

Faculté des sciences

*Mycorrhizal association dynamics between
Rhizophagus irregularis and Manihot
esculenta in a context of African Cassava
Mosaic Virus infection*

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Acronyms

ACMV	African Cassava Mosaic Virus
AMF	Arbuscular mycorrhizal fungi
RNA	ribonucleic acid
BCTIV	Beet curly top Iran virus
C	Carbon
CA	Arbuscular colonization
CBM	basic culture medium
CIDER-Seq	Circular DNA Enrichment Sequencing
CMD	Cassava mosaic disease
CMN	common mycelial networks
CP	Coat protein
Cq	quantification cycle
CT	Total Colonization
CTAB	cetyltrimethylammonium bromide
CuSO ₄	copper sulfate
CV	Vesicular Colonization
DI DNAs	Defective interfering DNAs
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
eccDNAs	Extrachromosomal circular DNAs
ECM	Ectomycorrhizae
FC	fold change
HC	Hyphal Compartment
HCl	hydrochloric acid
HiFi	High Fidelity
K	potassium
KOH	potassium hydroxide
MP	Movement protein
N	nitrogen
NaCl	sodium chloride
NSP	Nuclear shuttling protein
ORF	Open reading frame
P	phosphorus
PCR	Polymerase chain reaction
RC	Root Compartment
RCA	Rolling circle amplification
REn	Replication enhancer
ReP	Replication associated protein
SEGS	Sequences Enhancing Geminivirus Symptoms
SMRT	Single Molecule Real Time
ssDNA	Single stranded DNA
SWEET	Sugar Will Eventually be Exported Transporters
TDP	Thiamine diphosphate
TE	Transposable element

THI	Thiamine
TMP	thiamine monophosphate
TPK	Thiamine Pyrophosphate kinase
TPP	thiamine pyrophosphate
TrAP	Transcriptional activator protein
TYLCSV	Tomato Yellow Leaf Curl Sardinia Virus
UBQ10	ubiquitin gene
VOCs	volatile organic compound

Introduction

M. esculenta, the cassava plant, also called manioc or yucca, is a crop culture of great importance in Africa, Asia and south America. It is cultivated for its edible tuber roots and leaves, mainly in tropical regions, where it is the third calory source, after rice and corn. It is particularly resistant to drought and adapted to poor fertility soil, making it an excellent choice for marginal farmers that uses *M. esculenta* as subsistence agriculture. Moreover, it bears high yield harvests and the development and application of optimal farming practices promises to increase the worldwide importance of *M. esculenta* cultures and *M. esculenta*-derived products.

M. esculenta culture, however, is constantly threatened by viral diseases, such as the cassava mosaic disease, caused by viruses from the Begomovirus genus, which includes the *African Cassava Mosaic Virus*. The cassava mosaic disease induces a stunted growth and highly reduces the development of tuber roots in infected plants, which lead to highly reduced yields and huge economic losses.

Arbuscular mycorrhizal fungi are soil micro-organism that are known to establish an association with the roots of more than 80% of land plants, including numerous important crop species including *M. esculenta*. This association has been extensively studied and demonstrated to promote plant nutrition and growth, as the fungal symbiont provides its host plant with water and mineral nutrients such as nitrogen, phosphate, potassium. In exchange, the plants transfer a significant part of its photosynthetically reduced carbon to the symbiont under the form of sugars. In addition, this association has been shown to enhance tolerance against biotic and abiotic stresses.

In this master thesis, the impact of inoculation of the ACMV, mycorrhization by *Rhizophagus irregularis* and the *in vitro* conditions on the growth of *M. esculenta* is studied. *M. esculenta* plants have been grown under *in vitro* conditions, with the presence or absence of *R. irregularis* and with the infection by ACMV or as virus-free plants. The *in vitro* condition, even though hard-work demanding, allows to study the impact of several parameters on a system, while excluding cofounding factors that may otherwise have an impact. The expression levels of genes responsible of thiamine biosynthesis have been quantified, to assess the impact of mycorrhization and ACMV-infection on the metabolism of *in vitro* grown plants. And SWEET genes expression have also been quantified, as potentially differentially expressed in mycorrhized plants. The protective impact played by the mycorrhization against the viral infection is discussed.

State of the art

Manihot esculenta

Manihot esculenta (cassava, Fig. 1) is an angiosperm native from South America, more particularly to the western Amazonian Rim. This perennial plant forms edible tuber-shaped roots with high starch content. However, *M. esculenta* roots and leaves contain highly toxic cyanogen compounds such as cyanogenic glucoside, cyanohydrins and free cyanide¹. Therefore, the tissues have to be processed in order to be safe for consumption. Multiple processing exists, such as pounding or crushing, sun-drying, soaking in water, roasting and boiling. The efficiency at removing cyanogen compounds depends on the method, the application time and the type of tissue on which it is applied (i.e. root or leaf)¹.

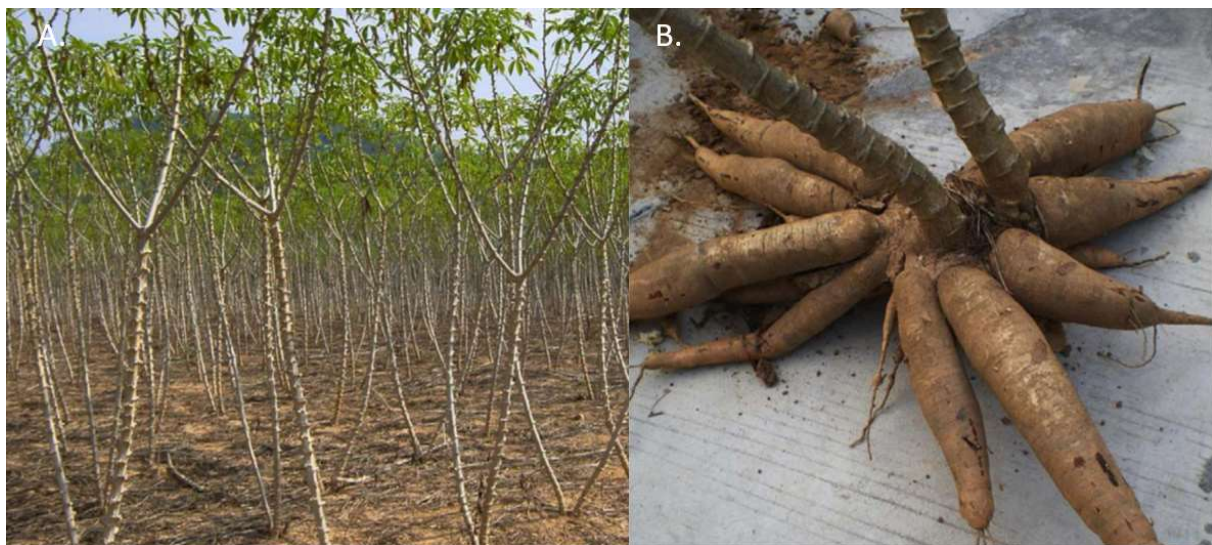


Figure 1. Healthy *M. esculenta*. A. 10-month old cassava plants in a field and B. storage roots harvested from 10-month old cassava plants. Taken from Q Liu *et al.* (2014)

Manihot esculenta is also present in Africa and Asia, where it was transported by Portuguese traders in the 16th century. The plant established in these regions, as the tropical climates mimicked its original environment. Nowadays, *M. esculenta* is cultivated in all tropical regions under temperatures above 20°C and annual rainfall above 1000 mm². This plant has a high tolerance to a variety of soils, including low fertile soils, but is restricted to well-drained soils².

In the past, *M. esculenta* was considered a staple crop for millions of peoples in tropical regions growing this plant only to meet their nutritional needs. The production of the plant was not sufficient for food industry or exportation³. Nowadays, the cultivation of *M. esculenta* has improved substantially with the development of several products such as fresh or starch-based foods, fodders for animal husbandry or various starchy industrial products² allowing export to regions or countries outside the production area. Currently, *M. esculenta* nourishes almost one billion people in 105 different countries, accounting for almost one third of their daily caloric intake^{4,5}. However, farming practices to grow *M.*

esculenta are often less developed and less effective than those applied to high-yield commercial crops². This leads to yields markedly below to what would be achieved under optimal farming practices, such as better weed control, planting of improved cultivars, soil and fertility management and control of pests and diseases². The adoption by farmers of mechanization as well as chemical weed control would lead to a gradual increase in *M. esculenta* production, and would eventually allow *M. esculenta* to reach an economic status equivalent to other current starch products-providing crops that are more efficient and which culture is more profitable due to the more developed and more controlled cultivation techniques².

