abundance: A% (**C**) in the root of *A. annua* plants (clone 7). Control: control plants, Pf7: plants inoculated with *P. protegens*; BEG12: plants inoculated with *F. mosseae,* Pf7+BEG12: plants co-inoculated with *F. mosseae* and *P. protegens*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

According to the two-way ANOVA, the “Fungus” factor was highly significant. The mycorrhizal percentage (M%, Figure 63 B) and the arbuscule abundance (A%, Figure 63 C A) had higher levels in the co-inoculated plants (Pf7+BEG12), about of 4% and 1% respectively, if compared with the plants inoculated with BEG12 alone, but no differences were reported to each other. Moreover, the plants inoculated with BEG12 alone were similar to the control plants and those inoculated with Pf7 alone. The “Fungus” factor only was significant (two-way ANOVA). Instead, vesicles (V%) were observed only in the co-inoculated plants (Pf7+BEG12), however no significant differences between all the plant treatments were observed (data are reported in Annex A, table 6.4).

6.4.2 - Morphometric and weight parameters

6.4.2.1 - Shoot fresh and dry weight, leaf fresh and dry weight, and ratio of shoot dry/fresh weight

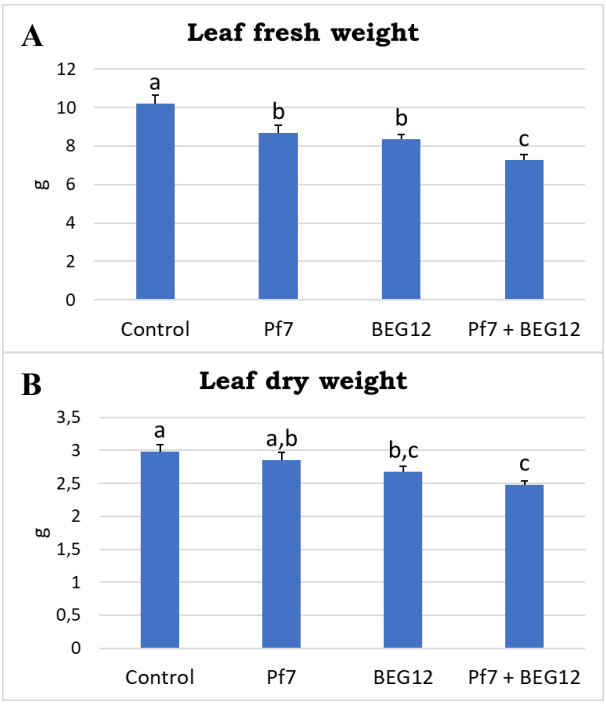
The shoot fresh weight (Figure 64) had the highest values in the control plants, and these plants were significantly different from all the other plant treatments. Slightly lower values were reported in the plants inoculated with Pf7 or BEG12 alone, and these two latter plant treatments were also similar to each other but significantly different to all the other plant treatments. Instead, co-inoculated plants (Pf7+BEG12) had the lowest values of fresh biomass, and they were, moreover, significantly different to all the other plant treatments. According to the two-way ANOVA, the “Bacterium” factor was significant, while the “Fungus” factor was highly significant. Regarding the shoot dry weight (data are reported in Annex A, table 6.4), no significant differences were reported between all the different plant treatments.



Factor	p value
F	***
B	**
F*B	ns

Figure 64. The figure shows the mean values and the relative standard error of the shoot fresh weight of *A. annua* plants (clone 7). Control: control plants, Pf7: plants inoculated with *P. protegens,* BEG12: plants inoculated with *F. mosseae*, Pf7+BEG12: plants co-inoculated with *F. mosseae* and *P. protegens*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

The leaf fresh weight (Figure 65 A) had the same trend observed in the shoot fresh weight; according to the two-way ANOVA, the "Bacterium" factor was significant, while the "Fungus" factor was highly significant. In the leaf dry weight (Figure 65 B) was confirmed the same abovementioned trend, but the plants inoculated with Pf7 alone and the control plants were similar to each other, and the plants inoculated with BEG12 alone and those co-inoculated with both microorganisms (Pf7+BEG12) were also similar to each other. In this case, as underlined by the two-way ANOVA, the "Fungus" factor only was significant.

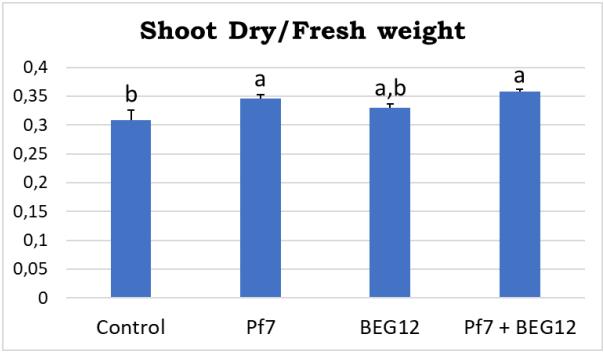


Factor	p value
F	***
B	**
F*B	ns

Factor	p value
F	**
B	ns
F*B	ns

Figure 65. The figure shows the mean values and the relative standard error of the leaf fresh (**A**) and of the dry (**B**) weight of *A. annua* plants (clone 7). Control: control plants, Pf7: plants inoculated with *P. protegens,* BEG12: plants inoculated with *F. mosseae*, Pf7+BEG12: plants co-inoculated with *F. mosseae* and *P. protegens*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

Looking at the ratio of shoot dry/fresh weight (Figure 66), the highest values were reported in the plants inoculated with Pf7 alone and in those co-inoculated with both microorganisms (Pf7+BEG12), while plants inoculated with BEG12 alone had intermediate values, and control plants had the lowest values of the ratio. Significant differences were reported between control plants and those inoculated with Pf7 alone and co-inoculated with both microorganisms (Pf7+BEG12). According to the two-way ANOVA, the "Bacterium" factor only was significant.



Factor	p value
F	ns
B	**
F*B	ns

Figure 66. The figure shows the mean values and the relative standard error of the shoot dry to fresh weight of *A. annua* plants (clone 7). Control: control plants, Pf7: plants inoculated with *P. protegens,* BEG12: plants inoculated with *F. mosseae*, Pf7+BEG12: plants co-inoculated with *F. mosseae* and *P. protegens*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

6.4.2.2 - Root fresh and dry weight, and ratio of root dry/fresh weight

The root biomass, both fresh and dry, and the ratio of root dry/fresh weight (data are reported in Annex A, table 6.4) did not show significant differences between all the plant treatments.

6.4.2.3 - Root/shoot ratio of fresh weight and root/shoot ratio of dry weight

Concerning the root/shoot ratio of fresh and dry weight (data are reported in Annex A, table 6.4), no significant differences were detected between the various plant treatments.

6.4.2.4 - Stem height, root length and their ratio

The stem height, the root length, and their ratio, did not show significant differences between all the plant treatments (data are reported in Annex A, table 6.4).

6.4.3 - Leaf photosynthetic pigment concentrations

Regarding the photosynthetic system, the highest concentration of chlorophyll *a* (Figure 67 A) was observed in the control and co-inoculated (Pf7+BEG12) plants, and these plants were significantly different from those inoculated with Pf7 alone. The two-way ANOVA underlined that the interaction between the two factors (F*B) was significant.

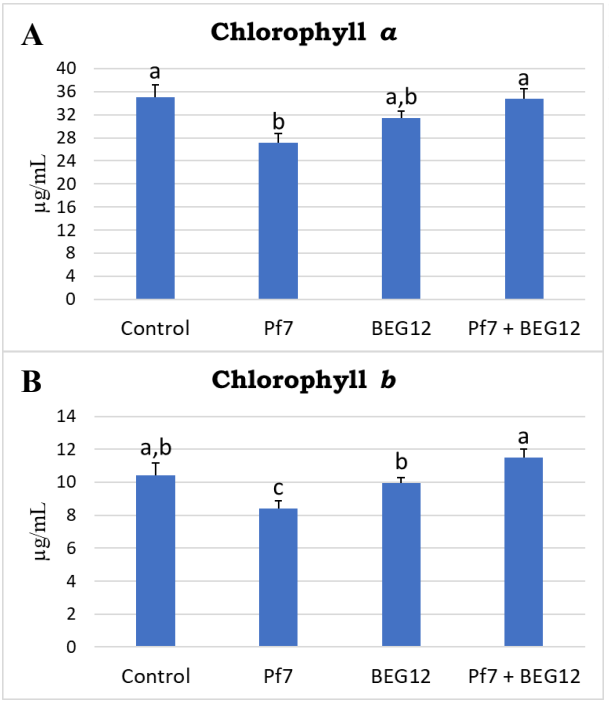


Figure 67. The figure shows the mean values and the relative standard error of the chlorophyll *a* (**A**) and of the chlorophyll *b* (**B**) in the leaves of *A. annua* plants (clone 7). Control: control plants, Pf7: plants inoculated with *P. protegens,* BEG12: plants inoculated with *F. mosseae*, Pf7+BEG12: plants co-inoculated with *F. mosseae* and *P. protegens*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

The concentration of chlorophyll *b* (Figure 67 B) showed the same trend of the chlorophyll *a*; however, plants inoculated with BEG12 alone were significant different from those co-inoculated and inoculated with Pf7. In this case, also the

"Fungus" factor, beyond the interaction between the two factors (F*B) was significant (two-way ANOVA).

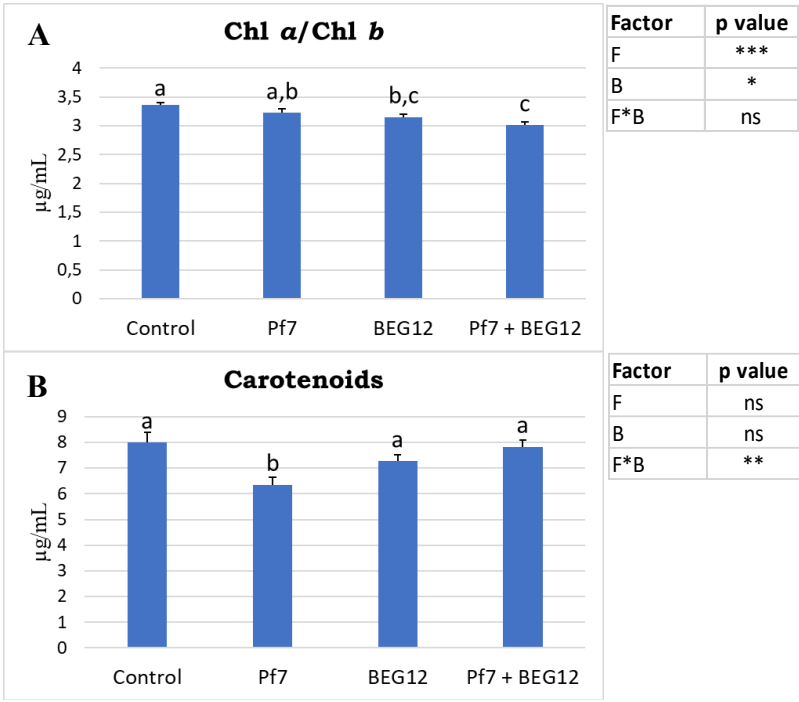


Figure 68. The figure shows the mean values and the relative standard error of the chlorophyll *a* / *b* ratio (**A**) and of the carotenoid concentration (**B**) in the leaves of *A. annua* plants (clone 7). Control: control plants, Pf7: plants inoculated with *P. protegens,* BEG12: plants inoculated with *F. mosseae*, Pf7+BEG12: plants co-inoculated with *F. mosseae* and *P. protegens*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

The chlorophyll ratio (Figure 68 A) had the highest values in the control plants, and these latter were significant different from plants inoculated with BEG12 alone and co-inoculated with both microorganisms (Pf7+BEG12). The lowest value was recorded in the co-inoculated plants, and they were different from plants inoculated with Pf7 alone but similar to those inoculated with BEG12 alone. Instead, these two last groups of plants showed intermediate values of the ratio. According to the two-way ANOVA, the "Bacterium" factor was

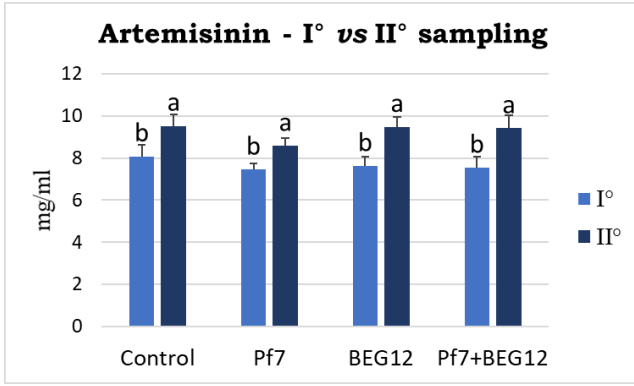
significant, and the "Fungus" factor was highly significant. Finally, the carotenoid concentrations (Figure 68 B) had the same trend recorded in the chlorophyll concentrations, however, in this case plants inoculated with Pf7 alone only were significantly different from all the other plant treatments. Also in this case, as reported for the chlorophyll *a* concentration, only the interaction between the two factors (F*B) was significant.

6.4.4 - Artemisinin concentration in the leaves

The artemisinin concentration did not vary in a significant manner among all the plant treatments (data are reported in Annex A, table 6.4). However, in the bacterial plants (Pf7) a reduction in its concentration compared to all the other plant treatments was noticed.

6.4.5 - Artemisinin concentration in the leaves: first versus second sampling

Looking at the comparison of the artemisinin concentration between the first and the second sampling (Figure 69), the concentration significantly increased over the time in all the plant treatments. According to the two-way ANOVA, the "Time" factor only was highly significant.



Factor	p value
Tr	ns
Ti	***
Tr*Ti	ns

Figure 69. The figure shows the mean values and the relative standard error of the artemisinin concentration in the leaves in the I° sampling (light blue) and in the II° sampling (dark blue) of *A. annua* plants (clone 7). Control: control plants, Pf7: plants inoculated with *P. protegens*; BEG12: plants inoculated with *F. mosseae*, Pf7+BEG12: plants co-inoculated with *F. mosseae* and *P. protegens*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which Tr: Treatment factor; Ti: Time factor; Tr*Ti: interaction between Treatment and Time factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

7 - Leaf volatile composition in the CL26 plants in the sixth experiment

7.1 - Main classes of leaf volatile molecules in the first sampling

In these plants, 260 molecules were detected. The 30.8% of these molecules was in common among all the plant treatments, while about 10% was exclusive of each single plant group (C, Pf7, AMF), with the exception for the plants co-inoculated with Pf7+AMF mix which showed a lower percentage (3.8%), if compared to the previous ones (Figure 70).

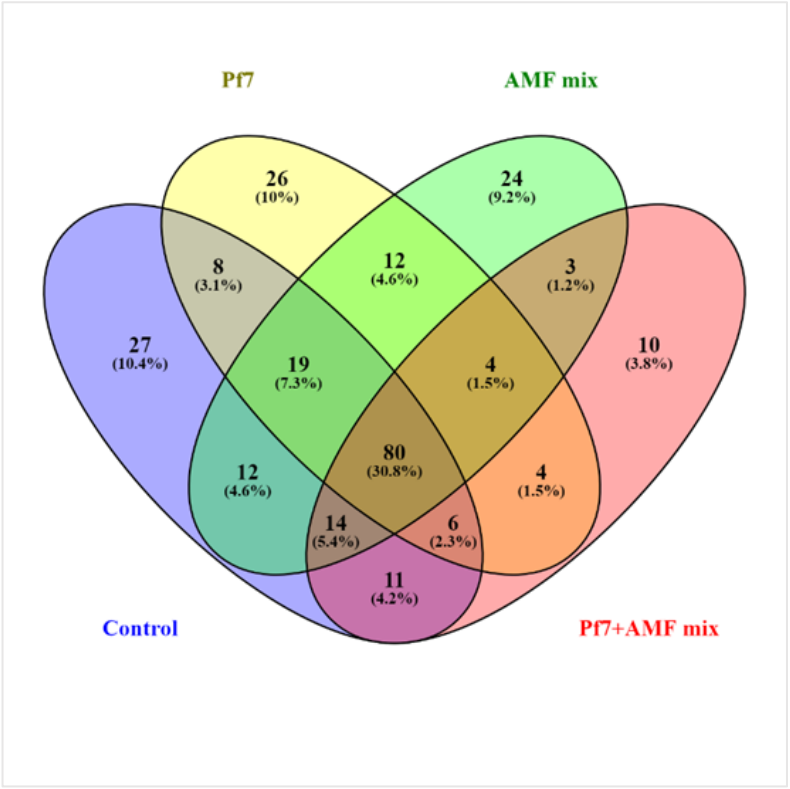


Figura 70. Eulero-Venn diagram shows the distribution of leaf volatile molecules in *A. annua* (clone 26) among the different plant treatments, after 30 days of cultivation. C: control plants, Pf7: plants inoculated with *P. protegens*, AMF mix: plants inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum*, Pf7+AMF mix: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. protegens*.

The molecules were classified into chemical classes and they had a different distribution, according to the different treatments (Figure 71). In comparison to the control plants (Figure 71 A), the percentage of alkanes increased in the plants inoculated with Pf7 (Figure 71 B) and AMF mix (Figure 71 C) alone, whereas this class of compounds were not present in the co-inoculated plants (Pf7+AMF mix; Figure 71 D). The alkenes increased in all the plants inoculated and co-inoculated with microorganisms; on the contrary, alcohols and ketones decreased in all the inoculated and co-inoculated plants (Figure 71 B, C, D), compared to the control ones.

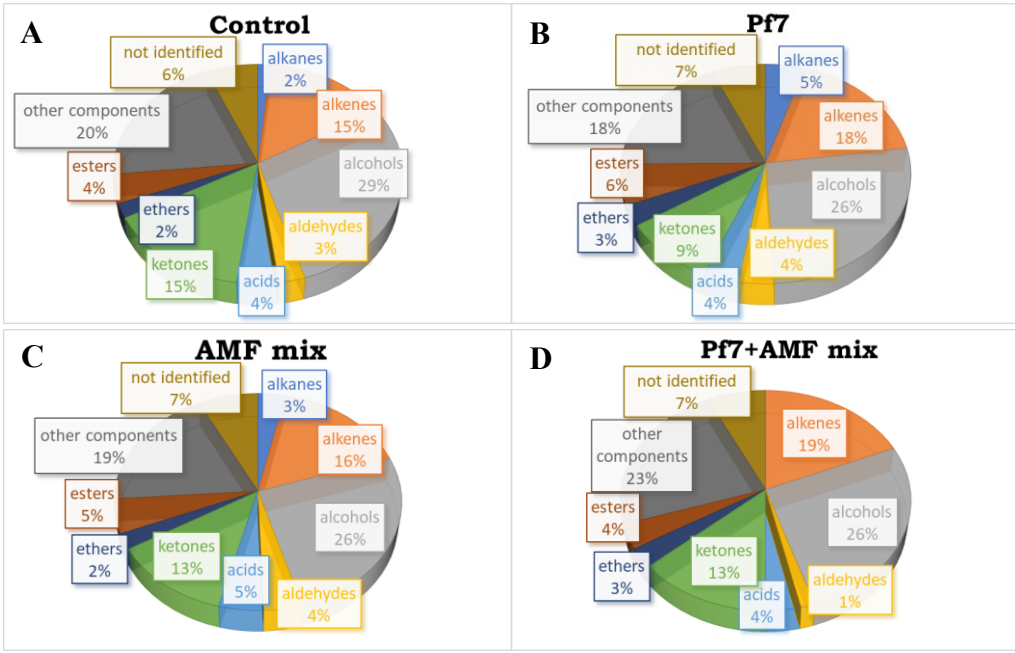


Figure 71. The figure shows the percentage distribution into chemical classes of the different components present in the leaves of *A. annua* plants (clone 26) after 30 days of cultivation in the sixth experiment. Control: control plants (**A**), Pf7: plants inoculated with *P. protegens* (**B**), AMF mix: plants inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* (**C**), Pf7+AMF mix: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. protegens* (**D**).

Aldehydes and esters increased in the plants inoculated with Pf7 (Figure 71 B) and AMF mix (Figure 71 C) alone, but diminished in those co-inoculated with

Pf7+AMF mix (Figure 71 D), in comparison to the control ones. Variations in the acids have been detected only in the plants inoculated with AMF mix alone (Figure 71 C). Finally, ethers enhanced in the plants inoculated with Pf7 alone (Figure 71 B) and in those co-inoculated with Pf7+AMF mix (Figure 71 D); while the other components increased in the plants co-inoculated with both microorganisms (Pf7+AMF mix; Figure 71 D), but decreased in plants inoculated with Pf7 alone (Figure 71 B) and AMF mix alone (Figure 71 D).

7.1.1 - Leaf volatile molecule identification

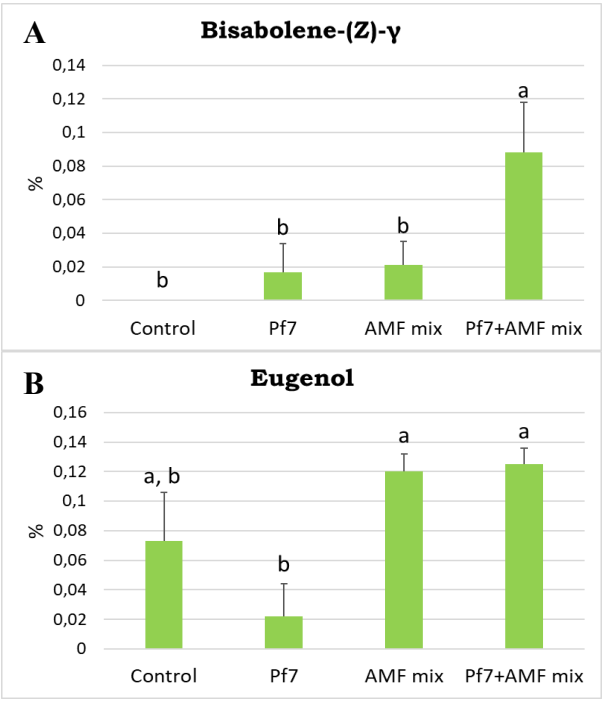
Among the molecules which have been identified, some of them were typical components of the *A. annua* essential oil and they have already been reported in literature (Table 3). Artemisia ketone, bicyclogermacrene, cadin-4en-7-ol<cis->, camphor, caryophyllene oxide, caryophyllene<(E)>, chrysantenol<cis>, copaene-α, copaene-β, cubebene-α, elemene-β, eugenol, farnesene<E-,β>, germacrene D, mentha-2,8-dien-1-ol<cis-,para>, phytol, Selina-3,11-dien-6-α-ol, selinene-β, spathulenol were detected in all the plant treatments. However, variations in the presence or in the absence of other components have been observed, when the plants were inoculated with the microorganisms. Aromadendrene, pinocarvone, and myrtenol were not present only in the plants inoculated with Pf7 alone, while bisabolol-α was present only in these latter plants. Bisabolene <(Z)-,γ> and Cubebene-β were detected in all the inoculated and co-inoculated plants (Pf7, AMF mix, Pf7+AMF mix). Caryophyllene oxide was found in the AMF mix plants only. Instead, eicosene was not found in the plants co-inoculated with Pf7+AMF mix, whereas lavandulyl acetate and valencene were detected in these latter plants only.

Table 3 | Components of *A. annua* essential oil known in the literature.

	Plant treatments			
Molecule name	**C**	**Pf7**	**AMF mix**	**Pf7+AMF mix**
Aromadendrene	✓	-	✓	✓
Artemisia ketone	✓	✓	✓	✓
Bicyclogermacrene	✓	✓	✓	✓
Bisabolene <(Z)-,γ>	-	✓	✓	✓
Bisabolol α	-	✓	-	-
Cadin-4en-7-ol<cis->	✓	✓	✓	✓
Camphor	✓	✓	✓	✓
Caryophyllene oxide	-	-	✓	-
Caryophyllene-(E)	✓	✓	✓	✓
Chrysantenol<cis>	✓	✓	✓	✓
Copaene α	✓	✓	✓	✓
Copaene β	✓	✓	✓	✓
Cubebene α	✓	✓	✓	✓
Cubebene β	-	✓	✓	✓
Eicosene	✓	✓	✓	-
Elemene β	✓	✓	✓	✓
Eugenol	✓	✓	✓	✓
Farnesene <E-,β>	✓	✓	✓	✓
Germacrene D	✓	✓	✓	✓
Lavandulyl acetate	-	-	-	✓
Mentha-2,8-dien-1-ol <cis-,para->	✓	✓	✓	✓
Myrtenol	✓	-	✓	✓
Phytol	✓	✓	✓	✓
Pinocarvone	✓	-	✓	✓
Selina-3,11-dien-6-α-ol	✓	✓	✓	✓
Selinene β	✓	✓	✓	✓
Spathulenol	✓	✓	✓	✓
Valencene	-	-	-	✓

It is showed the presence (✓) or the absence (-) of each molecule in the leaf extract analysed in GC-MS in *A. annua* plants (clone 26) after 30 days of cultivation. C: control plants, Pf7: plants inoculated with *P. protegens*, AMF mix: plants inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum*, Pf7+AMF mix: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. protegens*.

Moreover, in some of the before-mentioned components, significant variations in semi-quantitative terms were also reported. Bisabolene-(Z)-γ was not present in the control plants, whereas the plants co-inoculated with Pf7+AMF mix showed a significant higher value if compared to the plants inoculated with Pf7 and AMF mix alone (Figure 72 A). These differences were imputable to the "Fungus" and the "Bacterium" factor alone (two-way ANOVA).



Factor	p value
F	*
B	*
F*B	ns

Factor	p value
F	**
B	ns
F*B	ns

Figure 72. The figure shows the mean values and the relative standard error of the bisabolene-(Z)-γ (**A**) and of the eugenol (**B**) percentage in the leaves of *A. annua* plants (clone 26) analysed in GC-MS, after 30 days of cultivation. The percentage results from the ratio of the peak area of each molecule to the total peak area of the chromatogram and multiplied by 100. Control: control plants, Pf7: plants inoculated with *P. protegens*, AMF mix: plants inoculated with *Rhizophagus intraradices*, *R. aggregatus*, *Septoglomus viscosum*, *Claroideoglomus claroideum*, *C. etunicatum*, Pf7+AMF mix: plants co-inoculated with *Rhizophagus intraradices*, *R. aggregatus*, *Septoglomus viscosum*, *Claroideoglomus claroideum*, *C. etunicatum* and *P. protegens*. Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

Instead, eugenol showed the lowest values in the plants inoculated with Pf7 alone, and these plants were significantly different from those inoculated with AMF mix and co-inoculated with Pf7+AMF mix. These two latter groups of plants had higher values comparable to those of the control ones (Figure 72 B). According to the two-way ANOVA, the "Fungus" factor only had a significant influence.

Other series of components, that were not commonly reported in literature in the *A. annua* essential oil, have been detected (Table 4).

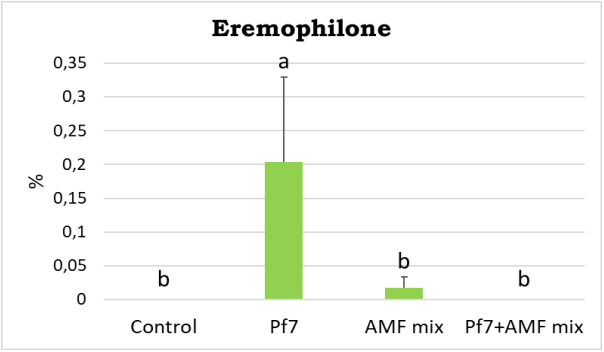
Table 4 | Components of *A. annua* essential oil not found in the literature.

	Plant treatments			
Molecule name	**C**	**Pf7**	**AMF mix**	**Pf7+AMF mix**
Allo-Cedrol	✓	✓	✓	-
Calarene	✓	✓	✓	✓
Cedroxyde	✓	✓	✓	✓
Cypertundone	✓	✓	✓	✓
Drim-8(12)-ene	✓	✓	✓	✓
Elemodiol <8-α-11->	✓	-	-	-
Eremophilone	-	✓	✓	-
Isocedranol	✓	✓	✓	✓
Thujopsenal	-	✓	✓	-
Tricos-(9Z)-ene	✓	✓	✓	✓
Vetivone	✓	✓	✓	-
γ-muurolene	✓	✓	-	✓

It is showed the presence (✓) or the absence (-) of each molecule in the leaf extract analysed in GC-MS in *A. annua* plants (clone 26) after 30 days of cultivation. C: control plants, Pf7: plants inoculated with *P. protegens*, AMF mix: plants inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum*, Pf7+AMF mix: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. protegens*.

Also in this case, some components were in common among all the plant treatments: calarene, cedroxyde, cypertundone, drim-8(12)-ene, isocedranol, tricos-(9Z)-ene. Whereas, variations were also registered in some other

components: allo-cedrol was not present in the plants co-inoculated with Pf7+AMF mix; elemodiol <8-α-> was not present in all the plants inoculated and co-inoculated with microorganisms (Pf7, AMF mix, Pf7+AMF mix); eremophilone and thujopsenal were present only in the plants inoculated with Pf7 or AMF mix alone; vetivone was not present in the co-inoculated plants only (Pf7+AMF mix); and finally γ-muurolene was absent in the AMF mix plants only. In this case, eremophilone also varied in semi-quantitative terms (Figure 73): it had the highest values in the plants inoculated with Pf7 alone, and these ones were significantly different from all the other plant treatments. Whereas, AMF mix plants showed lower values but they were not different from control and Pf7+AMF mix plants in which eremophilone was not detected. The interaction between the two factors (F*B) was responsible for these results (two-way ANOVA).



Factor	p value
F	ns
B	ns
F*B	*

Figure 73. The figure shows the mean values and the relative standard error of the eremophilone percentage in the leaves of *A. annua* plants (clone 26) analysed in GC-MS, after 30 days of cultivation. The percentage results from the ratio of the peak area of each molecule to the total peak area of the chromatogram and multiplied by 100. Control: control plants, Pf7: plants inoculated with *P. protegens*, AMF mix: plants inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum,* Pf7+AMF mix: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. protegens.* Different letters among treatments express statistically significant differences ($p < 0.05$). The box on the top right side reports the two-way ANOVA, in which F: Fungus factor; B: Bacterium factor; F*B: interaction between Fungus and Bacterium factors. ns: not significant; $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***.

Instead, some other molecules of biological interest have been reported in Table 5. Also in this case, the distribution of these ones was modulated by the presence of microorganisms. Benzoic acid and scopoletin were found only in the plants inoculated with Pf7 alone; deoxyartemisinin was present in the control plants only; and prim-O-glucosilcimifugin was observed only in the control and AMF mix plants. On the contrary, emetine was found in all the plants.

Table 5 | Bioactive molecules found in *A. annua* leaves.

	Plant Treatments			
Molecule name	**C**	**Pf7**	**AMF mix**	**Pf7+AMF mix**
Benzoic acid	-	✓	-	-
Deoxyartemisinin	✓	-	-	-
Emetine	✓	✓	✓	✓
prim-O-Glucosilcimifugin	✓	-	✓	-
Scopoletin	-	✓	-	-

It is showed the presence (✓) or the absence (-) of each molecule in the leaf extract analysed in GC-MS in *A. annua* plants (clone 26) after 30 days of cultivation. C: control plants, Pf7: plants inoculated with *P. protegens*, AMF mix: plants inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum*, Pf7+AMF mix: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. protegens*.