One of the most devastating and economically impacting threats on *M. esculenta* cultures are viral diseases⁶, particularly in sub-Saharan Africa, where viruses are a constant threat to food security, leading to annual economic losses of more than 1 billion US\$⁷. More specifically, one of the most impacting viral disease complex of *M. esculenta* is known as Cassava Mosaic Disease (CMD), which develops yellow mosaics, leaf curl or deformation, yellow veins and stunting (growth retardation) as symptoms (Fig. 2 D-G), leading to highly reduced yield^{8,9} (Fig. 2 A-C).



Figure 2. Symptoms of cassava mosaic disease (CMD) on *M. esculenta* plants in fields. (A-C) Comparison of healthy, moderately and severely ACMV-infected *M. esculenta* plant. (A) Healthy *M. esculenta* plant with tubers. (B) Plant moderately infected by CMD shows moderate growth and few tubers. (C) Severely infected plant shows poor growth and no tuber. (D) An infected plant showing severe stunting and distortion of leaves compared to (E) A healthy plant. (F-G) Leaves of CMD-infected plants produce misshapen and twisted leaflets with mosaic and mottling symptoms. Modified from Alabi *et al.* (2011).

Geminiviruses

Geminivirus is the plant virus family with the greatest evolutionary success. It is found both in monocotyledon and dicotyledon species and is transmitted via a vast range of insects as vectors⁸. Therefore, viruses of this family are a constant threat to tropical and temperate crops¹⁰. Geminiviruses are divided in nine genera, depending on their host range, the vector type, their genome organization and genetic relationships. The most studied genus of the Geminivirus family is the Begomoviruses genus, which causes the CMD¹¹. One of the most prevalent and economically devastating member of the Begomoviruses genus is the *African Cassava Mosaic Virus* (ACMV), which occurs at the level of the entire African continent¹⁰.

Geminivirus infection and life cycle completion (Fig. 3) begins with a whitefly feeding on a suitable host plant. The virions are injected into the cell cytoplasm by the whitefly⁸. They are uncoated and the single-stranded DNA (ssDNA) then enters the nucleus, where the complementary strand is synthesized, leading to dsDNA¹². A bidirectional transcription takes place on the dsDNA, and the transcripts move to the cytoplasm to be translated to proteins, which return back to the nucleus¹². The newly synthesized viral proteins then assist in the replication process. For instance, the Rep viral protein binds to the origin of replication where it creates a nick in a nucleotide conserved sequence (Fig. 4) and a rolling circular replication occurs¹². Once the genome replication is completed, a newly synthesized ssDNA molecule is released and can continue the cycle until viral DNA reaches a threshold¹². When the threshold is reached, coat proteins encapsulate the viral

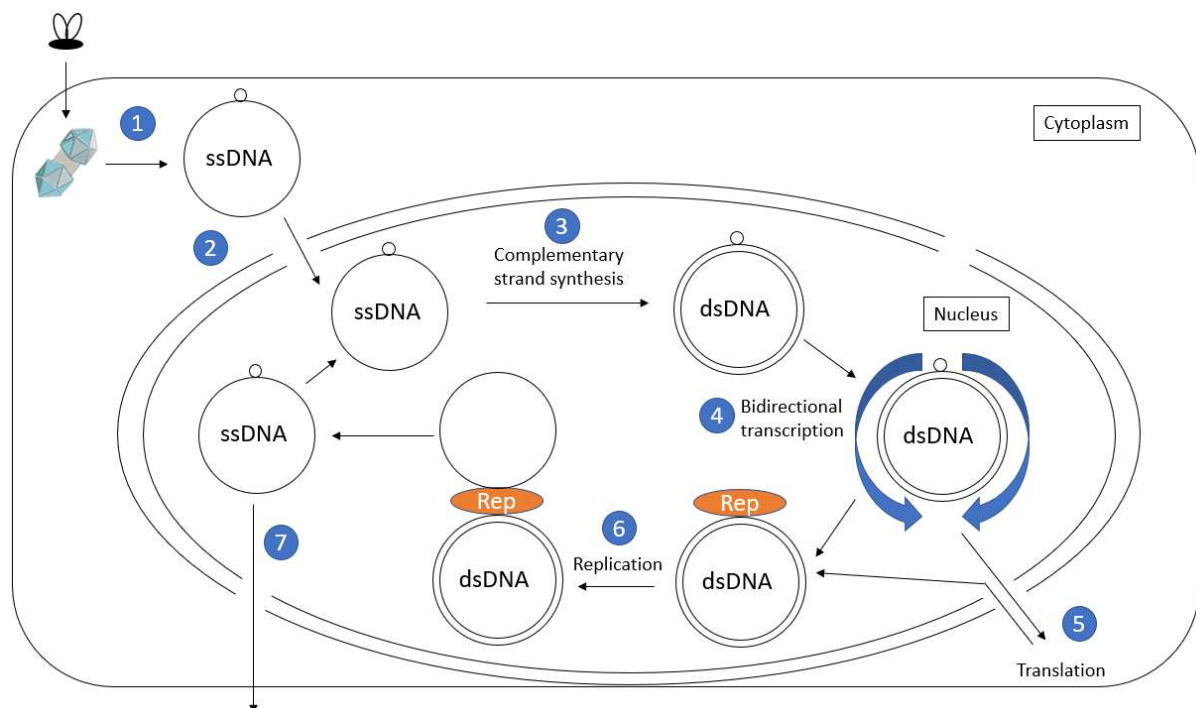


Figure 3. Geminivirus life cycle. See main text for details. 1. Whitefly-mediated infection and uncoating. 2. Entering nucleus. 3. Complementary strand synthesis. 4. Bidirectional transcription. 5. Viral protein translation. 6. Rolling circle replication. 7. Cell to cell movement or vector acquisition. (C. Gutierrez (1999), Navas-Castillo and Fiallo-Olivé (2020))

ssDNA molecules, which are then ready for cell to cell movement or acquisition by a feeding whitefly¹².

Geminiviruses have a small genome that encodes a limited number of proteins (Fig 4). They largely rely on the host replication machinery to carry out the replication of their ssDNA genome¹². All members of the Geminivirus family have a monopartite genome, at the exception of the Begomoviruses genus, which can have monopartite or bipartite genomes¹³. For bipartite genomes viruses, which is the case of the ACMV, the genome components are named DNA-A and DNA-B, each measuring between 2.5 and 2.7 kb in size¹⁴. Proteins encoded in the DNA-A component are in charge of replication, control of gene expression, overcoming host defenses, encapsidation and insect transmission, while DNA-B codes for proteins involved in intra and intercellular movement^{8,14–16}.

DNA-A has five open reading frames (ORF), which code for proteins involved in symptom development, genome replication and virus structure : coat protein (CP), a replication-associated protein (Rep), a transcriptional activator (TrAP), a replication enhancer (REn), and C4 protein⁸. DNA-B has two ORF, which code for proteins involved in viral movement inside the cell and between cells, respectively Nuclear Shuttling Protein (NSP) and Movement Protein (MP)⁸.

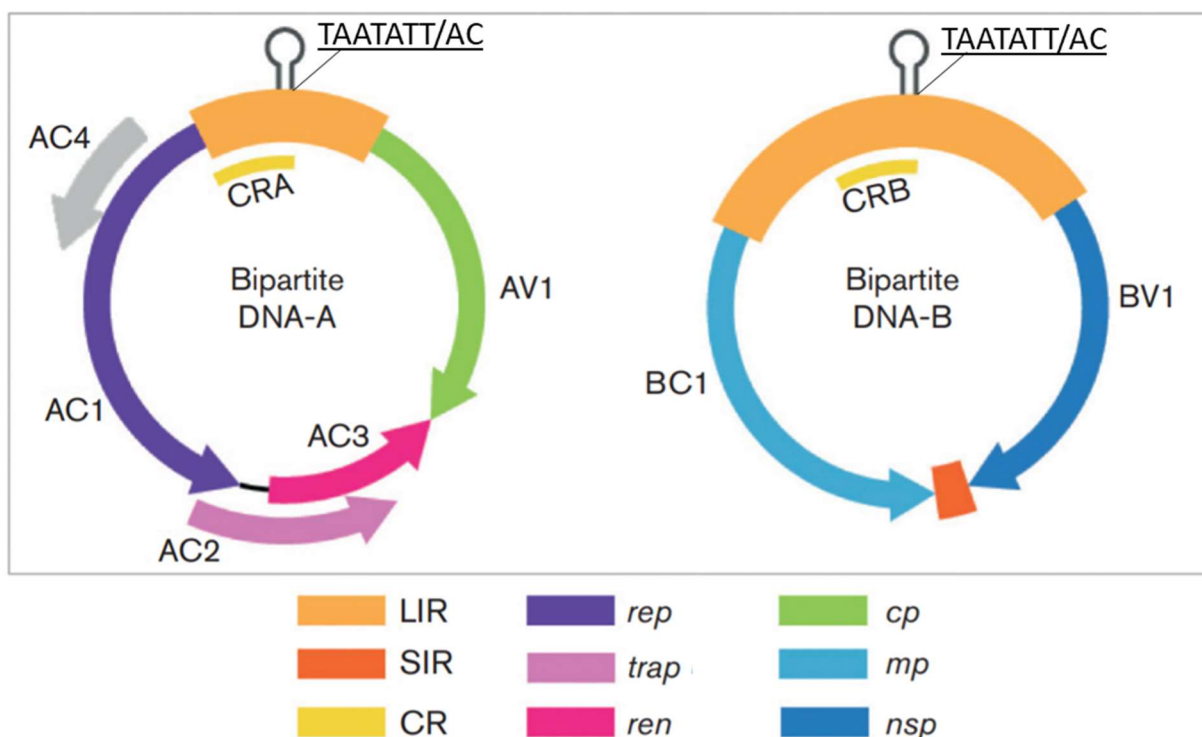


Figure 4. Genome organization of bipartite begomoviruses. The invariant TAATATT/AC nonanucleotide sequence located in the CR contains the initiation site (/) of rolling-circle DNA replication. LIR, Long Intergenic Region; SIR, Short Intergenic Region; CR, Common region; Rep, replication protein; TrAP, transcriptional activator; REn, replication enhancer; CP, coat protein; MP, movement protein; NSP, Nuclear Shuttling Protein. Modified from Murilo Zerbini et al. (2017).

Circular DNA molecules

The term Extrachromosomal circular DNAs (eccDNAs) describes circularized, genome-independent, double-stranded DNA molecules. eccDNAs length can vary widely, ranging from a few hundred base pairs to several million. Despite their ubiquitous presence in eukaryotes (animals¹⁷, plants^{18,19}, yeast²⁰), eccDNAs functions and roles in cellular biology still remains poorly studied. eccDNAs can derive from Transposable Elements (TE) or from coding or non-coding areas of the genome, and several putative mechanisms are proposed to explain the formation of eccDNAs of different sizes.

The genes present in eccDNAs can be transcribed, inducing the overexpression of the encoded genes²¹. For example, an eccDNA found in members of the plant species *Amaranthus palmeri* confers resistance to the herbicide glyphosate by amplification of the gene encoding the herbicide target, allowing its massive overexpression and rendering it ineffective¹⁹.

Some eccDNA have been found encoding the sequence of transposable elements (TE). Retrotransposons and DNA transposons use an extrachromosomal linear DNA form during their life cycle, in order to integrate in a genome. Given that eccDNAs have never been shown to be able to be reintegrated into the genome, this prompted Lanciano *et al.*, 2017²² to hypothesize that the eccDNAs containing TE sequences could be the result of a mechanism to limit the number of new insertions of active transposon into the genome.

Several putative mechanisms are proposed to explain the genesis of eccDNAs. All of these mechanisms are related to DNA repair, and are divided into 4 categories by Cao *et al.*, 2021²³: (i) Homologous recombination, (ii) non-homologous end joining, (iii) DNA replication and (iv) transcription.

The CIDER-Seq(Circular DNA Enrichment Sequencing) is a major technological advance allowing the accurate profiling of circular DNA molecules²³, which include eccDNAs and viral circular genomes. Until now, high-throughput sequencing was not suitable for virus identification and sequencing because of two issues ; (i) the sequence bias during virus enrichment, before sequencing²⁴ and (ii) the risk of assembling artificial chimeric viral genomes due to short sequencing read lengths²⁵⁻²⁷. CIDER-Seq overcome these two drawbacks respectively with the use of RCA instead of PCR²⁸ for the enrichment step and with the long-reads of the Single Molecule – Real time sequencing^{29,30}. PCR represented an issue, as the utilization of fixed templates as primers can lead to the disappearance of certain viral sequences, because of the different affinities of the primers templates. RCA settled this problem by using random primers, and is therefore less prone to the primer complementarity bias. However, the nucleic acid molecules produced by an RCA are (i) hyperbranched, (ii) of high molecular weight and (iii) concatenated³¹ products, which must be linearized. Restriction enzyme is a possible choice³², but it requires knowledge of the conserved cleavage sites, and this inevitably leads to the loss of sequences that lack them²⁴. To address this problem, CIDER-Seq uses the polymerase cloning (ploning) method. This technique employs endonucleases and DNA repair enzymes to linearize RCA products, as well as shotgun sequencing and whole genome assembly³³. Note that the RCA

is performed directly on the DNA sample without any removal of the linear DNAs. They are therefore also amplified, but because of the long-reads of the SMRT sequencing, it is possible to discriminate between amplified linear and circular DNAs.

Several circular DNA molecules that are related to viral genomes have been discovered. The *Beet curly top Iran virus* (BCTIV) of the Becurtovirus genus, Geminiviridae family has recently been shown to form hybrid DNA minicircles composed of viral and plant during infection by the BCTIV³⁴. Additionally, another type of circular DNA molecule has been identified in Geminiviruses, including in ACMV, and has been named Defective Interfering DNAs (DI DNAs)^{35,36}. No biological role has yet been found for these new type of biomolecules, although they have been associated with reduced viral replication³⁷. Finally, two Sequences Enhancing Geminivirus Symptoms (SEGS) have been identified in the *M. esculenta* genome³⁸. These sequences are present as episomes during ACMV infection, but are not present in healthy plants, and the formation of a circular DNA intermediate during their replication is known³⁹. As circular DNA molecules, DNA minicircles, DI DNAs and episomal forms of SEGS, cited above, can presumably be detected by CIDER-Seq.

Arbuscular mycorrhizal fungi and common mycorrhizal networks

Mycorrhiza is an intimate association between a Mycorrhizal Fungus (MF) and the roots of a host plant. After the encounter of the two symbiotic partners and once the association is established, the MF receives carbon molecules that have been fixed via photosynthesis by the host plant under the form of sugars and lipids. In exchange, the fungus supplies the plant with water and mineral nutrients such as nitrogen (N), phosphorus (P) and potassium (K). This nutrient exchange brings multiple benefits to the host plant, namely an enhanced growth and an improved tolerance to biotic and abiotic stresses^{40,41}. Mycorrhizal fungi are found in natural as well as agricultural environments, and they provide services to the ecosystems that host them; for example, sequestering carbon⁴², aggregating soil particles, and modulating microbial and plant communities⁴³.

Among the entire plant kingdom, between 320,000 and 340,000 plants, vascular or non-vascular, are expected to form mycorrhizae (around 75% of all existing plant species)⁴⁴, and angiosperms is the largest plant group that form mycorrhizas, as 85 to 95% of their members are believed to establish this association⁴⁵. Angiosperms include not only trees, shrubs and grasses, but also a large number of staple crops, such as rice, wheat, corn, potatoes, sweet potatoes, tomatoes and cassava⁴⁵. On the fungal side of the association, at least 50,000 species of fungi belonging to the groups of Ascomycetes, Basidiomycetes and Mucoromycota are mycorrhizal fungi⁴³.

Two major types of mycorrhizae exist: ectomycorrhizae and endomycorrhizae. Ectomycorrhizae (ECM) are mostly found in temperate forest, associated with trees. As their name suggest, ectomycorrhizal fungi do not penetrate the host plant cells. Conversely, endomycorrhizal fungi do penetrate in the host plant cells⁴⁶. The co-evolution between soil fungi and plants, which lasted for at least 400 million years⁴⁷, has generated 4 types of mycorrhizae. These 4 types emerged at different evolutionary time points, and have specific functional and morphological characteristics. In addition, these types do not

have the same overall prevalence: arbuscular mycorrhiza (AM) is the dominant type (72% of all plants), followed by orchid mycorrhiza (ORM, 10%), ECM (2%) and finally ericoid mycorrhizae (ERM, 1.4%)⁴⁸. AM and ECM belong to endomycorrhizae while ectomycorrhizae contains ORM and ERM

Arbuscular mycorrhizal fungi (AMF) can be considered the most important of the mycorrhizal types, as they are the most prevalent of the symbiosis types and because a vast majority of the crop cultures are colonized by AMF^{40,45}. Moreover, several studies highlighted the roles of AMF on plant biodiversity, productivity and community structure^{49–52}

The mycorrhizal dynamics of an ecosystem often follow three rules. (i) Several types of mycorrhizae coexist in the majority of ecosystems, (ii) certain plants and certain fungi can establish several types of mycorrhizae and (iii) a fungus can colonize multiple hosts, conspecific or heterospecific. It is now widely accepted that the result is the production of underground hyphal networks called common mycelial networks (CMNs) which connect several distinct plant individuals and are ubiquitous in ecosystems^{53–56}.