7.2 - Main classes of leaf volatile molecules in the second sampling

In these plants, 124 molecules were detected. The 47.6% of these molecules were shared among all the plant treatments, whereas about 7% was exclusive of each single plant treatment (C, Pf7, AMF mix), except for the plants co-inoculated with Pf7+AMF mix that showed a percentage of about 3% (Figure 74).

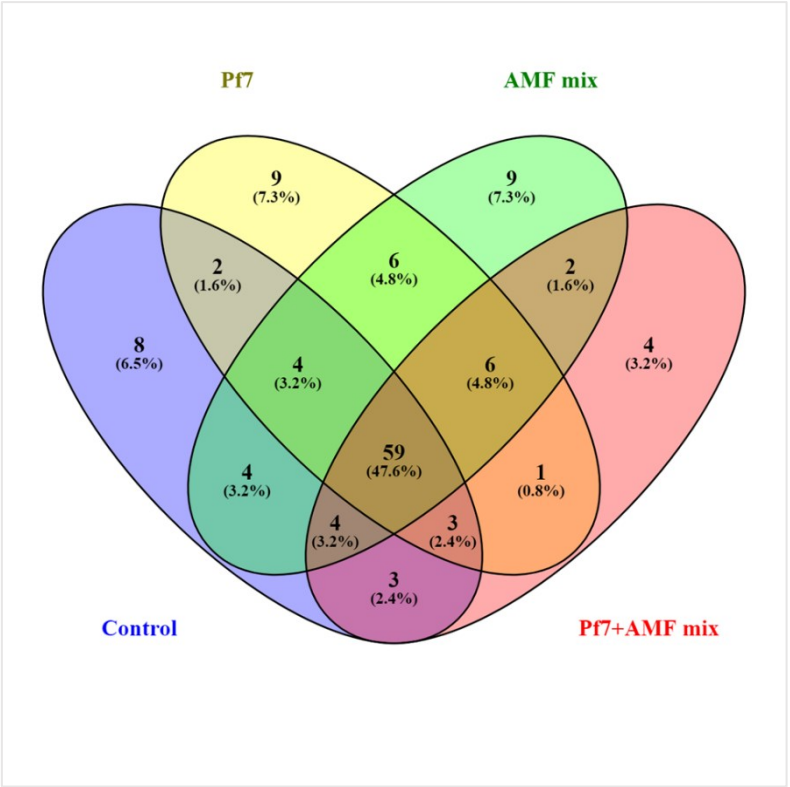


Figura 74. Eulero-Venn diagram shows the distribution of leaf volatile molecules in *A. annua* (clone 26) among the different plant treatments, after 60 days of cultivation. C: control plants, Pf7: plants inoculated with *P. protegens*, AMF mix: plants inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum*, Pf7+AMF mix: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. protegens*.

After 60 days of cultivation, also in this sampling the molecules were classified into chemical classes, and a different distribution, according to the different treatments, was noticed (Figure 75). Alkenes and aldehydes decreased in all the plants inoculated with microbes, alone or in combination, whereas alcohols and ethers increased in the same plant treatments (Figure 75 B, C, D), if compared to the control plants. On one hand, the plants inoculated with Pf7 alone (Figure 75 B) showed an improvement of acids, but a decrease in ketones; on the other hand, an increase in both acids and ketones was detected in the co-inoculated plants

(Pf7+AMF mix; Figure 75 D). In the end, ketones and esters increased only in the plants inoculated with AMF mix alone (Figure 75 C).

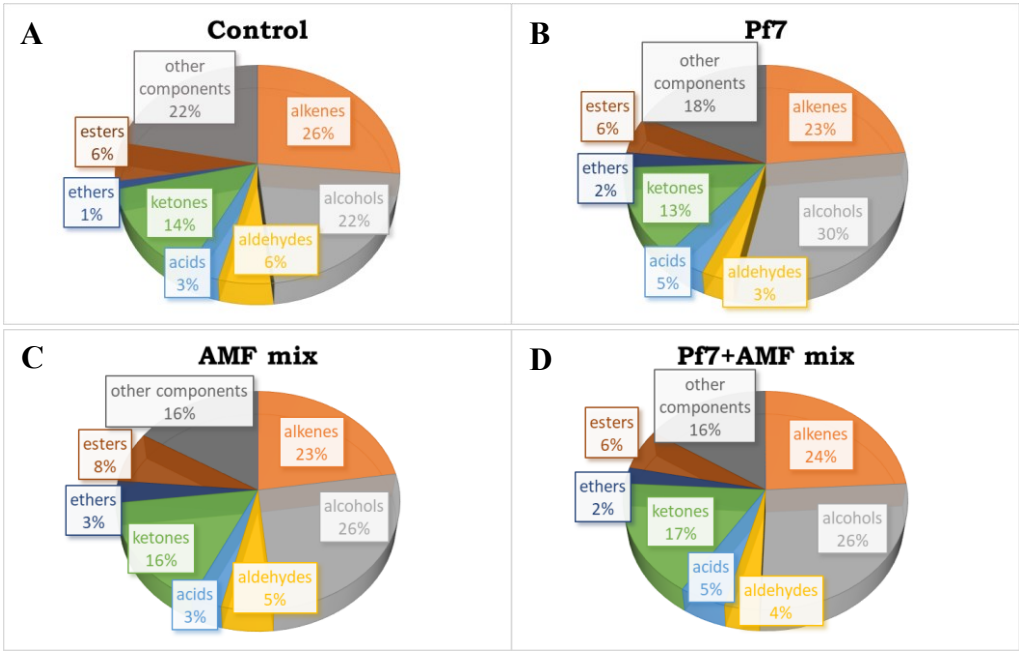


Figure 75. The figure shows the percentage distribution into chemical classes of the different components present in the leaves of *A. annua* plants (clone 26) after 60 days of cultivation in the sixth experiment. Control: control plants (**A**), Pf7: plants inoculated with *P. protegens* (**B**), AMF mix: plants inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* (**C**), Pf7+AMF mix: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. protegens* (**D**).

7.2.1 - Leaf volatile molecule identification

The typical components of the *A. annua* essential oil, that have already been reported in literature, are reported in the Table 6. Aromadendrene, bicyclogermacrene, cadin-4en-7-ol<cis->, camphor, caryophyllene-(E), chrysantenol<cis>, copaene-α, eicosene, eugenol, farnesene<E-,β>, germacrene D, mentha-2,8-dien-1-ol<cis-,para->, selina-3,11-dien-6-α-ol, selinene-β, spathulenol were detected in all the plant treatments, included the control plants.

Table 6 | Components of *A. annua* essential oil known in the literature.

	Plant treatments			
Molecule name	**C**	**Pf7**	**AMF mix**	**Pf7+AMF mix**
Aromadendrene	✓	✓	✓	✓
Artemisia ketone	-	✓	✓	-
Bicyclogermacrene	✓	✓	✓	✓
Bisabolol α	-	-	✓	✓
Cadin-4en-7-ol<cis->	✓	✓	✓	✓
Camphor	✓	✓	✓	✓
Caryophyllene oxide	-	-	✓	-
Caryophyllene-(E)	✓	✓	✓	✓
Chrysantenol<cis>	✓	✓	✓	✓
Copaene α	✓	✓	✓	✓
Copaene β	✓	-	✓	✓
Cubebene β	✓	✓	-	✓
Eicosene	✓	✓	✓	✓
Elemene β	✓	-	✓	-
Eugenol	✓	✓	✓	✓
Farnesene <E-,β>	✓	✓	✓	✓
Germacrene D	✓	✓	✓	✓
Mentha-2,8-dien-1-ol <cis-,para->	✓	✓	✓	✓
Phytol	-	✓	✓	✓
Selina-3,11-dien-6-α-ol	✓	✓	✓	✓
Selinene α	-	-	-	✓
Selinene β	✓	✓	✓	✓
Spathulenol	✓	✓	✓	✓

It is showed the presence (✓) or the absence (-) of each molecule in the leaf extract analysed in GC-MS in *A. annua* plants (clone 26) after 60 days of cultivation. C: control plants, Pf7: plants inoculated with *P. protegens*, AMF mix: plants inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum*, Pf7+AMF mix: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. protegens*.

On the contrary, other components varied according to the plant treatment, if compared to the control plants. Artemisia ketone was found only in the plants inoculated with Pf7 or AMF mix alone; bisabolol-α was present only in the plants inoculated with AMF mix alone and in those co-inoculated with Pf7+AMF mix;

caryophyllene oxide was detected in the Pf7 plants only, whereas copaene-β was not present in these latter plant treatment; cubebene-β was absent in the plants inoculated with AMF mix alone; elemene-β was not present in the Pf7 and Pf7+AMF mix plants; phytol was present in all the plants inoculated or co-inoculated with the microorganisms (Pf7, AMF mix, Pf7+AMF mix); and selinene-α was registered in the co-inoculated plants (Pf7+AMF mix) only.

Concerning the components that were not commonly detected in the *A. annua* essential oil (Table 7), some of them were shared among all the plant treatments, such as calarene, cedroxyde, cypertundone, eremophilone, thujopsenal and tricos-(9Z)-ene.

Table 7 | Components of *A. annua* essential oil not found in the literature.

	Plant treatments			
Molecule name	**C**	**Pf7**	**AMF mix**	**Pf7+AMF mix**
Calarene	✓	✓	✓	✓
Cedroxyde	✓	✓	✓	✓
Cypertundone	✓	✓	✓	✓
Drim-8(12)-ene	✓	-	✓	-
Elemodiol <8-α-11->	-	-	✓	-
Eremophilone	✓	✓	✓	✓
Isocedranol	-	✓	-	-
Thujopsenal	✓	✓	✓	✓
Tricos-(9Z)-ene	✓	✓	✓	✓
Vetivone	-	✓	✓	✓
γ-muurolene	✓	✓	✓	-

It is showed the presence (✓) or the absence (-) of each molecule in the leaf extract analysed in GC-MS in *A. annua* plants (clone 26) after 60 days of cultivation. C: control plants, Pf7: plants inoculated with *P. protegens*, AMF mix: plants inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum*, Pf7+AMF mix: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. protegens*.

Instead, some components varied according to the used microorganisms, if compared to the control plants. Drim-8(12)-ene was not present in the plants

inoculated with Pf7 and co-inoculated with Pf7+AMF mix; elemondiol<8-α-11-> was present in the AMF mix plants only; while isocedranol was detected only in the Pf7 plants; vetivone was present in all the inoculated or co-inoculated plants (Pf7, AMF mix, Pf7+AMF mix); and γ-muurolene was absent in the Pf7+AMF mix plants only.

Table 8 | Bioactive molecules found in *A. annua* leaves.

	Plant Treatments			
Molecule name	**C**	**Pf7**	**AMF mix**	**Pf7+AMF mix**
Deoxyartemisinin	-	✓	✓	✓
Emetine	-	✓	-	-
prim-O-Glucosilcimifugin	✓	✓	-	✓

It is showed the presence (✓) or the absence (-) of each molecule in the leaf extract analysed in GC-MS in *A. annua* plants (clone 26) after 60 days of cultivation. C: control plants, Pf7: plants inoculated with *P. protegens*, AMF mix: plants inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum*, Pf7+AMF mix: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. protegens*.

Finally, regarding the molecules of biological interest (Table 8), deoxyartemisinin was present in all the plants inoculated with microorganisms (Pf7, AMF mix, Pf7+AMF mix), compared to the control ones; emetine was only found in Pf7 plants; while prim-O-glucosilcimifugin was not present in the AMF mix plants only.

8 - Leaf volatile composition in the CL26 plants in the fourth experiment

8.1 - Main classes of leaf volatile molecules

In these plants, 121 molecules has been detected. The 52.9% of these molecules was shared among all the different plant treatments, whereas the 9.9% was exclusive of the control plants, the 10.7% was exclusive of the BEG12+SVB6R1 plants, the 2.3% was exclusive of the AMF mix+SVB6R1 plants, and the 0.8% was exclusive of SVB6R1 ones (Figure 76).

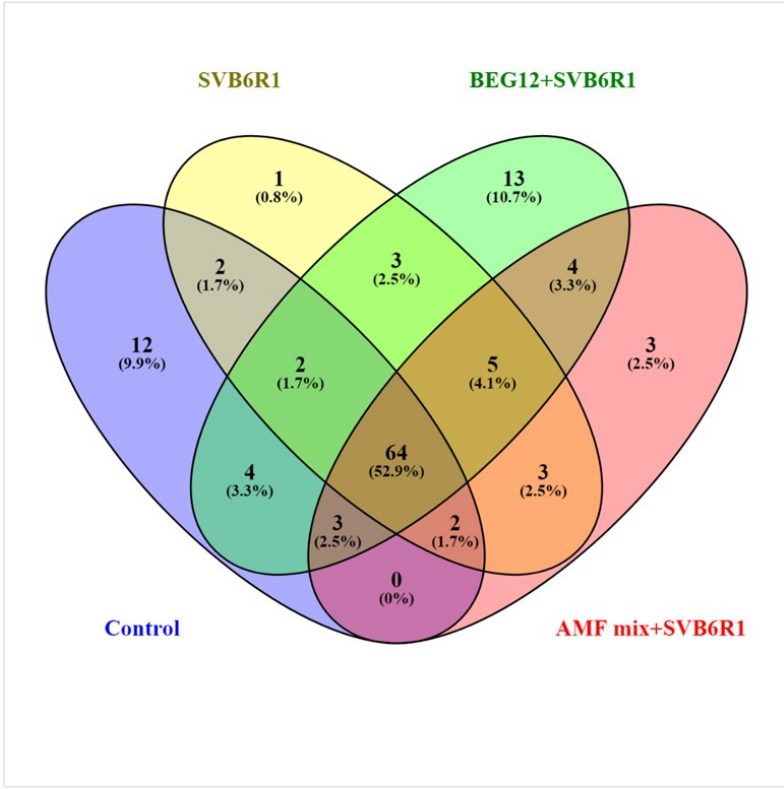


Figura 76. Eulero-Venn diagram shows the distribution of leaf volatile molecules in *A. annua* (clone 26) among the different plant treatments, after 60 days of cultivation in the fourth experiment. C: control plants; SVB6R1: plants inoculated with *P. brassicacearum*; BEG12+SVB6R1: plants co-inoculated with *F. mosseae* and *P. brassicacearum*; AMF mix+SVB6R1: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. brassicacearum.*

Regarding the plants of this experiment, they were grown for 60 days, and also in this case variations in the leaf chemical profile were reported, according to the used microorganisms. Alkanes were not present in all the plant treatments, included the control plants (Figure 77).