An interesting features of CMN which has been known for a long time is their ability to translocate nutrients between plant individuals⁵⁷. This feature has been investigated mostly in laboratory and under controlled environments. Therefore the real prevalence of CMN-mediated translocation of nutrients in natural ecosystem could be underestimated^{55,58,59}. CMNs formed by ECM fungi have been reported to mediate the translocation of mineral nutrient such as N and P^{60,61} and carbon (C)⁶². Oppositely, the exact implication of AMF-formed CMNs in C and other mineral nutrients translocation remains under debate. Several studies reported the transfer of N and P between plants^{42,63}, while C transfer seems well less widespread for AMF-constituted CMNs⁶⁴.

Additionally, it has been shown that CMNs could mediate an interplant communication, by acting as below-ground conduits that plants can use to send and receive putative enemy-warning signals to neighboring connected plants. Experiments aiming at studying this CMN-mediated interplant communication have, up to now, always involved an infected/infested “donor” plant which is connected via CMN to a “receiver” disease-free plant, on which parameters such as defense gene expression were investigated. A seminal study conducted on tomato (*lycopersicum esculentum*) plants linked by CMN of *Fulleniformis mosseae* revealed that healthy tomato plants upregulate six defense-related genes as well as harbor increased activities of enzymes putatively involved in defense when connected to a tomato plant infected by the fungal pathogen *Alternaria solani*⁶⁵. Similar results were obtained when replacing the fungal pathogen by herbivore insects. The herbivore caterpillar *Spodoptera litura*, when grazing on a donor tomato plant, upregulated defense-related genes in a receiver herbivore-free tomato plant connected by CMN of *F. mosseae*⁶⁶. The presence of the pea aphid *Acyrtosiphon pisum* on pea (*Vicia faba*) plants lead to the production of aphid-repulsing and aphid predators-attracting VOCs in aphid-free pea plants connected by CMN of *R. irregularis*⁶⁷. Interestingly, the authors of the last publication have, for the first time, investigated the rapidity of the

putative transmitted signal, and demonstrated that the responses in receiver plants is initiated before 24 hours⁶⁸. Moreover, it has recently been shown that CMN-linked receiver plants could harbor a response as shortly as 6 hours after wounding and submission of donor plants to *Manduca sexta* larval oral secretions to mimick herbivory⁶⁹. Recently, an *in vitro* experiment on potato (*Solanum tuberosum*) plants connected by a CMN of *R. irregularis* and in which donor plants where infected by the potato blight-inducing pathogen *Phytophthora infestans* revealed up-regulation of jasmonic acid and ethylene defense-related genes⁷⁰. No experiment of the type cited in this paragraph has ever been carried out with a viral agent as a source of infection in donor plants.

Importantly, only the handful studies cited in the preceding paragraph (Table 1) have been conducted on the putative interplant communication via hyphal networks^{65–68,70}. And despite the small number of studies, the hyphal-mediated interplant communication has already made a lot of ink to flow, both in the non-scientific and the scientific world, in which authors often extrapolate theories out of limited scientific evidence.

Table 1 : Features of the studies conducted on the CMN-mediated interplant communication

Receiver and donor plants	Fungal organism	<i>In vitro/in vivo</i>	Infection method	Effect on healthy receiver plants	Reference
<i>Solanum lycopersicum</i> (tomato plant)	<i>Fulleniformis mosseae</i>	<i>In vivo</i> (sterilized soil/sand mixture)	<i>Alternaria solanii</i> (fungal pathogen)	Increase in activities of putative defensive enzymes and 6 defense-related genes	Song <i>et al.</i> 2010
<i>Solanum lycopersicum</i> (tomato plant)	<i>Funneliformis mosseae</i>	<i>In vivo</i> (sterilized soil/sand mixture)	<i>Spodoptera litura</i> (herbivore caterpillar)	Increase in activities of putative defensive enzymes and defense-related genes	Song <i>et al.</i> 2014
<i>Vicia faba</i> (bean plant)	<i>Rhizophagus irregularis</i>	<i>In vivo</i> (initially sterile substrate)	<i>Acyrtosiphon pisum</i> (Pea aphids)	VOC profile modification, repulsion of aphids, attraction of Aphid predators	Babikova <i>et al.</i> 2013
<i>Nicotiana attenuate</i> (tabaco plants)	<i>Rhizophagus irregularis</i>	<i>In vivo</i>	Wounding and <i>Manduca sexta</i> larval secretions	Jasmonic acid bursts, higher level of several amino acids and higher glycoside metabolism in leaves	Song <i>et al.</i> 2019
<i>Solanum tuberosum</i> (potato plant)	<i>Rhizophagus irregularis</i>	<i>In vitro</i> (bicompartemented Petri plates)	<i>Phytophthora infestans</i>	Upregulated defense genes	Alaux <i>et al.</i> 2020

For instance, in his paper published in Current Commentary, Rhodes (2017) wrote “[...] the CMNs serve to transport [...] compounds that serve to defend the plant against parasitic attack [...] between different plants”⁵⁹. And, as unique reference for this sentence, use the paper of Song *et al.* (2010)⁶⁵. However, these authors effectively brought to light an increase in defense-related genes expression and putative defensive enzyme activities between healthy and fungal pathogen-infected plants linked by a CMN. But they did not prove that the CMN is directly responsible for the transfer of a putative signal nor do they even say that the interplant communication is mediated by a compound of any kind, which is factually extrapolated by Rhodes though wrong. What is brought to light by

Song *et al.* (2010) is that the presence of a CMN is correlated to a putative interplant communication. None of the studies on signal transmission between plants by AMF provided any data on the molecular or structural nature of the transferred signal. Moreover, there is no experimental data on how the putative signal transfer occurs, and the experiments only shows that CMNs are involved in the interplant communication process. Therefore, many questions remain unanswered about CMNs and their ecological roles. Is it transferred preferentially to the same species than the plant from which the signal is emitted ? Does the warning signal reach plants that are not sensitive to the original stress⁷¹ ?

Impacts of AM symbiosis on plant viral infection

The viral infection of AMF colonized plants has poorly been studied, and information about the effect of AM symbiosis on viral infection are limited and sometimes contradictory^{72,73}. Indeed, some studies reveal that AMF colonization may have a detrimental effect on plant defenses against viruses, aggravate symptoms and may promote the multiplication of viral particles^{74–79}, while other studies show that AMF seems to alleviate symptoms and reduce virus titer in colonized plants^{80–82} (Annex 1). However, the entirety of the experiments on the impacts of AMF colonization on viral infection in plants have only been conducted on positive ssRNA⁷³ virus from different viral families, at the except of Maffei *et al.* (2014), who worked on a ssDNA Begomovirus infected plant⁸¹. In this study, authors grew tomato plants mycorrhized with the AMF *F. mosseae* and infected with the Begomovirus *Tomato Yellow Leaf Curl Sardinia Virus* (TYLCSV).

Their results reveal that (i) mycorrhizal development and expression levels of mycorrhizal-responsive genes were not significantly modified between mycorrhized infected and non-mycorrhized infected plants, which indicates that the AM symbiosis is unaffected by the virus infection. And (ii) that, compared to non-mycorrhized infected plants, mycorrhized infected plants showed milder viral symptoms and reduced viral DNA concentrations. Therefore, they concluded that overall, AM symbiosis with *F. mosseae* lead to an attenuation of the disease caused by TYLCSV on tomato plants.

Thus, the results brought by experiments conducted on the interplay between AM-colonized plants and virus are not conclusive. In their review, Miozzi *et al.* (2019)⁷³ propose a model in order to explain such contradictory results. They argue that the result of such a tripartite interaction depends on several factors such as viral pathogen lifestyle, plant nutritional status and timing of interaction, and that hormones such as abscisic acid, jasmonate and ethylene may play a key role in the equilibrium reached by the system AMF-plant-virus⁷³. Therefore, AMF colonization could have a beneficial or detrimental effect, depending on the hormonal interaction induced by root colonization, that eventually lead to a favorable or unfavorable environment for the development and spread of the virus.