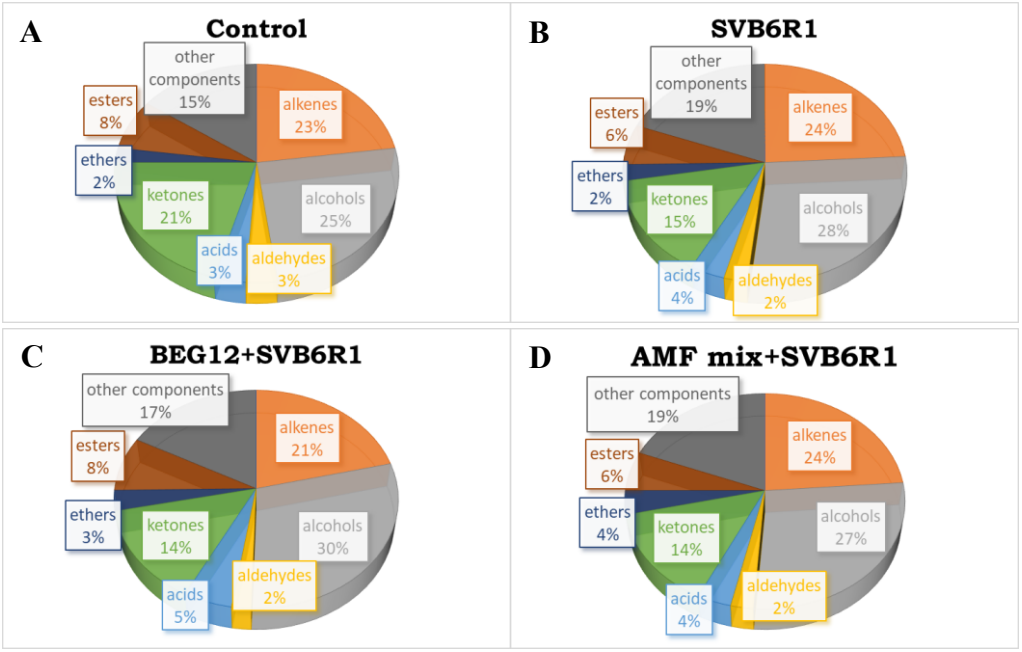


Figure 77. The figure shows the percentage distribution into chemical classes of the different components present in the leaves of *A. annua* plants (clone 26) after 60 days of cultivation in the fourth experiment. Control: control plants (**A**), SVB6R1: plants inoculated with *P. brassicacearum* (**B**), BEG12+SVB6R1: plants co-inoculated with *F. mosseae* and *P. brassicacearum* (**C**), AMF mix+SVB6R1: plants co-inoculated with *Rhizophagus intraradices, R. aggregatus, Septoglomus viscosum, Claroideoglomus claroideum, C. etunicatum* and *P. brassicacearum* (**D**).

In comparison with the control plants (Figure 77 A), alcohols, acids, and other components increased in all the plants inoculated with the microorganisms, both alone (77 B) or in combination (Figure 77 C, D). On the other hand, in the same beforementioned plants, a decreasing trend in aldehydes and ketones was registered, compared to the control ones (77 A). Instead, the percentage of ethers was higher both in the plants co-inoculated with BEG12+SVB6R1and AMF mix+SVB6R1 (Figure 77 C, D), if compared to the control and bacterial plants

(77 A, B); while, esters decreased in the plants inoculated with the bacterium alone (SVB6R1; Figure 77 B) and co-inoculated with AMF mix+SVB6R1 (Figure 77 D).

8.2 - Leaf volatile molecule identification

Most of the typical components of the *A. annua* essential oil (Table 9) were in common with all the plant treatments, such as artemisia ketone, bicyclogermacrene, cadin-4en-7-ol<cis->, camphor, caryophyllene-(E), chrysantenol<cis>, copaene-α, copaene-β, eicosane, elemene β, eugenol, farnesene <E-,β>, germacrene D, mentha-2,8-dien-1-ol <cis-,para->, phytol, pinocarvone, selina-3,11-dien-6-α-ol, selinene β, and spathulenol. However, some components varied according to the plant treatment compared to the control plants: aromadendrene was present in all the plants inoculated with the microorganisms, whereas cubebene-β was not present in these latter three treatments. Lavandulyl acetate was present in the plants co-inoculated with BE12+SVB6R1 only, while myrtenol was absent in the SVB6R1 plants only. Selinene-α was not registered in the plants inoculated with SVB6R1 and in those co-inoculated with AMF mix+SVB6R1, whereas valencene was found only in these two latter groups of plants.

Table 9 | Components of *A. annua* essential oil known in the literature.

	Plant treatments			
Molecule name	**C**	**SVB6R1**	**BEG12+SVB6R1**	**AMF mix+SVB6R1**
Aromadendrene	-	✓	✓	✓
Artemisia ketone	✓	✓	✓	✓
Bicyclogermacrene	✓	✓	✓	✓
Cadin-4en-7-ol<cis->	✓	✓	✓	✓
Camphor	✓	✓	✓	✓
Caryophyllene-(E)	✓	✓	✓	✓
Chrysantenol<cis>	✓	✓	✓	✓
Copaene α	✓	✓	✓	✓
Copaene β	✓	✓	✓	✓
Cubebene β	✓	-	-	-
Eicosene	✓	✓	✓	✓
Elemene β	✓	✓	✓	✓
Eugenol	✓	✓	✓	✓
Farnesene <E-,β>	✓	✓	✓	✓
Germacrene D	✓	✓	✓	✓
Lavandulyl acetate	-	-	✓	-
Mentha-2,8-dien-1-ol <cis-,para->	✓	✓	✓	✓
Myrtenol	✓	-	✓	✓
Phytol	✓	✓	✓	✓
Pinocarvone	✓	✓	✓	✓
Selina-3,11-dien-6-α-ol	✓	✓	✓	✓
Selinene α	✓	-	✓	-
Selinene β	✓	✓	✓	✓
Spathulenol	✓	✓	✓	✓
Valencene	-	✓	-	✓

It is showed the presence (✓) or the absence (-) of each molecule in the leaf extract analysed in GC-MS in *A. annua* plants (clone 26) after 60 days of cultivation in the fourth experiment. C: control plants, SVB6R1: plants inoculated with *P. brassicacearum*, BEG12+SVB6R1: plants co-inoculated with *F. mosseae* and *P. brassicacearum*, AMF mix+SVB6R1: plants co-inoculated with *Rhizophagus intraradices*, *R. aggregatus*, *Septoglomus viscosum*, *Claroideoglomus claroideum*, *C. etunicatum* and *P. brassicacearum*.

Regarding the components which were not commonly reported in the *A. annua* essential oil (Table 10), calarene, cedroxyde, cypertundone, drim-8(12)-ene, eremophilone, tricos-(9Z)-ene, vetivone, and γ-muurolene were shared among all

the plant treatments, included the control plants. On the other hand, only two components varied between the plant treatments: isocedranol was found in the plants co-inoculated with BEG12+SVB6R1 only, and thujopsenal was not present in all the plants inoculated with the microorganisms (SVB6R1, BEG12+SVB6R1, AMF mix+SVB6R1).

Table 10 | Components of *A. annua* essential oil not found in the literature.

			Plant treatments	
Molecule name	**C**	**SVB6R1**	**BEG12+SVB6R1**	**AMF mix+SVB6R1**
Calarene	✓	✓	✓	✓
Cedroxyde	✓	✓	✓	✓
Cypertundone	✓	✓	✓	✓
Drim-8(12)-ene	✓	✓	✓	✓
Eremophilone	✓	✓	✓	✓
Isocedranol	-	-	✓	-
Thujopsenal	✓	-	-	-
Tricos-(9Z)-ene	✓	✓	✓	✓
Vetivone	✓	✓	✓	✓
γ-muurolene	✓	✓	✓	✓

It is showed the presence (✓) or the absence (-) of each molecule in the leaf extract analysed in GC-MS in *A. annua* plants (clone 26) after 60 days of cultivation in the fourth experiment. C: control plants, SVB6R1: plants inoculated with *P. brassicacearum*, BEG12+SVB6R1: plants co-inoculated with *F. mosseae* and *P. brassicacearum*, AMF mix+SVB6R1: plants co-inoculated with *Rhizophagus intraradices*, *R. aggregatus*, *Septoglomus viscosum*, *Claroideoglomus claroideum*, *C. etunicatum* and *P. brassicacearum*.

In this case, variations were reported in semi-quantitative terms for γ-muurolene: the control plants had a lower content if compared to all the other plant treatments, but they were significantly different only from the co-inoculated plants (BEG12+SVB6R1, AMF mix+SVB6R1; Figure 78).

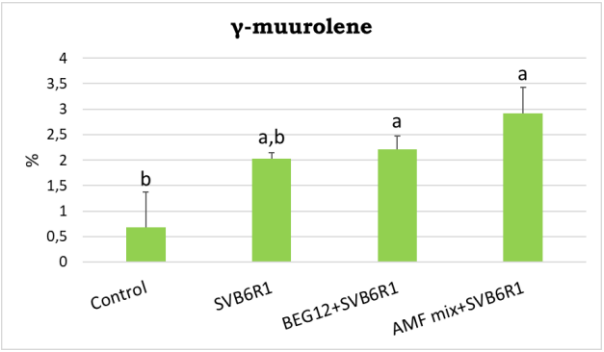


Figure 78. The figure shows the mean values and the relative standard error of the γ-muurolene percentage in the leaves of *A. annua* plants (clone 26) analysed in GC-MS, after 60 days of cultivation in the fourth experiment. The percentage results from the ratio of the peak area of each molecule to the total peak area of the chromatogram and multiplied by 100. Control: control plants, SVB6R1: plants inoculated with *P. brassicacearum*, BEG12+SVB6R1: plants co-inoculated with *F. mosseae* and *P. brassicacearum*, AMF mix+SVB6R1: plants co-inoculated with *Rhizophagus intraradices*, *R. aggregatus*, *Septoglomus viscosum*, *Claroideoglomus claroideum*, *C. etunicatum* and *P. brassicacearum*. Different letters among treatments express statistically significant differences ($p < 0.05$).

In the end, looking at the molecules of biological interest (Table 11), emetine was registered in all the co-inoculated plants only, and prim-O-glucosilcimifugin was detected in all the plants inoculated with the microorganisms, compared to the control ones.

Table 11 | Bioactive molecules found in *A. annua* leaves.

	Plant Treatments			
Molecule name	**C**	**SVB6R1**	**BEG12+SVB6R1**	**AMF mix+SVB6R1**
Emetine	-	-	✓	✓
prim-O-Glucosilcimifugin	-	✓	✓	✓

It is showed the presence (✓) or the absence (-) of each molecule in the leaf extract analysed in GC-MS in *A. annua* plants after 60 days of cultivation in the fourth experiment. C: control plants, SVB6R1: plants inoculated with *P. brassicacearum*, BEG12+SVB6R1: plants co-inoculated with *F. mosseae* and *P. brassicacearum*, AMF mix+SVB6R1: plants co-inoculated with *Rhizophagus intraradices*, *R. aggregatus*, *Septoglomus viscosum*, *Claroideoglomus claroideum*, *C. etunicatum* and *P. brassicacearum*.

Chapter 5

Discussion

In this work, the micropropagation technique proved to be a good method to obtain a uniform plant population in terms of genetic variability, also in an aromatic and medicinal plant like *A. annua* (1). The clones selected and supplied by CREA showed different morphologies: dwarf or tall, and a tighter or broader leaf lamina, according to the specific plant clone. However, some aspects related to the difficulty in the maintenance of plant culture *in vitro* conditions emerged. Among the 5 clones selected by CREA, only the CL26 proved to be stable both during the micropropagation steps and *in vivo*. This is a well-known problem of this technique, in which the majority of plant loss is related to the poor stability of the plant culture *in vitro* (2), and even more when seedlings are transferred *in vivo* conditions (3-5).

1 - Mycorrhizal colonization

A. annua plant, belonging to the *Asteraceae* family, can establish the symbiosis with AMF, as reported in some papers (6, 7). On the contrary, in this work a general low rate of mycorrhizal colonization in the root system was observed. Despite the low mycorrhization degree, we could observe variations among the different plant clones and the microorganisms used in each experiment. The highest levels of colonization were observed with *R. irregularis* (Ri) with the clone CL26 (10%), while the consortium of AMF was the most effective in all the clones, especially if compared to *F. mosseae* (BEG12). In this case, mycorrhizal colonization reached 2% at most, and in some cases it did not show significant differences to the uninoculated plants. These results are very different if compared with what is reported in literature, both using a single fungal species

and a consortium of fungi, in which mycorrhizal colonization in *A. annua* plant reached percentages ranged from 40% (8-13) to 85% (14). In some of these studies, inoculation was performed with the same fungi used in the present study: in Awasthi et al. (10), plants of *A. annua* (cv. CIM-Arogya) inoculated with *F. mosseae* showed a percentage of mycorrhization of 52%, which is a higher value if compared to our 2% of colonization rate detected in the root of our clones inoculated with the same fungus; in Mandal et al. (11) and Domokos et al. (12, 13) it was used the fungus *R. irregularis* which reached a root colonization of 56% and 50% respectively, and also in this case these values were at a fair distance from those detected in our experiments with the same fungus, which showed a colonization rate near 10%. However, it must be underlined that the plants used in the before-mentioned studies were genetically different from our plants, which in turn were different from each other because they were distinct plant clones derived from *in vitro* micropropagation. Moreover, the different results observed in these mentioned studies could be due to the different growth conditions and substrate of cultivation. Mycorrhizal symbiosis is described as an aspecific mutualistic relationship (15) widely diffused among most of the *Angiospermae* (16), but a sort of compatibility between the two symbionts has been noticed (17, 18). In the literature, fluctuations in the mycorrhizal colonization in different accessions of *A. annua* inoculated with the same species of arbuscular mycorrhizal fungi have been reported (9). Moreover, also in other plant species, the mycorrhizal colonization can vary according to the plant and the fungus species (19), and even to the plant and the fungus genotypes (20). The great differences in root colonization, reported among and within many varieties of the same plant species (21), highlight the great importance of the compatibility between the two symbionts possibly during the first phases of the symbiosis establishment (22). In fact, despite they are not host specific, these fungi exhibit a host preference (23-27). This is probably related to the genetic differences in

the common symbiosis signalling, which is a pathway associated with the symbiosis establishment and the plant-fungus recognition process (28). This could involve root exudates, such as strigolactones (28, 29), flavonoids and sesquiterpenes, that are released from the roots as signalling compounds that intercede the root perception by the fungal mycelium, also altering the architecture of the root (30), that is in turn altered also by fungal exudates that influenced root hormone balance (31-33). In recent studies on wheat plants, some significant markers on wheat chromosomes and related to root architecture regions were detected, such as the Quantitative Trait Locus (QTL) (34), which could be involved in the establishment of the early genetic differences in the symbiosis (35). On the other hand, the establishment and the development of the mycorrhizal symbiosis are strongly influenced by different external environmental factors (36). They are very sensitive toward the availability and the amount of nutrients present in the soil, (particularly P and N; 9, 32, 37, 38). It has been widely recognised that arbuscular mycorrhizal development is heavily repressed in high phosphate conditions (38, 39). However, in our work, we used a nutrient solution containing a reduced concentration of phosphate (32 μM of P) for watering the plants. So another possibility, in order to explain the low mycorrhizal colonization detected in the roots of our plants, may be that the nutrients present in the peat, which was used to make the substrate of cultivation in all our experiments, cancelled the P nutrient-deficiency, thus making the cultivation substrate more rich in essential macronutrients. In fact, a granular slow-releasing mineral fertilizer was present in the peat and it was not subjected to leach out, with a NPK of 15-9-15 (w/w/w), and these essential macro-nutrients were all in soluble forms (Vigor Plant's agronomist personal communication). Since *A. annua* is a ruderal plant which has not particular nutritional requirements (40, 41), despite the reduced P concentration in the used Long Ashton nutrient solution, a NPK of 15-9-15 in the used peat could be enough for its optimal

growth. In this respect, it is well known that plants have a direct pathway to take up macronutrients from the soil (42). Regarding P, they have a direct pathway (DP) responsible for the uptake of the above-mentioned macronutrients independently from AM fungi (43), due to the presence of specific transporters in the membrane of the root cells (P or N transporters; 44, 45). Instead, when P is present in low amounts in the soil or it is not available for plant uptake by DP, plants can establish symbiosis with AM fungi releasing strigolactones that give rise to the mutualistic relationship between them (46). In this latter case, plants can take up P through the mycorrhizal pathway (MP), thus restoring the macronutrient loss in the soil, and providing photosynthates to the fungus as a trade-off (47). So, if the plant is able to take up essential nutrients by itself, it does not require the symbiosis benefits and will not establish the interaction with AM fungi (38, 48). On the fungus side, this microbe is an obligate symbiont that needs a host plant to complete its life cycle (22, 46). Therefore, it is its interest to find a host and try to establish symbiosis with it. Furthermore, in some studies it was observed that high P supply can suppress the early stages of the plant-fungus interaction; in fact, the fungal hyphopodium formation is arrested as a result of the treatment with high P concentrations (48). Nevertheless, also under high P conditions, AM fungi can colonize the plant root depending on the combination of the P concentration, and the combination of plant and fungal species (21, 27, 49-51). The plant-fungus exchange of nutrients takes place in the periarbuscular membrane, an intermediate surface between the plant cell and the arbuscule of the fungus (52). The arbuscule presence in the root is a sign that the symbiosis is active. Concerning this latter aspect, in our experiments the arbuscules were always detected in the roots, therefore the symbiosis was actually active, but to a lesser extent compared to what was reported in other studies on *A. annua* plant (12, 13) and in different plant species (20, 53), also inoculated with the same fungi used in our experiments (10, 11, 53) or with a consortium of AM fungi (14,