Thiamine biosynthesis-related plant genes

Thiamine (vitamin B1) is an essential molecule for living organisms (cofactor necessary in reactions of the photosynthesis, respiration, pentose phosphate pathway, Calvin cycle and Krebs cycle processes)^{83,84}. Thiamine is a bicyclic compound composed of a pyrimidine and a thiazole moiety and its biosynthesis occurs in bacteria⁸⁵, fungi^{86,87} (including yeast⁸⁸) and plants⁸⁹, though from different biosynthetic pathway. In plants, three forms of thiamine co-exist; thiamine, thiamine monophosphate (TMP) and thiamine pyrophosphate (TPP), although almost all thiamine in *M. esculenta* is found under TMP or TDP forms⁹⁰. In the vegetal biosynthetic pathway of thiamine, the pyrimidine and thiazole moieties are synthesized separately and eventually fused together, forming TMP. The enzyme Thiamine Pyrophosphate kinase (TPK) and putative phosphatases are involved in the different phosphorylated states of the thiamine molecules. In addition to the TPK, the pyrimidine synthase (THIC), the thiazole synthase (THI1) and the thiamine phosphate synthase (TH1) are key enzymes of the thiamine biosynthetic pathway in plants⁹⁰. By using the *A. thaliana* THIC and THI1 protein sequences as search parameters in Phytozome, in the *M. esculenta* genome, Mangel *et al.* (2017) discovered two THIC and two THI1 genes, and named them THIC1, THIC2 and THI1a, THI1b, respectively⁹⁰. Moreover, the amino acids sequence of both THIC *M. esculenta* proteins were found to be identical at 95%. The same trend was observed for both THI1 *M. esculenta* proteins, that were identical at 94%.

In nature, thiamine is produced by bacteria associated with plant roots, so that plants can rely on an exogenous supply of thiamine instead of producing *de novo* the entirety of its thiamine needs. In exchange of the exogenous thiamine supplied by bacteria, plants exude between 12 and 40% of their photosynthetically fixed carbon, thus highlighting a mutualistic interaction between a plant and soil bacteria⁹¹.

SWEET genes

SWEETs (Sugar Will Eventually be Exported Transporters) are a relatively recently discovered family of plant genes which encode sugar transporters. They are integral proteins that have up to 7 transmembrane domains and whose function is to selectively transport sugars, such as sucrose, glucose or fructose⁹². The family of SWEETs generally contain many genes; 35 SWEETs were identified in potato⁹³; 17 in the model organism *A. thaliana*, 36 in *Hevea brasiliensis*⁹⁴ and 28 in *M. esculenta*⁹⁵. SWEETs are versatile and intervene in different processes of plant metabolism, such as the selective transport of glucose, the production of nectar, the loading of the phloem and the export of fructose from the vacuole to the cytosol⁹⁴. By definition, because they transport sugars, SWEETs have a major impact on the glycometabolism of plants. But, as the efflux of sugar is also an essential function of several crucial processes, they also participate in these crucial processes, such as growth, development, stress responses and plant-microbe interactions⁹⁴.

SWEET genes have been divided in 4 different clades, originally established in regards to the SWEET family gene of *A. thaliana*. Clades I and II contains SWEETs that preferentially

transport hexose molecules while SWEETs of the clade III transports sucrose molecules⁹⁶⁻⁹⁸. Moreover Clade IV SWEET genes are fewer in number and have been shown to participate in the control of the fructose flux across the tonoplast⁹⁸⁻¹⁰⁰. The great importance of SWEETs is represented by (i) the high number of processes in which they are involved, (ii) the high number of genes present in this family in different organisms, (iii) the fact that they are the target of many pathogen effectors, and (iv) by their involvement in mycorrhizal interaction⁹³. However, the family of SWEETs genes has been given very little interest, and only partial genomic information and annotations are available.

Objectives

This master thesis takes place in the scope of the MyVIP project, which aims at identifying a CMN-mediated interplant communication from a virus-infected plant to a healthy plant. Within this general objective, the current thesis was focused on (1) performing the first eccDNA sequencing on *M. esculenta* and *R. irregularis*, under *in vitro* conditions, in presence and absence of ACMV and (2) characterize the impact of symbiosis with *R. irregularis* and ACMV infection on the *in vitro* growth of *M. esculenta* via phenotypic observations and quantification of the expression of SWEET genes and thiamine biosynthesis-related genes.

Material and methods

To achieve these objectives, the experimental conditions of Maffei *et al.* (2016) were applied to *M. esculenta* plants, colonized with *R. irregularis* and infected with ACMV and were extended to *in vitro* conditions. Thus, four treatments were considered: (1) plants colonized by the AMF and infected by the ACMV (+Myc+ACMV), (2) plants colonized by the AMF and not infected by the ACMV (+Myc-ACMV), plants not colonized by the AMF and infected by the ACMV (-Myc+ACMV) and (4) plants not colonized by the AMF and not infected by the ACMV (-Myc-ACMV). The plants were cultured under *in vitro* conditions. Using these biological conditions, we investigated the impact of mycorrhization by *R. irregularis* and of ACMV infection on the growth parameters of *M. esculenta* plants under *in vitro* conditions, via phenotypic observations and gene expression quantification.

Biological material and growth conditions

Manihot esculenta (cv. 604444, originally obtained from the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria) was grown *in vitro* in the laboratory of mycology at UCLouvain and used as the mycorrhizal and viral host in the experiments. It was maintained in jars filled with basic culture medium (CBM): 4.4 g L⁻¹ Murashige & Skoog (MS) which contains salts with vitamins, 20 g L⁻¹ sucrose, 1mL L⁻¹ CuSO₄ 2mM, 3.2 g L⁻¹ Gelrite, pH 5.8,) sterilized at 121 °C for 15 min. The plants were incubated at 28°, 16h day length and >50% humidity. The plants stock was renewed every three to four months by transferring cuttings in new jars.

Rhizophagus irregularis (MUCL 49134) was grown in association with Ri T-DNA transformed carrot roots in the laboratory of mycology at UCLouvain and used for the

colonization of *M. esculenta*. Inoculum of *R. irregularis* was produced in Petri plates filled with the Modified Strullu-Romand (MSR) medium (0.594 g L⁻¹ Strullu-Romand Modified (MSR) powder, 0.256 g L⁻¹ Ca(NO₃)₂, 10 g L⁻¹ sucrose, pH 5.5, sterilized at 121 °C for 15 min. (Declerck *et al.*, 1998, modified from Strullu and Romand, 1986). Petri plates were incubated for two to three months in an inverted position, and in the dark at 25°.

An ACMV isolate from Nigeria have been used in this work. This ACMV isolate have been modified by Lentz *et al.* (2018) to develop an agroclone for virus-induced gene silencing in *M. esculenta* leaves and roots¹⁰¹. Agroclones are viral clones used in *Agrobacterium tumefaciens*-mediated inoculation of plants. The DNA-A and DNA-B genomes of the initial ACMV isolate are contained in two separate pCAMBIA plasmids (Fig 5). These plasmids are the same as those used by Zaidi *et al.* (2020), except that our DNA-A plasmid does not contain a multiple cloning site. These plasmids were transformed and multiplied in the AGL1 *A. tumefaciens* strain and stored in 50/50 glycerol at -80°, see Zaidi *et al.* (2020) for details¹⁰².

Agrobacterium inoculum preparation and inoculation

The infection of *M. esculenta* plants with the ACMV was mediated by *Agrobacterium tumefaciens* containing DNA-A or DNA-B in a pCAMBIA plasmid. DNA-A and DNA-B agroclones starter culture were made by plating 100 µl of bacterial glycerol stock of each agroclone separately on sterile Petri plates containing LB medium supplemented with Rifampicin and Kanamycin (final concentration: 50 mg L⁻¹ for both). Cultures were grown at 27° for 36h until a consequent biofilm is formed. The lids of the Petri plates were used to harvest the DNA-A and DNA-B *Agrobacterium* biofilms and the needle of a 1 mL insulin syringe was used to mix them, creating an inoculum containing both plasmids. The same syringe was used to puncture the younger nodes five times, with dipping of the needle tip in the biofilm mixture between each puncturation. *A. tumefaciens* with plasmid without