54) in different plant growth conditions. In fact, it was evident that the arbuscule abundance never overcame the threshold of 1%, except in the case of the CL26 plants inoculated with *R. irregularis* and with a consortium of AM fungi (AMF mix). Once more, these observations led us to support the great importance of the compatibility between the partners involved in this symbiotic relationship (18, 27, 53), but it could be a symptom of something that limited, not the establishment of the symbiosis, but its development after that the mutualistic dialogue was started. According to the above-mentioned considerations on the mycorrhization level in relation to NPK supply, also the extension of hyphae and the arbuscule formation in the root cells could be negatively influenced by high-P levels in the growth substrate (38, 39, 55, 56). It could be plausible that root colonization by AMF is highly influenced by different factors which can easily overlap: sensitivity to nutrients (57), soil mineral content (58, 59), and genetic differences between and within the plant species (21, 27). In some cases, PGPB can support the mycorrhizal colonization and facilitate the fungal colonization of the roots (60, 61), also in *A. annua* plant as reported in several papers (10, 14). In our experiments, the two used bacteria (*P. protegens* strain Pf7 and *P. brassicacearum* strain SVB6R1) gave different effects on the mycorrhizal development according to the fungus species and the plant clone. *F. mosseae* (BEG12) colonization increased in the presence of Pf7 only in the CL26, CL6 and CL7 clones, whereas it decreased in the CL10 plants. Instead, the same fungus was more responsive in the presence of SVB6R1: in fact, the co-inoculation of BEG12 with SVB6R1 showed the highest values of colonization in the root. Also *R. irregularis* enhanced its colonization rate in the presence of Pf7, but more than in the presence of SVB6R1. On the contrary, the use of a consortium of different AMF species in the presence of SVB6R1 showed an improved fungal colonization in the root of all the clones, except for the CL24 plants; while, the co-inoculation with Pf7 resulted in a significant reduction in

root colonization in all clones, except for the CL10 plants, in which an increase of this parameter was registered. These data highlight the importance of the compatibility not only between plant and fungus (62, 63), but also along the tripartite interaction fungus-bacterium-plant (64, 65), in order to increase the mycorrhizal colonization degree. It has clearly been observed that in some cases *P. protegens* and *P. brassicacearum* had a helper behaviour, according to the fungus species and the plant clone. This phenomenon was previously reported in studies on *A. annua* in which the mycorrhizal colonization improved in the presence of different PGPB (10, 14), even if they were inoculated with different bacterial genera comparing to those used in our experiments, and also in other plant species (66-69). It is well known that bacteria belonging to the *Pseudomonas* genus are often associated with *Glomus* sp. in natural soils (62, 70-72), thus showing a good reciprocal compatibility and acting as Mycorrhiza Helper Bacteria (MHB; 73-76). These bacteria can use root exudates and soil nutrients, also providing nutrient uptake to the fungus (like P and N) and stimulating the release of root exudates by plants (77, 78). Furthermore, a molecular dialogue with AM fungi has been reported (79) and it leads to a stimulation of spore germination (80), hyphal branching (81), fast mycelium elongation (82) and root colonization (83), releasing metabolites, such as IAA (84, 85). Moreover, in some experiments the presence of other molecules, as flavonoids and Nod factors, could interfere between the fungus and plant root communication, acting as fungal growth regulators and leading to an enhancement of the AM colonization into the plant roots (86). MHB can indirectly stimulate higher mycorrhizal colonization by stimulating lateral root formation thanks to the above-mentioned auxin-like molecules (67, 87), thus increasing the number of plant-fungus interaction sites (88). Probably, bacteria interact with plant roots earlier than fungi, therefore they would release enzymes that digest the root cell wall (89, 90), making infiltration points that facilitate AM

fungi penetration and colonization (91). On the other hand, a neutral (92, 93) or negative effect on the mycorrhizal colonization rate can occur (51, 70, 71). This was reported, not only in the case of a single AM fungus species co-inoculated with one bacterium (94), but also in the presence of a AMF consortium and bacteria (51, 95). Rapparini et al. (14) detected a reduction in the mycorrhizal colonization in *A. annua* plants co-inoculated with a consortium of AMF (*F. mosseae*, *G. intraradices*, *G. viscosum*) and PGPB (*P. fluorescens* 2 strains, *B. subtilis*, *Streptomyces* sp., *Radiobacter*), if compared to plants inoculated with the AMF consortium alone. Also in other studies, in different plant species, a reduction of the mycorrhizal colonization in the presence of PGPB was reported (51). Negative effects on the mycorrhizal colonization due to the co-inoculation with the two different *Pseudomonas* strains used in our experiments, could be related to the before-mentioned compatibility and specificity of the interaction between the microbes (62-65), but also to the release of compounds by bacteria, that could have a negative influence on the fungal growth (96, 97). In fact, this genus of bacteria can produce a wide variety of secondary metabolites characterized by many different functions, such as anti-microbial activities (97, 98). In particular, it produces metabolites like 2,4-diacetylphloroglucinol, polyketides, pyoluteorin, pyrrolnitrin and hydrogen cyanide, that are toxic to fungi (97, 99). In order to verify the mycorrhizal colonization development in the root over the time, we monitored it making an intermediate sampling at 30 days on the CL26 and CL7 plants, in the last experiment. The mycorrhizal colonization had lower values compared to the sampling at 60 days and, as we expected, it increased over the time according to what was reported in some works (51, 92, 100). However, the absolute values of the mycorrhizal parameters (F%, M%, and A%) were lower if compared to other studies (100) probably because of the different plant species, and the mineral fertilizer present in our used peat. Instead,

after 60 days, the percentage of colonization in the root system was in line with what has been formerly reported in the text.

2 - Plant growth parameters

The plants of each clone showed very different growth responses, in accordance to the used microorganisms. Regarding the aboveground part of the plant, in general, it has been observed that the inoculum composed by a consortium of arbuscular mycorrhizal fungi (AMF mix) resulted to be the most effective to induce positive responses in the plants whatever the clone, showing an overall positive effect on the plant growth and, in some cases, specifically increasing the shoot biomass production (both fresh and dry). The same effect, in plants of the same clone co-inoculated with the AMF consortium and the two *Pseudomonas* species has been obtained, as underlined by the two-way ANOVA in which also the "Bacterium" factor assumed a significant positive influence on plant growth. It is known, from several studies on *A. annua* plants (101 and references therein), that the use of a AMF consortium or AMF and PGPB consortia can lead to an enhancement of plant growth parameters (14); but also in other studies, on different plant species, the same effect has been reported (102, 103), probably due to the synergism between fungi and bacteria (104). It is well known that mycorrhizal fungi have the ability to improve water uptake, water management of the plant and plant nutrition of essential minerals (105). These nutrients could be used as substrates in plant metabolism leading to an improvement of plant growth (106). Furthermore, PGPB produce many hormones such as IAA (107) and the capacity of AMF to change the hormonal balance of the plant (108) could have influenced plant development and thus its biomass production. However, in some cases a low response to the before-mentioned consortium of microorganisms has been observed. In fact, the co-inoculation with the AMF consortium and *P. brassicacearum* led to a reduction of biomass in CL10 and

CL24 plants. This observation has highlighted the key role of the plant genotype, a phenomenon well reported in the scientific literature (18) and related to a genetic compatibility between the plant and the used microorganisms (109). This compatibility would be crucial in order to have positive effects on plant productivity, as reported in Rapparini et al. (14), in which a consortium of either mycorrhizal fungus or PGPB improved plant growth compared to uninoculated plants. Looking at the plants inoculated with a single species of AMF, we observed a more variable trend. In the plants inoculated with *F. mosseae* (BEG12) alone, a neutral effect in three clones (CL26, CL10 and CL6) and a negative impact in one clone (CL7) were observed. Also the inoculation with *R. irregularis* (Ri) in CL26 plants resulted in a neutral effect on the plant growth. When the same plant clones were co-inoculated with the same above-mentioned fungi and with *P. protegens* (Pf7) or *P. brassicacearum* (SVB6R1), the results did not change, with the exception of the Ri+Pf7 plants of CL26 in which a significant reduction of leaf dry biomass was registered, and in Pf7+BEG12 plants of CL7 in which the shoot biomass reduction was even more pronounced. These data are quite different if compared to other studies in which *A. annua* plants inoculated with a single fungal species showed an increase in the plant growth and thus in the shoot biomass production (8-10, 12, 13). In some cases, as Domokos et al. (12) and Awasthi et al. (10), the same fungi as ours were used, *R. irregularis* and *F. mosseae* respectively, but probably the different plant genotype and fungal strain have been the cause of the different results; this hypothesis is also supported by the corresponding low rate of *R. irregularis* and *F. mosseae* root colonization and arbuscule abundance in our experiments. This underlined the essential role of the genotypic compatibility between the two symbionts. In fact, it is widely recognised that various AM fungi can colonize plant roots to different degrees and hence they can have variable effects on the plant growth, called “effectiveness” (9). These properties are under genetic

control of the actors of this interaction: the host plant and the AM fungus (110). Accordingly, in Awasthi et al. (10) the fungus *G. fasciculatum* was not able to stimulate significant variations in plant growth, if compared to the control plants and the plants inoculated with *G. mosseae*, or *G. aggregatum*, or *G. intraradices*, consistently with the data of the present study. Looking at other plant species, there are many papers that showed the positive effect on the plant growth due to the mycorrhizal fungi over the last 15 years (111 and references therein). Other studies underlined significant variations, in a negative, neutral, or positive way, on plant productivity depending on the two partners of the symbiosis (19, 20, 23). Furthermore, always Awasthi et al. (10) observed that plants co-inoculated with *F. mosseae* and *Bacillus subtilis* or *Stenotrophomonas spp*. had an increase in shoot biomass compared to the plants inoculated with the fungus alone. Despite these results are in contrast with our work, in the same study, neutral and negative effects on the *A. annua* biomass production were reported, in depending on the combination of the used fungus and bacterium (10). Similar contrasting results were also reported in studies on other plant species (19, 112, 113). Currently, not much is known on the cross-talk between PGPB and AMF, but they would share a significant homology in some receptors for signal molecules produced and released by themselves and, in some cases, these receptors could be able to perceive both signal types (104). Among these signal molecules, beneficial bacteria produce exopolysaccharides that are good candidates as an important factor for the establishment of the association with AM fungi (114). Furthermore, in van Buuren et al. (115), genes of exoribonuclease were also detected in some soil bacteria that would be required to develop a positive association with *Gigaspora margarita*. In other studies, MHB can stimulate AMF growth also thanks to a wide range of active metabolites (vitamins, amino acids) and growth substances, that may directly stimulate AMF growth (116, 117).

Instead, regarding the fungi, they can release organic compounds from hyphae which can support the nutrition of the rhizospheric microbes (95). Some studies have observed that AM fungi can increase bacterial populations in the soil, and these interactions can be commensalistic as well as amensalistic (118); but in some cases the presence of an AM fungus can supress the population of different bacterial species (119). It could also be important to consider that in all the plant clones, inoculated with AMF alone or co-inoculated with AMF and bacteria, the mycorrhizal colonization was low, as already underlined in the specific section of this discussion. Therefore, it could be possible that AMF colonization at low levels has not growth promotion effects on each plant clone, as observed in Xie et al. (19). However, few data are available in the scientific literature about the threshold level of hyphae and mainly of arbuscules required for a significant promoting effect on plant shoot biomass production. But in some cases it was reported a significant increase in plant growth (also in field conditions) despite a low mycorrhizal colonization degree at root level (93). On the other hand, a biomass reduction in the presence of AMF colonization was reported (120-122). In these cases, fungi could act as a "hitch-hiker" profiting from the symbiosis network established with the plant without returning benefits back to the host (123). This way has been confirmed by experiments that underlined the AMF capacity to change the carbon, nitrogen and phosphorus stoichiometry in plant tissues (124). Another possible explanation for the neutral and negative effects on the plant biomass production could also be related to the nutrient levels of the mineral fertilizer present in the used peat (NPK 15-9-15 all in soluble form); in fact, many studies reported that, when the environmental conditions (in particular the nutrient availability) are good enough for the requirements of the specific cultivated plant species, the AMF effect tends to have a minor importance on the growth and development of the host plant (13). The principal advantage furnished to the plant by AMF is the translocation of mineral nutrients not available for

plant uptake, i.e. those in insoluble forms (125, 126). Therefore, if these nutrients are all in soluble form and not in limited amount, as it probably happened in the present work, the plant might not need the fungus help, and the fungus could have suppressed the direct P uptake by the plant roots (27, 127, 128). Considering the used bacteria, *P. protegens* and *P. brassicacearum,* alone or in combination, their effects on the shoot growth of *A. annua* clones were substantially neutral. In fact, the vast majority of the shoot growth parameters had the same trend and values detected in the control plants. These observations are in contrast with what reported in many studies on *A. annua* plants inoculated and co-inoculated with beneficial soil bacteria, in which an improvement of the shoot growth parameters was always reported (10, 129-132). However, in strawberry plants, Morais et al. (133) observed no beneficial effect on the plant growth in all the bacterial treatments. In addition, also in other studies, a lack of positive effects on the basis of the specific strain was reported (134, 135). Instead, in our experiments, *P. protegens* and *P. brassicacearum* had a negative effect on the shoot growth: the first one in two clones (CL7 and CL10), while the second bacterium only in one clone (CL10). Since the control plants had almost always the best values of shoot biomass, it could be plausible that, beyond the plant genotype-bacterial strain compatibility (136), there might have been a competition for the nutrients present in the substrate of cultivation between plants and bacteria. Therefore, since plants can independently take up soluble nutrients by specific transporters present in the root cells (25), and soil bacteria also use these soluble elements for their own growth (137, 138), it is possible a sort of plant-bacteria competition for the main macronutrients (139, 140). Chemical fertilizers, which are rich in readily available nutrients, can strongly influence the soil microbial population (138, 141) that have the capacity to use NPK in soluble forms for their own growth and reproduction (138, 142). On the other hand, it has also been reported that in soils with a great availability of nutrients (particularly N and P) the belowground

communities can be strongly influenced in their composition (143, 144) due to their sensitive to NPK fertilization, and in this context microbes and plants can compete for the nutrient resources (145). In our experiments we used two species of the *Pseudomonas* genus, which are Gram negative bacteria, and interestingly in a study of Peacock et al. (146) it was observed that high rates of N significantly lowered the abundance of the Gram negative bacteria more than the Gram positive ones, comparing to unfertilized soil. So it is possible that the amount of N in the mineral fertilizer present in the peat could have had an influence on the mentioned used bacteria. However, other studies report no differences in the bacterial community composition between fertilized and unfertilized soils (147-149). Therefore, according to the available data, it is very difficult to deduce the multiple interrelated interactions among the fertilizer type and amount, the soil microorganism groups, and the plant species (150). Plant-microorganism interaction is strongly driven by the chemotactic response of bacteria in respect to the organic compounds secreted by the roots (151-153). For instance, Hawes et al. (154) reported that a specific group of root cells, such as the root border cells, on one hand it stimulates the growth and chemoattraction of bacteria and fungi, and on the other hand it can release compounds in the rhizosphere, that enhance plant growth and inhibit several bacteria and fungi, when the nutrient conditions are not limited for the plant growth (155). This phenomenon has also been observed by Walker et al. (156), in which the secretion of anti-microbial metabolites, as root exudates, suppressed *Xanthomonas* sp. and *P. fluorescens* strains. In another study, on *Mentha piperita*, root exudates reduced the *Pseudomonas* population in the soil (157): it is known that some plants can release a broad range of secondary metabolites with anti-microbial activity (158), and *A. annua* plant has many potential root compounds for this purpose (159, 160). During the sampling, after 30 days of cultivation, beyond the lower values of all the growth parameters related to the age of the plants, no differences in the

results have been reported if compared to the sampling after 60 days on the CL26 plants; whereas the shoot biomass already decreased in a significant manner in the CL7 plants inoculated with *F. mosseae* also in the co-presence of *P. protegens*, thus showing that some microorganism influence on the plant growth can also manifest in the first phases of the plant-microbes interaction (100).

Concerning the belowground part of the plant, a few differences have been detected among all the plant treatments in every clone during each single experiment. In fact, the best values were always registered in the control plants if compared to all the other plant treatments. A significant improvement in root biomass was observed only in CL26 plants inoculated with the fungal consortium (AMF mix), that have a higher rate of arbuscule abundance compared to all the plant clones inoculated with AMF. The same result was reported by Domokos et al. (12) that observed an increase in root growth in *A. annua* plants inoculated with AMF. In addition, different studies showed that plant root increased its own biomass due to AM colonization (31, 161-165). In this sense, it is well known that mycorrhizal fungi can release macronutrients, like nitrogen and phosphate, directly inside the root cortex and afterwards specific plant ion transporters, present in the periarbuscular membrane, can transfer these nutrients into the plant cell (166-168), thus improving the root growth. However, the same inoculum led to a reduction of root fresh biomass in the CL10 plants but not in the dry one, thus underlining an important role of the AM fungi in helping the plant for a better water management (128). In addition, both bacterial strains gave rise to a decreasing root growth in two clones (CL24 and CL10), when inoculated in combination too. These results differ from those reported in two studies on *A. annua* plants inoculated with PGPB, in which the root biomass significantly increased compared to the control ones (131, 132), and differ from observations in which several species of the *Pseudomonas* genus had the same before-mentioned effect, but in different plant species (169-171). On the other hand, our

results are in accordance with other studies in which a decreased root growth was observed (172, 173). The inoculum composed by *F. mosseae* and *P. protegens* or *P. brassicacearum* gave a significant increase in root growth comparing to the control plants, only in the CL26 clone; whereas, the same bacteria used in combination with *R. irregularis*, in the same clone, have an opposite response compared to the previous one. Instead, the dual inoculation with the AMF consortium and *P. protegens* increased some root parameters only in CL26 plants, but significantly decreased the root growth in CL24 plants, in this latter case also by substituting *P. protegens* with *P. brassicacearum*. Furthermore, a significant decrease in root biomass, using the AMF consortium in combination with *P. brassicacearum*, was also observed in the CL10 plants, if compared to the control ones. Unfortunately, the effect on *A. annua* root growth is poorly investigated, scanty information is available, and our results are partially in accordance with studies that reported an improvement in the root growth when the plants were co-inoculated with PGPB and an endophytic fungus (132, 133). Also in other studies on different plant species, the combined use of AMF and PGPB has been reported: this leads to an enhancement in the root biomass production (172, 174-176). Moreover, some papers reported that the root growth increased in trifoliate orange plants inoculated with AMF under drought conditions, whereas it decreased in well-watered conditions (177). Therefore, different AMF can give rise to different influences on the root growth, on the basis of their specific compatibility with the specific plant genotype and according to the growth conditions (122). The increases in root biomass observed in our study, in the presence of the AMF consortium, could be related to the presence of different fungus species, some of which could be more efficient in high soil nutrient conditions (178). Concerning bacteria, also in this case, many factors such as plant genotype, bacterium species, bacterium strain, and available soil nutrients, could influence the plant response and the positive effect due to

the inoculation (169, 179, 180). In fact, different PGPB species can have several effects also on the root growth (135). These microbes, included the *Pseudomonas* genus, can produce IAA that stimulates the root growth (171, 181), but also cytokinins, like zeatin (182), and DAPG at low concentrations (183), could inhibit the root growth (185). According to some papers, the plant would drive the selection of microorganisms (185, 186), this could be due to the composition of the root exudates which is in turn dependent on the developmental stage of plant (187), soil abiotic factors such as nutrient availability (172), and the intra- and interspecific genetic variability (188). So, the beneficial association establishment needs a mutual recognition and coordinated responses between plant and microbes, and since the PGPB (like *Pseudomonas* species) require a physical contact with the host root, in order to stimulate the plant growth, the composition of the root exudates could be a crucial factor that may have influenced the interaction between bacteria and plants (189, 190).

3 - Photosynthetic pigments

Regarding the photosynthetic pigment concentration in the leaves, each clone showed different responses according to used microorganisms. In most cases, no differences were reported among all the plant treatments in each clone, thus showing a neutral effect. Instead, significant differences were detected in the CL26 plants, in which both chlorophyll *a* and *b* increased in the presence of *F. mosseae* and *P. protegens*, and when *F. mosseae* were co-inoculated with *P. brassicacearum* or *P. protegens*. The inoculation with the AMF consortium resulted in an improvement, whereas the combined use of the same inoculum with *P. protegens* or *P. brassicacearum* led to a decrease in the chlorophyll concentrations. Instead, the use of *P. protegens* alone decreased both the chlorophyll *a* and *b* concentration in the CL7 plants, but increased the chlorophyll *b* concentration only when these plants were co-inoculated with the above-

mentioned bacterium and *F. mosseae*. On the other hand, *P. protegens* alone gave rise to an improvement in the chlorophyll *a* concentration in the CL24 plants, also when inoculated in combination with *P. brassicacearum*. This latter combination also led to an enhancement of the chlorophyll *b* concentration only in the CL10 plants. According with what has been above discussed, there are many papers on *A. annua* plants that reported no differences in the chlorophyll concentrations in the presence of AMF and PGPB, used alone or in combination (8, 12, 14). This trend was also observed in other plant species, such as strawberry (53, 133), common bean and maize (190, 191). Nevertheless, many studies on *A. annua* plant inoculated with beneficial soil microorganisms showed a significant increase in chlorophyll concentrations (129, 131, 192), a trend also observed in other plant species (19, 126, 190, 193, 194-197). Moreover, PGPB can produce siderophores that facilitate plant iron acquisition, and since it is an important element in many key biochemical processes, this could improve the photosynthetic efficiency (198-201). Looking at the chlorophyll *a*/*b* ratio, only in one clone (CL10) an increase of this parameter was observed, while in all the other clones a reduction of this ratio in the plants inoculated with microorganisms was detected. This was probably due to the variation in the proportion of the chlorophyll *a* and *b* concentrations in each single treatments. It would have seemed that an equilibrium between costs and benefits was combined with the effect of both mycorrhizal symbiosis and PGPB on plant growth (202). Plants paid a higher cost in terms of photosynthates for the symbiosis, but it would be balanced by an improved photosynthetic capacity (203). Therefore, the result and the extent of the effects on the photosynthetic pigments are greatly dependent on the AMF species, the PGPB strain and the plant genotype compatibility (126, 204, 205), and also on the synergism between AMF and PGPB (18). Instead, the carotenoid concentrations increased only in the CL26 plants co-inoculated with *F. mosseae* and *P. protegens*, and decreased in CL7 plants inoculate with *P.*

protegens alone. An increase of carotenoids in *A. annua* plants in the presence of a dual microorganism inoculation was also reported by Arora et al. (131). In fact, it is known that AMF and PGPB can stimulate the antioxidant compound production in the plants (132), and protect plants from abiotic and biotic stress (206), for instance against oxidative damage as a prevention for the generation of ROS (207).

4 - Artemisinin concentration in the leaves

The analysis of artemisinin concentration in the clones used in the sixth experiment, CL26 and CL7, revealed that the concentration in the plant can be influenced by the clonal variability, as reported in other studies (1), but slight variations and no differences among all the plant treatments have been registered, both after 30 and 60 days of cultivation. On the contrary, significant differences were recorded between the two samplings in each single plant treatment, thus underlining the importance of the time to increase the artemisinin content in the *A. annua* leaves, as already reported in Towler and Weathers (208) and observed in our previous study (unpublished data). These findings disagree with many papers that showed an increase in the artemisinin content in response to plant inoculation with beneficial soil microorganisms (8-13, 129-132, 209, 210). In fact, the mycorrhizal symbiosis and the interaction between plant and PGPB can induce the methyl erythritol phosphate (MEP) pathway, thus enhancing the availability of substrates used for the artemisinin biosynthesis (211, 212). On the other hand, some studies revealed no significant variations in the terpenoid pathway (14), also in other plant species (54) in presence of beneficial microorganisms. In addition, in some works different species of AMF and PGPB were used and, in some cases, not all the microorganisms gave rise to an increase in the artemisinin content. In this regard, for instance, the fungus *F. mosseae* has poorly been used, and in Awasthi et al. (10) the plants inoculated with this fungus

alone did not result in a significant difference in the artemisinin content, whereas the difference became significant when the same fungus was co-inoculated with PGPB. In another study, the artemisinin content dramatically increased in the inoculated plants, according to the used fungus and the plant accession (9); furthermore, in the same study a plant treatment supplied with only a mineral fertilizer was also present, and it resulted in a similar amount of artemisinin concentration if compared to the plants inoculated with microbes. Therefore, the plant-microbes compatibility could play a crucial role in order to have a stimulatory effect on the artemisinin production (9, 10).

5 – Leaf metabolites

Concerning the leaf metabolites, in CL26 plants in the sixth experiment, the larger number of molecules detected in the young plants, compared to the plants grown for 60 days, reflects the fact that younger plants have a wide range of leaf secondary metabolites as a protection from all the types of biotic stresses (213). In the two considered experiments, the use of different microorganisms resulted in leaf volatile profile variations, in terms of chemical classes. Some of which, such as alkanes, were even not present in plants co-inoculated with AMF consortium and *P. protegens*. This phenomenon has only partially been reported in few studies on different cultivar of *A. annua* plants without the presence of microorganisms (214), but it has been well reported only in strawberry fruits in plants inoculated with bacteria and fungi (53). In fact beneficial soil microbes, such as AMF and PGPB, can modulate plant secondary metabolism as recorded in many studies on other plant species (7, 113, 215-220). *A. annua* is an aromatic/medicinal plant, therefore the essential oil is the most appreciated product, beyond the artemisinin, also for its anti-microbial properties (221, 222). In both the experiments, all the typical components already reported in literature of the *A. annua* essential oil were detected, such as aromadendrene, artemisia

ketone, byciclogermacrene, bisabolene <(Z)-,γ>, bisabolol α, cadin-4en-7-ol<cis->, camphor, caryophyllene oxide, caryophyllene-€, chrysantenol<cis>, copaene α, copaene β, cubebene α, cubebene β, eicosane, elemene β, eugenol, farnesene <E-,β>, germacrene D, lavandulyl acetate, mentha-2,8-dien-1-ol <cis-,para->, myrtenol, phytol, pinocarvone, 264ivari-3,11-dien-6-α-ol, selinene β, spathulenol, valencene (214, 222-225). In addition, other essential oil components, detected in other plant species but not yet reported in the *A. annua* leaves, were found: allo-cedrol (226), calarene (227), cedroxyde (228), cypertundone (229), drim-8(12)-ene (230), elemodiol <8-α-11-> (231), eremophilone (232), isocedranol (233), thujopsenal (234), tricos-(9Z)-ene (235), vetivone (236), γ-muurolene (237, 238). In both cases and experiments, qualitative and, for some components such as bisabolene, eugenol, eremophilone and γ-muurolene, also quantitative variations among the different plant treatments were found, according to other studies both on *A. annua* (9, 13, 14, 224) and other plant species (239, 240). The different microorganisms modulated plant secondary metabolite production in a different degree. The precise mechanisms involved in this effect on the plant secondary metabolism are not still completely understood, and three different hypothesis, concerning these variations provided by the beneficial microbes, are the most accredited: an improved uptake of nutrients (241-242), an activation of specific metabolic pathway (244, 245), or a defensive response toward bacteria and fungi (246). In reference to this last hypothesis, some molecules of biological interest and bioactive against microorganisms, such as benzoic acid, deoxyartemisinin, emetine, prim-O-glucosilcimifugin, and scopoletin were detected among the identified ones. Their presence varied according to the plant age and the used microbes. Emetine was present in all the young plants inoculated or not, whereas its presence was reported only in the adult plants inoculated with *P. protegens* alone, and in those co-inoculated with SVB6R1 and BEG12 or AMF mix. This

situation could be considered as a response displayed by the plant toward the specific bacterium alone over the time, in which this alkaloid molecule has a wide spectrum of anti-microbial activities (247, 248). These findings could also be supported by the fact that the use of *P. brassicacearum* alone did not stimulate a similar plant response, but only when it was inoculated in combination with fungi. Benzoic acid (BA) was found only in the young plants inoculated with *P. protegens* alone; it is an aromatic carboxylic acid with a wide range of anti-bacterial activities (249), that can even work both as a precursor of primary and secondary metabolites (250) and as a participant in the internal signals which are involved in the defence response against several stress conditions (biotic and abiotic; 251). Furthermore, BA can also play a crucial role in the chemical modification of root exudates (252), thus acting as an allochemical and a mediator of stress responses associated with plant-pathogen interactions when mineral nutrients accumulate in high concentrations in the soil (253). Scopoletin is a coumarin which possesses anti-microbial activities (254, 255) and it is related to disease resistance in many plants (256, 257). As BA, its presence was registered only in the younger plants inoculated with *P. protegens* alone, thus highlighting a PGPB-mediated induced systemic resistance (ISR; 258). Deoxyartemisinin, a molecule with a chemical structure like artemisinin without the endoperoxide bridge (259), also varied in accordance with the plant age and the microorganism presence: it was present only in the young control plants and in the adult inoculated plants of the sixth experiment. Unfortunately, no data are available in literature regarding this molecule in the presence of beneficial microbes. However, these findings underline the possibility that some metabolic changes need different times to be manifested, also in the presence of microorganisms (260). Prim-O-glucosilcimifugin (POG) is a chromone and one of the major effective components in *Saposhnikovia divaricata* root (261, 262), but it has never been detected in *A. annua* plants until now. POG also showed

variations over the time and according to the used microbes: in the sixth experiment, it was present in the young uninoculated plants and in those inoculated with the AMF mix alone; whereas in the older plants it was found in the controls and in those inoculated with *P. protegens*, even when it was in combination with the AMF consortium. Probably, either the time of cultivation or the bacterium re-inoculation performed in all the experiments after 30 days contributed to modify its production. On the other hand, POG was found in all the plants treated with other microorganisms in the fourth experiment (*P. brassicacearum*, *F. mosseae* and *P. brassicacearum*, AMF mix and *P. brassicacearum*), and this molecule is well known for its anti-bacterial and anti-fungal properties (263, 264). This further data highlights a specific interaction between plant and microbe species/combination, that revealed a wide range of plant metabolic responses probably due to the relationship established between the before-mentioned actors (265).