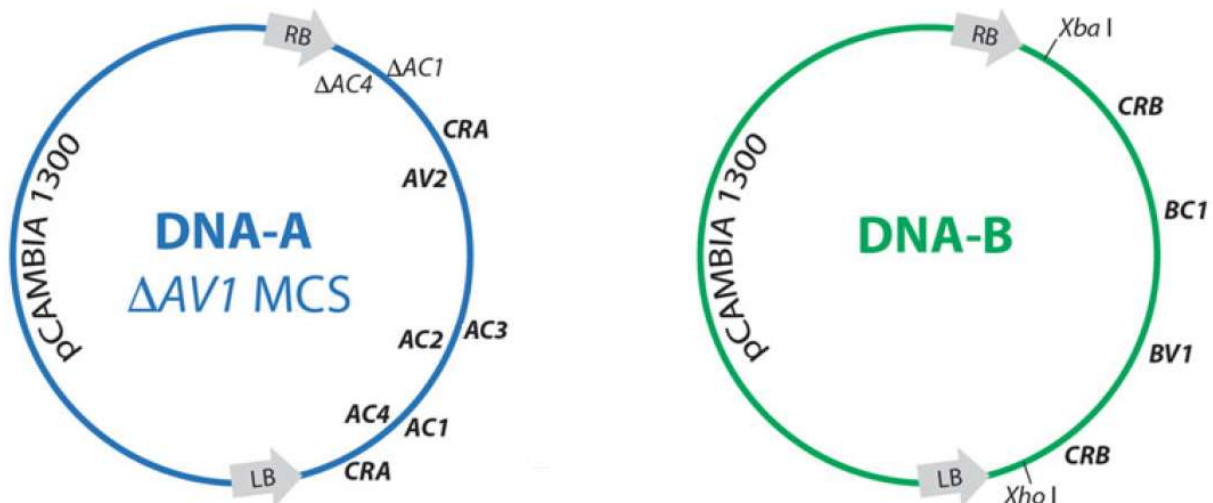


Figure 5. Schematic representation of the pCAMBIA 1300 plasmids modified with DNA-A or DNA-B. Viral coding sequences (AC1, AC2, AC3, AC4, AV1, AV2, BC1, BV1), viral common regions A and B (CRA, CRB) and left and right border (LB, RB), modified from Zaidi *et al.*, 2020

the ACMV genome was cultivated and inoculated in the same way. An additional treatment was considered, with mycorrhized plants that were inoculated with *A.tumefaciens* that contained the plasmid without the ACMV genome, in order to insure that potential symptoms would be induced by ACMV and not by the *Agrobacterium*-mediated inoculation process itself.

In vitro system design and establishment

In vitro bi-compartmented Petri plates were used to host the two symbiotic partners *R. irregularis* and *M. esculenta* (Fig 6). The *in vitro* bi-compartmented system consists of the lid of a 5 cm Petri plate placed in a large (15 cm) Petri plate, both being filled with MSR medium without sugar and vitamins (Fig 6, 7). Two weeks-old *M.vesculenta* plantlets were transferred in the bi-compartmented systems and inoculated with 100 spores of *R. irregularis*. *M. esculenta* roots are thus contained in the small Petri plate, named Root Compartment (RC) and were trimmed if needed to avoid their proliferation in the large Petri plate. The AMF hyphae were allowed to cross the partition wall and to develop in the large Petri plate, named Hyphal Compartment (HC). A lateral hole allowed the plant stems to grow outside the Petri plate, and a 1 cm hole in the lid allowed regular feeding of the system by adding fresh sterilized (121 °C for 15 min) MSR medium (no sugar, no vitamins). The *in vitro* systems were sealed with strips of parafilm soaked in ethanol going above and below the stem exiting the system. A layer of silicone grease around the stem and strips of cellophane again going above and below the stem was also added. An adhesive aluminum foil was used to cover the feeding hole. The *in vitro* systems were opened at regular intervals root trimming and feeding the plants.

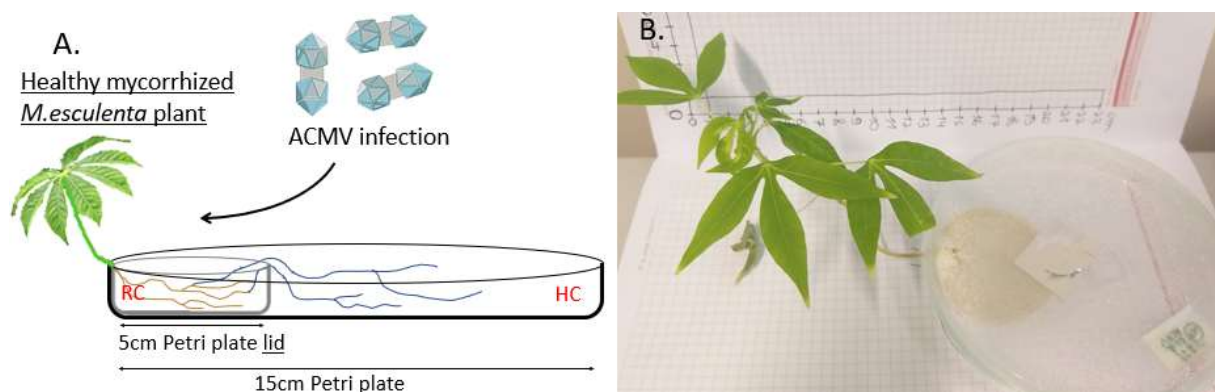


Figure 6. General illustration of the *in vitro* systems used in this work. (A) schematic representation and (B) example. RC, root compartment; HC, hyphal compartment.

Biological conditions and course of the experiment

The *in vitro* systems were divided in four treatments as explained above (Fig 7A): -Myc-ACMV, -Myc+ACMV, +Myc-ACMV and +Myc+ACMV. Three replicates were used per treatment, but only two could be used in -Myc-ACMV and +Myc+ACMV treatments.

The transfer in the bi-compartmented systems of the two weeks-old *M. esculenta* plantlets associated to the spores of *R. irregularis* was considered as week 0 (Fig. 8). During the time-course of experiment, the *in vitro* systems were monitored and provided with MSR medium (no sugar, no vitamins) whenever necessary, along with performing phenotypic observations and parameters recording, i.e. stem height and leaf numbers. The *M. esculenta* plants were inoculated with the ACMV after 10 weeks of development in the *in vitro* systems. Parameters recording continued with particular attention at observing, evaluating and describing any symptoms that would develop on the plants. In addition, control systems (not illustrated in figure 7) have been inoculated with *A. tumefaciens* containing an empty plasmid (without ACMV genome), to insure that symptoms are induced by the ACMV and not by *Agrobacterium*-mediated inoculation. The systems were harvested four weeks after infection (15 weeks in total) for +Myc treatments and three

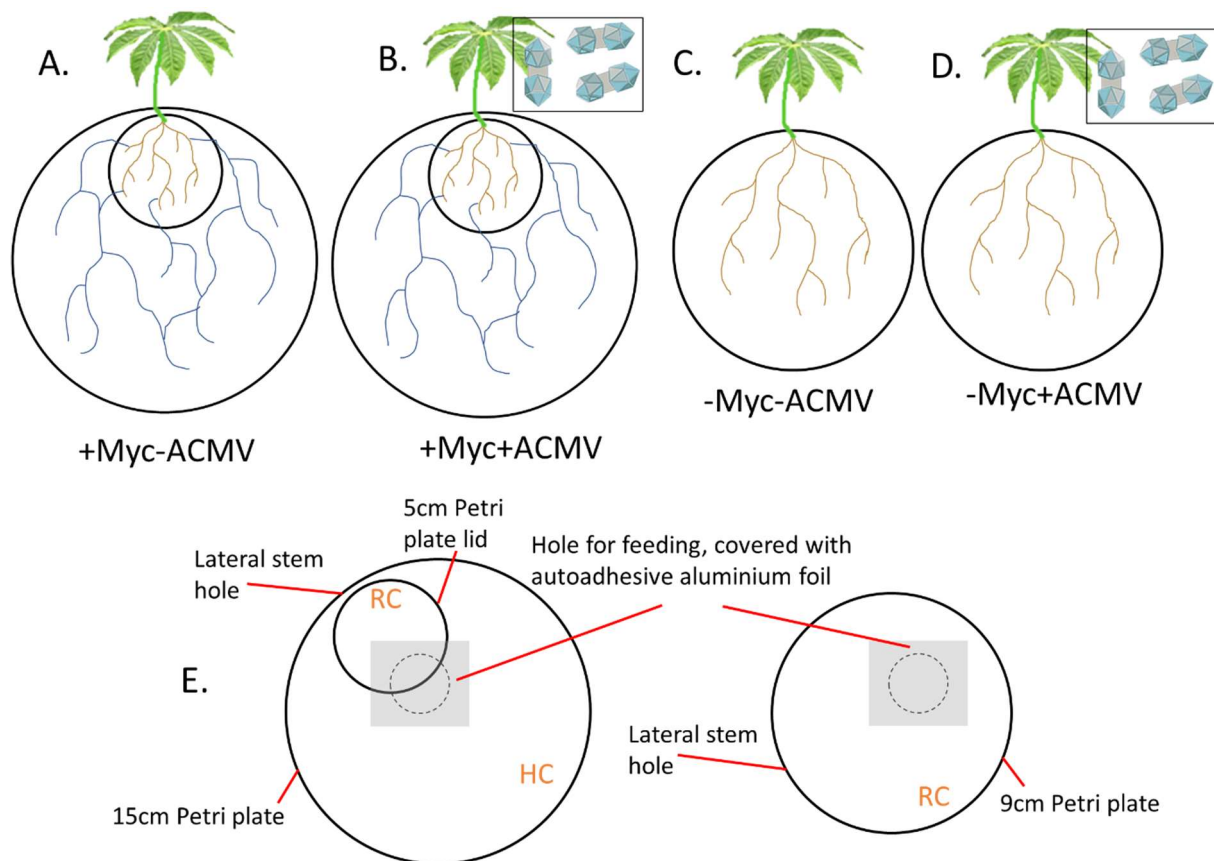


Figure 7. Biological conditions and *in vitro* system design. Biological treatments involve AMF-mycorrhized non-infected (A), AMF-mycorrhized virus-infected (B), non-mycorrhized non-infected (C) and non-mycorrhized virus infected (D) plants. E : General design of mycorrhized (left) and non-mycorrhized (right) *in vitro* systems involves a lateral hole allowing stem growth outside of the Petri plate and a feeding hole (dotted circle) that allows regular feeding. RC, Root compartment; HC, hyphal compartment.

weeks (14 weeks in total) for -Myc treatment. In addition, fungal root colonization of each mycorrhized system was evaluated. Leaves, roots and mycelium were harvested, freeze-dried and reduced to a fine powder, then submitted to DNA and RNA extractions. Leaves, roots and mycelial samples were then subjected to nucleic acid extraction. Samples were then used for (i) CIDER-Sequencing, (ii) virus titting and (iii) gene expression quantification.