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Chapter 6

Conclusions

In conclusion, the micropropagation technique has proved to be a good, rapid and safe method to select new plant genotypes in a short period of time. The method has some limits due to the stability of the new plant clones both *in vitro* and *in vivo* conditions. However, it has the advantages to provide a uniform plant population in terms of genetic variability and a large number of available plants. The mycorrhizal colonization showed generally low values in all the cultivated clones, with some slight fluctuations according to the different used microorganisms and their combination. Thus it is underlined the crucial role of the plant-fungus-bacterium compatibility, but also the equally crucial role of the available essential nutrients in the growth substrate.

The used microorganisms resulted in a wide range of effects on the above- and below-ground of the different plant clones, underlining the importance of the plant-microbes compatibility in order to have positive responses in terms of plant productivity. Beyond this latter observation, also the substrate of growth, and particularly, its available content of nutrients played a crucial role on the relationship that established among the actors of these symbiotic relationships. Furthermore, also the interaction between the microbes, AMF-PGPB, can contribute to the improvement of plant growth.

The artemisinin content showed different concentrations in each single clone, thus demonstrating that in vitro culture is a good tool in order to select high artemisinin genotypes. Its concentrations did not vary in the presence of the used microorganisms, so these microbes have proved to be poorly effective in order to modulate artemisinin content with the used plant clones. However, the time of cultivation also represents another important factor to reach its maximum yield.

The leaf metabolite analysis revealed that beneficial soil microorganisms have the potential to stimulate plant defence and modulate plant secondary metabolism, even in a different manner over the time, thus changing the qualitative characteristics of the plant material. Furthermore, new essential oil components and bioactive molecules, have been detected in the *A. annua* leaves for the first time in this plant species.

Future perspectives

In the future, it could be interesting to use other beneficial soil microorganisms in order to find the best compatibility/effectiveness in terms of mycorrhizal root colonization, plant biomass and artemisinin production, with the clone selected in this work.

It could also be worthwhile to make use of a different growth substrate in order to reduce the factors that could influence the experiments.

Considering the wide range of bioactive molecules which have been found in the *A. annua* clone leaves, it could be interesting to test the effectiveness of the leaf extract or the essential oil as an anti-microbial, an anti-parasitic or in the medical field, and also the potential role of these molecules as ecological mediators.

Annex A

1 - First experiment | Clone 26 (CL26)

Table 2 | Data of the different plant parameters that did not show significant differences between the various treatments.

	Plant treatment			
Parameter	**Control**	**Pf7**	**AMF mix**	**AMF mix+Pf7**
Shoot fresh weight (g)	14.390 ± 0.286**a**	15.221 ± 0.379**a**	14.822 ± 0.324**a**	14.651 ± 0.234**a**
Shoot dry weight (g)	3.899 ± 0.089**a**	4.189 ± 0.174**a**	4.030 ± 0.139**a**	4.246 ± 0.114**a**
Leaf fresh weight (g)	8.299 ± 0.219**a**	8.966 ± 0.206**a**	8.645 ± 0.320**a**	8.497 ± 0.186**a**
Leaf dry weight (g)	2.133 ± 0.046**a**	2.189 ± 0.029**a**	2.137 ± 0.041**a**	2.223 ± 0.045**a**
Shoot dry/fresh weight	0.271 ± 0.005**a**	0.275 ± 0.008**a**	0.272 ± 0.008**a**	0.290 ± 0.005**a**
Root fresh weight (g)	13.278 ± 0.639**a**	13.192 ± 0.676**a**	13.943 ± 0.489**a**	12.007 ± 0.491**a**
Root/Shoot fresh weight	0.920 ± 0.031**a**	0.871 ± 0.048**a**	0.947 ± 0.046**a**	0.819 ± 0.031**a**
Stem height (cm)	41.000 ± 1.145**a**	40.800 ± 1.103**a**	39.500 ± 1.698**a**	39.710 ± 1.079**a**
Root length (cm)	30.538 ± 0.974**a**	28.470 ± 1.574**a**	30.390 ± 1.129**a**	29.300 ± 0.864**a**
Stem height/root length	1.350 ± 0.042**a**	1.465 ± 0.076**a**	1.327 ± 0.088**a**	1.368 ± 0.062**a**

2 - Second experiment | Clone 10 (CL10) and Clone 24 (CL24)

2.1 - Clone 10 (CL10)

le 2.1 | Data of the different plant parameters that did not show significant differences between the various treatments.

	Plant treatment						
rameter	**Control**	**Pf7**	**SVB6R1**	**Pf7+SVB6R1**	**BEG12**	**BEG12+Pf7**	**BEG12+SVB6R1**
oot fresh ght (g)	20.012 ± 0.478a	19.970 ± 0.291a	20.803 ± 0.379a	20.381 ± 0.527a	19.228 ± 0.405a	20.761 ± 0.605a	20.164 ± 0.379a
f fresh ght (g)	12.238 ± 0.336a	12.190 ± 0.091a	12.618 ± 0.320a	12.590 ± 0.378a	12.227 ± 0.238a	12.236 ± 0.314a	12.057 ± 0.281a
ot fresh ght (g)	17.682 ± 0.655a	16.206 ± 1.103a	16.290 ± 0.484a	14.581 ± 0.982a	15.310 ± 0.874a	13.767 ± 1.415a	14.704 ± 0.558a
ot dry ght (g)	2.390 ± 0.103a	1.792 ± 0.077a	2.237 ± 0.130a	1.857 ± 0.151a	1.947 ± 0.146a	1.974 ± 0.200a	2.114 ± 0.107a
ot/Shoot sh weight	0.886 ± 0.049a	0.814 ± 0.067a	0.785 ± 0.030a	0.719 ± 0.049a	0.793 ± 0.041a	0.664 ± 0.069a	0.729 ± 0.027a
m height a)	36.000 ± 1.733a	38.460 ± 2.482a	43.067 ± 0.767a	41.329 ± 1.528a	35.683 ± 1.587a	37.971 ± 2.935a	40.800 ± 1.357a
l *a* **/mL)**	46.721 ± 2.460a	44.499 ± 2.673a	39.413 ± 1.298a	44.918 ± 2.966a	40.671 ± 1.446a	40.476 ± 2.259a	39.615 ± 2.245a
l *b* **/mL)**	13.844 ± 0.789a	13.437 ± 0.856a	10.835 ± 0.366a	12.548 ± 0.910a	11.328 ± 0.517a	11.377 ± 0.658a	11.574 ± 0.812a
rotenoids /mL)	9.666 ± 0.458a	9.211 ± 0.453a	8.505 ± 0.246a	9.243 ± 0.593a	8.424 ± 0.303a	8.871 ± 0.397a	8.809 ± 0.393a

2.2 - Clone 24 (CL24)

Table 2.2 | Data of the different plant parameters that did not show significant differences between the various treatments.

	Plant treatment						
Parameter	**Control**	**Pf7**	**SVB6R1**	**Pf7+SVB6R1**	**AMF mix**	**AMF mix+Pf7**	**AMF mix+SV**
Shoot fresh weight (g)	17.435 ± 0.341**a**	17.767 ± 0.375**a**	16.774 ± 0.224**a**	17.759 ± 0.458**a**	18.027 ± 0.312**a**	18.446 ± 0.514**a**	18.132 ± 0.
Shoot dry weight (g)	4.665 ± 0.111**a**	4.112 ± 0.059**a**	4.503 ± 0.137**a**	4.191 ± 0.118**a**	4.583 ± 0.094**a**	4.360 ± 0.193**a**	4.230 ± 0.2
Leaf fresh weight (g)	11.043 ± 0.206**a**	11.402 ± 0.284**a**	10.839 ± 0.285**a**	11.674 ± 0.308**a**	11.082 ± 0.149**a**	11.603 ± 0.313**a**	11.262 ± 0.
Leaf dry weight (g)	2.792 ± 0.077**a**	2.512 ± 0.032**a**	2.754 ± 0.097**a**	2.603 ± 0.055**a**	2.667 ± 0.066**a**	2.681 ± 0.104**a**	2.586 ± 0.1
Stem height (cm)	51.383 ± 0.530**a**	53.150 ± 0.518**a**	49.900 ± 0.848**a**	50.900 ± 1.621**a**	51.000 ± 1.104**a**	52.371 ± 1.210**a**	53.680 ± 0.
Chl *a* (µg/mL)	40.328 ± 2.011**a**	43.592 ± 2.825**a**	37.333 ± 1.413**a**	42.363 ± 2.451**a**	40.749 ± 3.578**a**	37.989 ± 3.434**a**	42.127 ± 3.
Chl *b* (µg/mL)	11.601 ± 0.583**a**	13.312 ± 0.910**a**	11.373 ± 0.460**a**	12.971 ± 1.018**a**	11.820 ± 0.947**a**	12.098 ± 1.114**a**	12.414 ± 1.
Carotenoids (µg/mL)	8.514 ± 0.363**a**	9.065 ± 0.426**a**	7.402 ± 0.254**a**	8.518 ± 0.476**a**	8.431 ± 0.639**a**	7.687 ± 0.572**a**	9.244 ± 0.6

3 - Third experiment | Clone 10 (CL10) and Clone 6 (CL6)

3.1 - Clone 10 (CL10)

e **3.1** | Data of the different plant parameters that did not show significant differences between the various treatments.

	Plant treatment						
meter	Control	Pf7	SVB6R1	Pf7+SVB6R1	AMF mix	AMF mix+Pf7	AMF mix+SVB6R1
	0**a**	0**a**	0**a**	0**a**	0.057 ± 0.036**a**	0.114 ± 0.067**a**	0.430 ± 0.179**a**
fresh nt (g)	13.250 ± 0.176**a**	14.077 ± 0.606**a**	13.270 ± 0.250**a**	12.813 ± 0.264**a**	13.598 ± 0.799**a**	14.467 ± 0.262**a**	14.371 ± 0.205**a**
dry nt (g)	2.910 ± 0.332**a**	2.223 ± 0.370**a**	1.827 ± 0.062**a**	2.667 ± 0.613**a**	2.775 ± 0.222**a**	2.964 ± 0.244**a**	2.544 ± 0.155**a**
/Shoot veight	0.530 ± 0.029**a**	0.423 ± 0.052**a**	0.373 ± 0.018**a**	0.537 ± 0.090**a**	0.445 ± 0.039**a**	0.497 ± 0.024**a**	0.457 ± 0.020**a**
height	47.833 ± 1.764**a**	46.233 ± 1.220**a**	41.933 ± 2.576**a**	45.800 ± 3.710**a**	45.617 ± 2.344**a**	47.829 ± 1.894**a**	45.200 ± 2.114**a**
(µg/mL)	41.717 ± 3.453**a**	48.836 ± 1.024**a**	48.803 ± 1.696**a**	54.434 ± 3.415**a**	43.320 ± 1.690**a**	43.677 ± 3.194**a**	42.438 ± 3.732**a**
tenoids nL)	8.979 ± 0.404**a**	9.942 ± 0.406**a**	9.944 ± 0.216**a**	10.871 ± 0.651**a**	9.060 ± 0.319**a**	8.631 ± 0.492**a**	8.657 ± 0.663**a**

3.2 - Clone 6 (CL6)

able 3.2 | Data of the different plant parameters that did not show significant differences between the various treatments.

	Plant treatment						
Parameter	**Control**	**Pf7**	**SVB6R1**	**Pf7+SVB6R1**	**BEG12**	**BEG12+Pf7**	**BEG12+**
hoot fresh weight (g)	22.168 ± 0.499**a**	20.788 ± 0.816**a**	21.790 ± 0.561**a**	21.681 ± 0.617**a**	20.753 ± 0.575**a**	21.077 ± 0.690**a**	21.073 ±
hoot dry weight (g)	5.635 ± 0.179**a**	5.195 ± 0.245**a**	5.291 ± 0.205**a**	5.471 ± 0.100**a**	5.123 ± 0.200**a**	5.143 ± 0.274**a**	5.034 ±
eaf fresh weight (g)	12.377 ± 0.401**a**	11.452 ± 0.662**a**	12.629 ± 0.317**a**	11.907 ± 0.436**a**	11.810 ± 0.624**a**	11.791 ± 0.453**a**	12.030 ±
eaf dry weight (g)	2.903 ± 0.092**a**	2.857 ± 0.111**a**	2.881 ± 0.061**a**	2.870 ± 0.069**a**	2.753 ± 0.043**a**	2.747 ± 0.104**a**	2.771 ±
hoot dry/fresh weight	0.255 ± 0.010**a**	0.253 ± 0.019**a**	0.241 ± 0.006**a**	0.254 ± 0.006**a**	0.248 ± 0.015**a**	0.244 ± 0.011**a**	0.240 ±
oot fresh weight (g)	15.367 ± 0.695**a**	15.692 ± 0.978**a**	14.716 ± 1.094**a**	16.183 ± 0.535**a**	17.460 ± 1.153**a**	15.013 ± 0.764**a**	14.146 ±
oot dry weight (g)	2.780 ± 0.128**a**	2.625 ± 0.230**a**	2.583 ± 0.263**a**	2.597 ± 0.070**a**	3.207 ± 0.333**a**	2.870 ± 0.200**a**	2.607 ±
oot dry/fresh weight	0.180 ± 0.004**a**	0.165 ± 0.006**a**	0.176 ± 0.011**a**	0.161 ± 0.008**a**	0.183 ± 0.008**a**	0.190 ± 0.012**a**	0.180 ±
oot/Shoot fresh weight	0.693 ± 0.027**a**	0.760 ± 0.056**a**	0.681 ± 0.058**a**	0.751 ± 0.040**a**	0.845 ± 0.061**a**	0.714 ± 0.036**a**	0.676 ±
oot/Shoot dry weight	0.125 ± 0.004**a**	0.127 ± 0.011**a**	0.119 ± 0.012**a**	0.119 ± 0.006**a**	0.155 ± 0.018**a**	0.134 ± 0.009**a**	0.123 ±
tem height (cm)	60.117 ± 0.964**a**	58.967 ± 1.273**a**	59.729 ± 1.376**a**	60.357 ± 0.979**a**	58.450 ± 0.823**a**	57.529 ± 1.253**a**	58.071 ±
hl *a* (µg/mL)	46.064 ± 2.434**a**	43.998 ± 1.286**a**	47.592 ± 2.184**a**	47.162 ± 2.422**a**	43.994 ± 1.182**a**	42.412 ± 1.720**a**	46.214 ±
hl *b* (µg/mL)	14.121 ± 0.955**a**	13.683 ± 0.524**a**	14.733 ± 0.987**a**	14.404 ± 0.745**a**	14.365 ± 0.602**a**	12.915 ± 0.556**a**	14.251 ±
hl *a*/Chl *b*	3.285 ± 0.105**a**	3.221 ± 0.034**a**	3.259 ± 0.085**a**	3.275 ± 0.020**a**	3.073 ± 0.064**a**	3.289 ± 0.060**a**	3.252 ±
arotenoids (µg/mL)	9.329 ± 0.409**a**	8.768 ± 0.232**a**	9.572 ± 0.347**a**	9.695 ± 0.395**a**	8.833 ± 0.181**a**	8.993 ± 0.358**a**	9.477 ±

4 - Fourth experiment | Clone 26 (CL26)

Table 4.1 | Data of the different plant parameters that did not show significant differences between the various treatments.