AMF root colonization assessment

In order to assess the level of root colonization of virus-infected and virus-free *M. esculenta* plants, the roots were first decolorized by a 10% KOH bath for 1h15 at 70 °C in Falcon tubes. The KOH was then drained and the roots rinsed 3 times with distilled water. Two % HCl was added for a 5 min soak to neutralize any trace of KOH. The roots were then stained by adding a 2 % ink – 1 % HCl solution for 30 min at 70 °C. After staining, 15 sections of 1 cm length were randomly cut off from the stained roots and mounted between slides and coverslips. Root colonization parameters of Arbuscular colonization (CA), Vesicular Colonization (CV) and Total Colonization (CT) were then evaluated by microscopy by counting arbuscules, vesicles and hyphae according to the method of McGonigle *et al.* (1990). CA represents the proportion of root length containing arbuscules, while CT determines the proportion of root length containing vesicles and CT the proportion of root length containing hyphae.

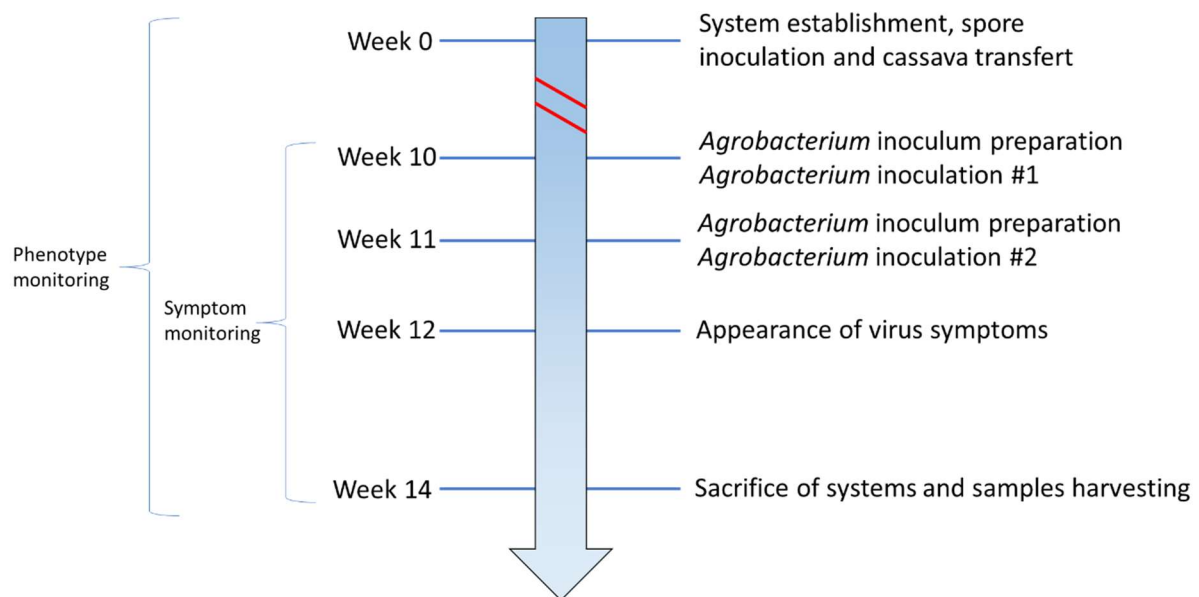


Figure 8. Timeline showing timing of development and infections of *in vitro* systems, from design to harvest. Systems were designed, *M. esculenta* plants introduced and inoculated with AMF spores. After 10 weeks of growth, systems were inoculated twice with one week interval. Symptoms of ACMV infection appeared two weeks after the first infection. Systems were sacrificed for RNA and DNA extraction.

DNA and RNA extraction

Each sample, (e.g. leaves, roots or mycelium) were ground in liquid nitrogen using pestles and mortars to a fine powder. For DNA extraction from roots and leaves samples, the

following protocol was applied : in a 1.5 mL microcentrifuge tube, 100 mg of root or leaf sample powder was incubated with 600 μ L CTAB isolation buffer and 12 μ L beta-mercaptoethanol at 65 °C for 30 min and mixed by tube inversion every 10 min. The tubes were centrifuged 10 min at max speed and around 600 μ L of supernatant was transferred to new 1.5 mL tubes and the same volume of cold chloroform (-20 °C) was added and mixed in a back and forth movement for 5 min then centrifuged 10 min at max speed. The supernatant was transferred to new 1.5 mL tubes. The chloroform step was performed one time again. The supernatant was transferred to a new 1.5 mL microcentrifuge tube with 0.7 volume of cold isopropanol (-20 °C) to precipitate nucleic acids, during 15 min of incubation at -80 °C. The samples were centrifuged at max speed for 30 min and at 4°C, and the supernatant was discarded. The pellets were air-dried and then resuspended in 50 μ L of DEPC-treated water. The mycelium samples consisted of the entirety of the medium in the HC and the mycelium that had been developing inside. These samples were therefore much more consequent and poorly concentrated in nucleic acids. So, we used the entirety of the mycelium-medium sample instead of a fixed amount. For DNA extraction from the mycelium sample, the same protocol was thus adapted for a much larger quantity: All sample mass was used and distributed in 4 Falcon tubes, 40ml of CTAB per tube, 12 μ L beta-mercaptoethanol, 40 ml of supernatant harvested after centrifugation, 10 ml chloroform. For RNA extraction, the same protocol was followed except for the isopropanol-mediated precipitation step, where 0.5 cold isopropanol volume and 0.5 volume of a high salt solution (NaCl 1.2 M, Citrate sodium dihydrate 0.38 M) were added instead of chloroform only, as RNA is more sensible to impurities.

Circular DNA sequencing : CIDER-Seq

Following DNA extraction, the samples were subjected to the CIDER-Seq technique, which allows to enrich and accurately sequence circular DNA without the need of PCR amplification, as described in Mehta et al., 2020²⁴. Due to the laborious work and waiting time of the CIDER-Sequencing, a limited number of samples were used : One system from the -Myc-ACMV treatment (leaf and root samples), one system from +Myc+ACMV (leaf, root and mycelium samples), one system from +Myc-ACMV (leaf, root and mycelium samples), and one system mycorrhized and inoculated with *A. tumefaciens* with plasmids without ACMV genome (leaf, root and mycelium samples).

CIDER-Seq involves 3 major steps. First, an enrichment step aiming at amplifying circular DNAs via a rolling circle amplification performed by the phi29 polymerase. Linear DNA was not removed, but was not amplified either. Secondly, CIDER-Seq requires long-read sequencing on a Pacific Biosciences SMRT-Seq instrument. The high precision and long-read sequences produced by this sequencing technology allow the differentiation between circular and linear DNA molecules by the DeConcat software (refs). DeConcat is part of the CIDERseq analysis software, written in Python (python ref if exist?).

Single Molecule Real Time library preparation and sequencing

To perform the assembly-free Single Molecule Real Time (SMRT; Pacific Biosciences Inc.) long-read sequencing, our samples were sent to the genetics platform of Functional

Genomics Center Zurich. They used the SMRTBell™ Barcoded Adapter Complete Prep Kit-96 and followed manufacturer's instructions (100-514-900, Pacific Biosciences) in order to construct our sequencing HiFi (High Fidelity) libraries. Libraries were subsequently loaded in a SMRT Cell 8M Tray and sequenced with the PacBio SEQUEL Ile system.

Bioinformatic analysis of CIDER-sequencing reads

After SMRT-sequencing, the Zurich sequencing platform performed the demultiplexing of the obtained sequences, producing one fastq file containing the raw sequence data of each sample. A quality control was first performed on these files with fastqc (Andrews, S. (2010); <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>).