	Plant treatment			
Parameter	**Control**	**SVB6R1**	**BEG12+SVB6R1**	**AMF mix+SVB6R1**
Shoot dry weight (g)	4.560 ± 0.097**a**	4.868 ± 0.158**a**	4.456 ± 0.231**a**	5.034 ± 0.185**a**
Leaf fresh weight (g)	10.956 ± 0.250**a**	10.830 ± 0.185**a**	10.904 ± 0.144**a**	11.586 ± 0.259**a**
Leaf dry weight (g)	2.462 ± 0.026**a**	2.498 ± 0.057**a**	2.374 ± 0.061**a**	2.570 ± 0.084**a**
Shoot dry/fresh weight	0.240 ± 0.008**a**	0.252 ± 0.011**a**	0.238 ± 0.010**a**	0.240 ± 0.011**a**
Root fresh weight (g)	13.704 ± 1.349**a**	13.110 ± 1.035**a**	11.654 ± 0.334**a**	12.978 ± 0.634**a**
Root dry weight (g)	2.478 ± 0.260**a**	2.284 ± 0.283**a**	2.186 ± 0.224**a**	2.692 ± 0.154**a**
Root dry/fresh weight	0.182 ± 0.006**a**	0.170 ± 0.012**a**	0.186 ± 0.016**a**	0.208 ± 0.006**a**
Root/Shoot fresh weight	0.718 ± 0.071**a**	0.686 ± 0.061**a**	0.620 ± 0.022**a**	0.624 ± 0.038**a**
Root/Shoot dry weight	0.542 ± 0.054**a**	0.466 ± 0.050**a**	0.488 ± 0.028**a**	0.534 ± 0.025**a**
Stem height (cm)	53.900 ± 1.105**a**	54.800 ± 0.771**a**	53.680 ± 1.788**a**	54.960 ± 0.533**a**

Table 4.2 | One-way ANOVA of the different plant parameters that showed significant differences between the various treatments.

	One-way ANOVA
Parameter	**p value**
F%	**
M%	*
A%	*
V%	*
Shoot fresh weight (g)	**
Root length (cm)	*
Stem height/Root length	*
Chl *a* (µg/mL)	*
Chl *b* (µg/mL)	*
Chl *a*/Chl *b*	*
Carotenoids (µg/mL)	*

5 - Fifth experiment | Clone 26 (CL26)

Table 5.1 | Data of the different plant parameters that did not show significant differences between the various treatments.

	Plant treatment			
Parameter	**Control**	**Ri**	**Ri+Pf7**	**Ri+SVB6R1**
Shoot fresh weight (g)	21.533 ± 0.428**a**	20.143 ± 0.668**a**	20.919 ± 0548**a**	21.106 ± 0.437**a**
Shoot dry weight (g)	6.163 ± 0.176**a**	5.672 ± 0.227**a**	5.493 ± 0.213**a**	5.941 ± 0.052**a**
Leaf fresh weight (g)	11.022 ± 0.259**a**	10.927 ± 0.496**a**	11.163 ± 0.488**a**	10.924 ± 0.301**a**
Shoot dry/fresh weight	0.287 ± 0.011**a**	0.282 ± 0.011**a**	0.263 ± 0.013**a**	0.281 ± 0.006**a**
Root dry weight (g)	4.075 ± 0.213**a**	4.320 ± 0.342**a**	3.560 ± 0.215**a**	3.710 ± 0.276**a**
Root dry/fresh weight	0.218 ± 0.006**a**	0.245 ± 0.012**a**	0.241 ± 0.007**a**	0.241 ± 0.009**a**
Root/Shoot fresh weight	0.510 ± 0.008**a**	0.543 ± 0.013**a**	0.533 ± 0.015**a**	0.519 ± 0.006**a**
Root/Shoot dry weight	0.662 ± 0.035**a**	0.763 ± 0.056**a**	0.644 ± 0.018**a**	0.623 ± 0.043**a**
Stem height (cm)	57.017 ± 0.442**a**	53.517 ± 0.865**a**	54.286 ± 1.228**a**	55.943 ± 1.046**a**
Chl *a* (µg/mL)	42.929 ± 3.249**a**	42.309 ± 3.859**a**	44.631 ± 2.514**a**	38.572 ± 1.298**a**
Chl *b* (µg/mL)	12.592 ± 0.983**a**	13.632 ± 1.230**a**	15.241 ± 0.986**a**	12.862 ± 0.495**a**
Carotenoids (µg/mL)	9.321 ± 0.720**a**	8.711 ± 0.676**a**	9.155 ± 0.444**a**	7.847 ± 0.360**a**

Table 5.2 | One-way ANOVA of the different plant parameters that showed significant differences between the various treatments.

	One-way ANOVA
Parameter	**p value**
F%	***
M%	***
A%	**
V%	*
Leaf dry weight (g)	**
Root fresh weight (g)	*
Root length (cm)	**
Stem height/Root length	*
Chl *a*/Chl *b*	***

6 - Sixth experiment | Clone 26 (CL26) and Clone 7 (CL7)

6.1 - I° sampling | Clone 26 (CL26)

Table 6.1 | Data of the different plant parameters that did not show significant differences between the various treatments.

	Plant treatment			
Parameter	**Control**	**Pf7**	**AMF mix**	**Pf7+AMF mix**
A%	0**a**	0**a**	1.653 ± 0.757**a**	0.485 ± 0.449**a**
V%	0**a**	0**a**	0.046 ± 0.035**a**	0**a**
Shoot fresh weight (g)	11.800 ± 0.327**a**	12.677 ± 0.373**a**	12.733 ± 0.301**a**	12.955 ± 0.330**a**
Shoot dry weight (g)	2.628 ± 0.061**a**	2.795 ± 0.091**a**	2.816 ± 0.079**a**	2.758 ± 0.151**a**
Leaf fresh weight (g)	6.223 ± 0.172**a**	6.608 ± 0.201**a**	6.239 ± 0.094**a**	6.743 ± 0.148**a**
Leaf dry weight (g)	1.365 ± 0.035**a**	1.357 ± 0.029**a**	1.356 ± 0.028**a**	1385 ± 0.068**a**
Shoot dry/fresh weight	0.223 ± 0.009**a**	0.220 ± 0.011**a**	0.220 ± 0.004**a**	0.215 ± 0.008**a**
Root fresh weight (g)	7.955 ± 0.673**a**	9.195 ± 0.502**a**	8.931 ± 0.436**a**	8.560 ± 0.498**a**
Root dry weight (g)	1.248 ± 0.073**a**	1.402 ± 0.127**a**	1.446 ± 0.057**a**	1.272 ± 0.071**a**
Root dry/fresh weight	0.160 ± 0.009**a**	0.150 ± 0.011**a**	0.161 ± 0.004**a**	0.148 ± 0.008**a**
Root/Shoot fresh weight	0.672 ± 0.040**a**	0.732 ± 0.053**a**	0.707 ± 0.041**a**	0.660 ± 0.035**a**
Root/Shoot dry weight	0.477 ± 0.032**a**	0.497 ± 0.035**a**	0.517 ± 0.030**a**	0.463 ± 0.021**a**
Stem height (cm)	35.200 ± 0.832**a**	37.517 ± 2.122**a**	37.671 ± 1.076**a**	38.667 ± 1.889**a**
Root length (cm)	36.467 ± 4.030**a**	30.333 ± 1.680**a**	28.643 ± 2.168**a**	27.783 ± 1.008**a**
Stem height/Root length	1.022 ± 0.103**a**	1.248 ± 0.087**a**	1.369 ± 0.115**a**	1.398 ± 0.074**a**
Chl *a* (µg/mL)	46.496 ± 4.480**a**	41.844 ± 3.694**a**	36.487 ± 1.202**a**	38.224 ± 1.972**a**
Chl *b* (µg/mL)	13.734 ± 1.556**a**	12.988 ± 1.107**a**	11.159 ± 0.466**a**	11.667 ± 0.605**a**
Chl *a*/Chl *b*	3.425 ± 0.079**a**	3.222 ± 0.074**a**	3.283 ± 0.080**a**	3.276 ± 0.023**a**
Carotenoids (µg/mL)	10.014 ± 1.044**a**	8.509 ± 0.828**a**	7.757 ± 0.280**a**	7.924 ± 0.395**a**
Artemisinin (mg/mL)	5.135 ± 0.454**a**	5.112 ± 0.444**a**	5.276 ± 0.361**a**	4.083 ± 0.344**a**

6.2 - I° sampling | Clone 7 (CL7)

Table 6.2 | Data of the different plant parameters that did not show significant differences between the various treatments.

	Plant treatment			
Parameter	**Control**	**Pf7**	**BEG12**	**Pf7+BEG12**
A%	0**a**	0**a**	0.095 ± 0.060**a**	0.080 ± 0.046**a**
V%	0**a**	0**a**	0**a**	0.002 ± 0.002**a**
Leaf fresh weight (g)	7.007 ± 0.220**a**	7.252 ± 0.132**a**	6.562 ± 0.236**a**	6.892 ± 0.131**a**
Leaf dry weight (g)	1.675 ± 0.046**a**	1.744 ± 0.020**a**	1.555 ± 0.068**a**	1702 ± 0.061**a**
Shoot dry/fresh weight	0.242 ± 0.006**a**	0.240 ± 0.005**a**	0.237 ± 0.007**a**	0.242 ± 0.010**a**
Root fresh weight (g)	9.098 ± 0.359**a**	9.504 ± 0.772**a**	8.100 ± 0.702**a**	8.768 ± 0.992**a**
Root dry weight (g)	1.235 ± 0.054**a**	1.310 ± 0.025**a**	1.295 ± 0.051**a**	1.430 ± 0.020**a**
Root dry/fresh weight	0.137 ± 0.008**a**	0.140 ± 0.011**a**	0.163 ± 0.010**a**	0.162 ± 0.011**a**
Root/Shoot fresh weight	0.698 ± 0.031**a**	0.728 ± 0.063**a**	0.698 ± 0.045**a**	0.726 ± 0.081**a**
Root/Shoot dry weight	0.390 ± 0.026**a**	0.420 ± 0.010**a**	0.480 ± 0.019**a**	0.482 ± 0.049**a**
Stem height (cm)	38.067 ± 2.995**a**	38.560 ± 1.319**a**	36.117 ± 1.327**a**	37.460 ± 1.761**a**
Root length (cm)	26.017 ± 0.661**a**	26.040 ± 1.497**a**	28.767 ± 0.817**a**	25.980 ± 0.863**a**
Stem height/root length	1.468 ± 0.127**a**	1.504 ± 0.109**a**	1.258 ± 0.049**a**	1.446 ± 0.062**a**
Chl *a* (µg/mL)	33.671 ± 1.183**a**	33.500 ± 0.990**a**	37.106 ± 1.983**a**	35.999 ± 0.829**a**
Chl *b* (µg/mL)	9.693 ± 0.386**a**	10.141 ± 0.358**a**	11.340 ± 0.666**a**	11.340 ± 0.344**a**
Carotenoids (µg/mL)	7.742 ± 0.170**a**	7.434 ± 0.195**a**	8.170 ± 0.341**a**	7.987 ± 0.160**a**
Artemisinin (mg/mL)	8.074 ± 0.546**a**	7.440 ± 0.315**a**	7.611 ± 0.457**a**	7.533 ± 0.530**a**

6.3 - II° sampling | Clone 26 (CL26)

Table 6.3 | Data of the different plant parameters that did not show significant differences between the various treatments.

	Plant treatment			
Parameter	**Control**	**Pf7**	**AMF mix**	**Pf7+AMF mix**
Shoot dry weight (g)	5.225 ± 0.547**a**	5.452 ± 0.111**a**	5.850 ± 0.266**a**	6.085 ± 0.084**a**
Root/Shoot dry weight	0.525 ± 0.070**a**	0.430 ± 0.026**a**	0.558 ± 0.020**a**	0.493 ± 0.013**a**
Stem height (cm)	54.650 ± 0.421**a**	53.040 ± 2.203**a**	55.880 ± 0.767**a**	56.750 ± 0.801**a**
Root length (cm)	26.475 ± 0.826**a**	27.300 ± 1.181**a**	26.540 ± 1.052**a**	29.417 ± 0.630**a**
Stem height/root length	2.070 ± 0.081**a**	1.956 ± 0.097**a**	2.118 ± 0.073**a**	1.932 ± 0.017**a**
Chl *a* (µg/mL)	31.666 ± 2.866**a**	34.084 ± 1.133**a**	34.728 ± 1.748**a**	29.097 ± 2.611**a**
Chl *b* (µg/mL)	9.653 ± 0.602**a**	11.068 ± 0.481**a**	11.161 ± 0.565**a**	9.784 ± 0.803**a**
Chl *a*/Chl *b*	3.268 ± 0.143**a**	3.087 ± 0.057**a**	3.112 ± 0.022**a**	2.965 ± 0.054**a**
Carotenoids (µg/mL)	7.524 ± 0.568**a**	8.225 ± 0.441**a**	7.563 ± 0.273**a**	6.436 ± 0.538**a**
Artemisinin (mg/mL)	7.677 ± 0.817**a**	8.261 ± 0.576**a**	7.804 ± 0.260**a**	6.903 ± 0.313**a**

6.4 - II° sampling | Clone 7 (CL7)

Table 6.4 | Data of the different plant parameters that did not show significant differences between the various treatments.

	Plant treatment			
Parameter	**Control**	**Pf7**	**BEG12**	**Pf7+BEG12**
V%	0**a**	0**a**	0**a**	0.006 ± 0.006**a**
Shoot dry weight (g)	5.484 ± 0.301**a**	5.628 ± 0.127**a**	5.260 ± 0.173**a**	5.014 ± 0.063**a**
Root fresh weight (g)	15.456 ± 0.793**a**	16.732 ± 1.049**a**	17.238 ± 1.042**a**	16.086 ± 0.680**a**
Root dry weight (g)	2.520 ± 0.115**a**	2.928 ± 0.182**a**	3.108 ± 0.288**a**	2.666 ± 0.090**a**
Root dry/fresh weight	0.164 ± 0.010**a**	0.174 ± 0.005**a**	0.180 ± 0.010**a**	0.166 ± 0.002**a**
Root/Shoot fresh weight	0.876 ± 0.066**a**	1.038 ± 0.086**a**	1.097 ± 0.089**a**	1.138 ± 0.044**a**
Root/Shoot dry weight	0.462 ± 0.018**a**	0.524 ± 0.039**a**	0.598 ± 0.066**a**	0.532 ± 0.018**a**
Stem height (cm)	53.960 ± 2.416**a**	54.980 ± 1.590**a**	54.750 ± 0.812**a**	51.900 ± 0.522**a**
Root length (cm)	28.180 ± 2.228**a**	26.780 ± 0.925**a**	25.533 ± 0.651**a**	27.700 ± 1.097**a**
Stem height/root length	1.966 ± 0.171**a**	2.068 ± 0.111**a**	2.150 ± 0.042**a**	1.886 ± 0.069**a**
Artemisinin (mg/mL)	9.528 ± 0.562**a**	8.570 ± 0.486**a**	9.471 ± 0.486**a**	9.408 ± 0.609**a**

List of publications

Massa N., Cesaro P., Todeschini V., Capraro J., Scarafoni A., Cantamessa S., Copetta A., **Anastasia F.**, Gamalero E., Lingua G., Berta G., Bona E. (2020). Selected autochthonous rhizobia, applied in combination with AM fungi, improve seed quality of common bean cultivated in reduced fertilization condition. *Applied Soil Ecology*, 148 (2020) 103507 ISSN 0929-1393. https://doi.org/10.1016/j.apsoil.2020.103507

Acknowledgements

Ringrazio il Professor Guido Lingua per la grande disponibilità, professionalità e competenza dimostratami durante questo percorso.

Desidero inoltre ringraziare la Dott.ssa Valeria Todeschini per la sua pazienza, cortesia e professionalità.

Ringrazio anche il Dott. Andrea Copetta e la Prof.ssa Valentina Gianotti.

Infine, un ringraziamento importante va alla mia famiglia ed a Serena che mi hanno sempre supportato durante questo percorso.