The de-concatenation algorithm, “DeConcat”, was then used on each file in order to identify the sequences of circular DNA molecules (i.e. eccDNAs) in the SMRT sequencing datasets. Running the DeConcat algorithm lead to the production of a statistics file and a fasta file. The statistic file contains a table with two columns, the first one indicates the name of the sequence, and the second indicates the number of concatemers in the corresponding sequence (if the value is ≥ 1 , the sequence arises from an eccDNA). This value corresponds to the number of times that the DeConcat algorithm has run in order to resolve the eccDNA sequence. The fasta file contains the de-concatenated form of each read. The software used for the analysis of CIDER-sequencing data and its detailed description are available at <https://github.com/devang-mehta/ciderseq2#input-files>.

On the basis of the statistic files, the sequence of each eccDNA was filtered from the DeConcat fasta output and registered in a new file, which will be further annotated via the ncbi blastn program. The sequence length of each eccDNA sequence was determined using the SeqKit toolkit (Shen et al., 2016; <https://bioinf.shenwei.me/seqkit/>)¹⁰³, and R (R Core Team, 2020; <https://www.R-project.org/>) was used to create histograms of the sequence length distribution analysis.

Gene expression quantification

Candidates chosen for gene expression quantification via qPCR include two *M. esculenta* genes involved in thiamine biosynthesis (TH1a and THIC1) and five *M. esculenta* SWEETs genes (SWEET1, 2, 5, 10, 16). Leaf and root samples from all treatments were subjected to DNase treatment, using the Invitrogen TURBO DNA-free Kit from Thermo Fischer Scientific to insure no genomic DNA amplification along the cDNA and bias of the results. Samples were then subjected to reverse transcription using the Transcriptor High Fidelity cDNA Synthesis Kit from Roche and qPCR analysis was realized with the LightCycler® FastStart DNA Master^{PLUS} SYBR Green I kit from Roche and a LightCycler96 machine.

The Roche LightCycler96 qPCR system allows to perform 96-well real-time qPCR at the same time. The SYBR Green dye, on which qPCR assay is based, emits a low fluorescence in solution that drastically increase upon binding to double-stranded (ds) DNA. The qPCR machine measures the fluorescence emission as the PCR assay occurs. A threshold fluorescence value is fixed, and the quantification cycle (Cq) parameter is defined as the number of cycles required to exceed the fluorescence threshold.. Standard curves are

produced from known dilutions and allow to extrapolate the transcript quantity of a sample from the Cq value.

Primers used in this work are available in annex table 2. The qPCR primers specific for the *M. esculenta* SWEETs genes were designed with the help of the primer-BLAST tool, available on the NCBI website (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>), following the recommendations for gene analysis by qPCR of Thornton and Basu, (2011)¹⁰⁴. qPCR primers specific to the two thiamine biosynthesis-related and a ubiquitin *M. esculenta* genes were taken from Mangel *et al.* (2017)⁹⁰. Primers were blasted on the *R. irregularis* genome, to ensure that no unexpected genomic DNA is amplified by chance. Using the LightCycler96, standard curves were established for each primer on serial triplicate dilutions of a pool containing 5 µL from each cDNA sample (1, 1:5, 1:10, 1:50, 1:100). In addition, melting curve analysis were realized to identify the potential dimerization of the primer pairs, which can lead to parasitic fluorescence thus introducing biases in the results. SWEET genes and thiamine-biosynthesis related genes expression was evaluated respectively on samples from -Myc-ACMV and +Myc-ACMV, and on all treatments. qPCR assays were realized on all selected samples in triplicate.

However, there are biases such as differences in cDNA synthesis or the initial quality of nucleic acids, that induce variations between samples that are not related to the experimental treatments. It is possible to handle these variations via normalization by dividing the amount of the target gene transcripts in a sample by the transcripts amount of a reference gene in the same sample. The reference gene used in this experiment was the ubiquitin gene UBQ10. After normalization, it is no longer a quantity of transcript, but the ratio between the quantity of the target transcripts over the reference transcripts. The fold change (FC) was then calculated by dividing each sample ratio by the ratio of a chosen control sample of the corresponding type (i.e. leaf or root).

Virus titrating PCR

A PCR was realized to confirm virus titrating results of qPCR and investigate potential cross-contamination among samples, with the GoTaq® G2 Hot Start Colorless Master Mix from Promega. The DNA-A sequence of the ACMV was targeted with the following F and R primers : respectively 5'-GGGAAAATCACGATTACGAACAATC-3' and 5'-GTATACACGCCATTCATTGCTTG-3'. The PCR products were run on a 1% agarose gel for 20 minutes at 100 volts.

Results

eccDNA sequencing in *R. irregularis* and *M. esculenta* grown *in vitro* and in presence of ACMV

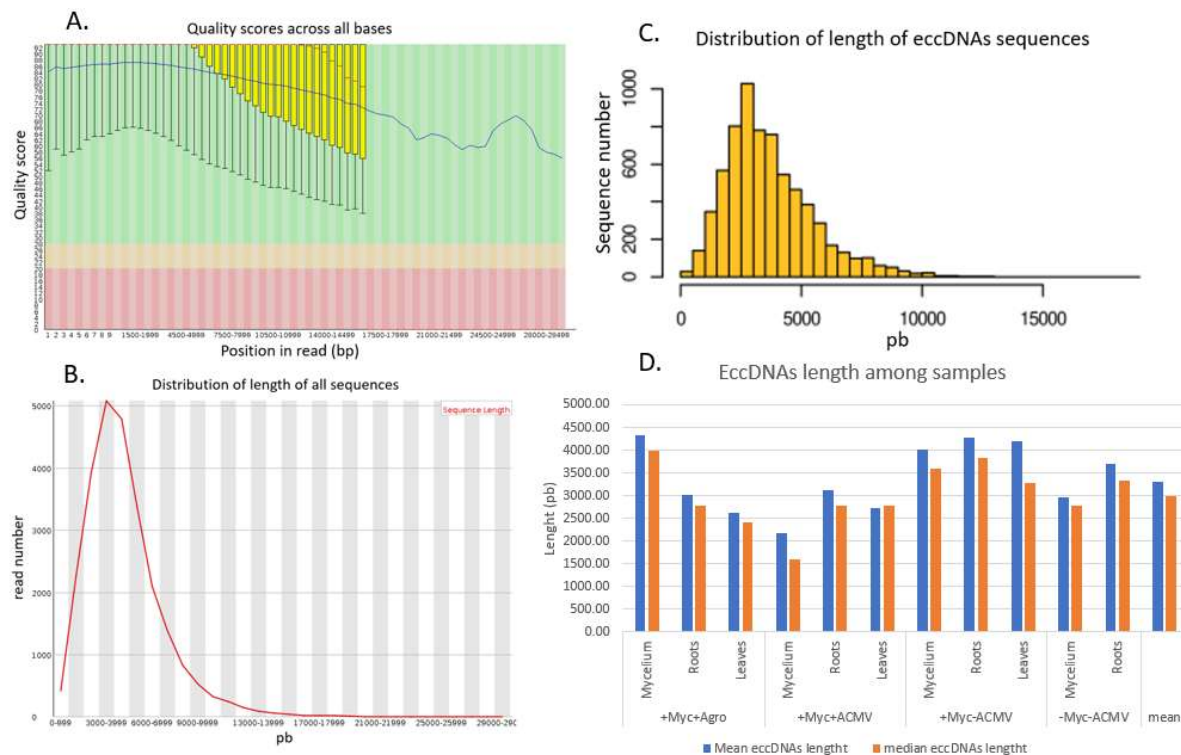


Figure 9. Illustration of CIDER-Seq results. (A) sequencing quality scores across all bases of the Pac Bio reads from a sample. (B) Histogram of Pac Bio sequencing read lengths (C) Histogram of eccDNAs sequence lengths. (D) Mean and median lengths of eccDNAs among all samples submitted to CIDER-Seq

The results of the CIDER-Seq sequencing (annex 4) indicate, for all the samples, an average of 96,786 reads, and among these, an average of 5845 eccDNAs, or 23.63% of eccDNAs on average per sample. The mean and median size of the eccDNAs identified here are similar, with 3293.26 and 2988 bp respectively (Fig. 9).

Impact of AMF colonization on the *in vitro* growth of *M. esculenta*

In both treatments, stem height at transfer to the bi-compartmented systems was around 4 cm. It then rapidly diverged (Fig 10). Over the monitored period, non-mycorrhized plants grew at a rate of 1.3 cm per week and reached a height of 13.9 ± 1.91 cm after 11 weeks, while mycorrhized systems grew at a rate of 0.86 cm per week and reached a height of 10.5 ± 2.17 cm. The means are significantly different $P < 0.01$.

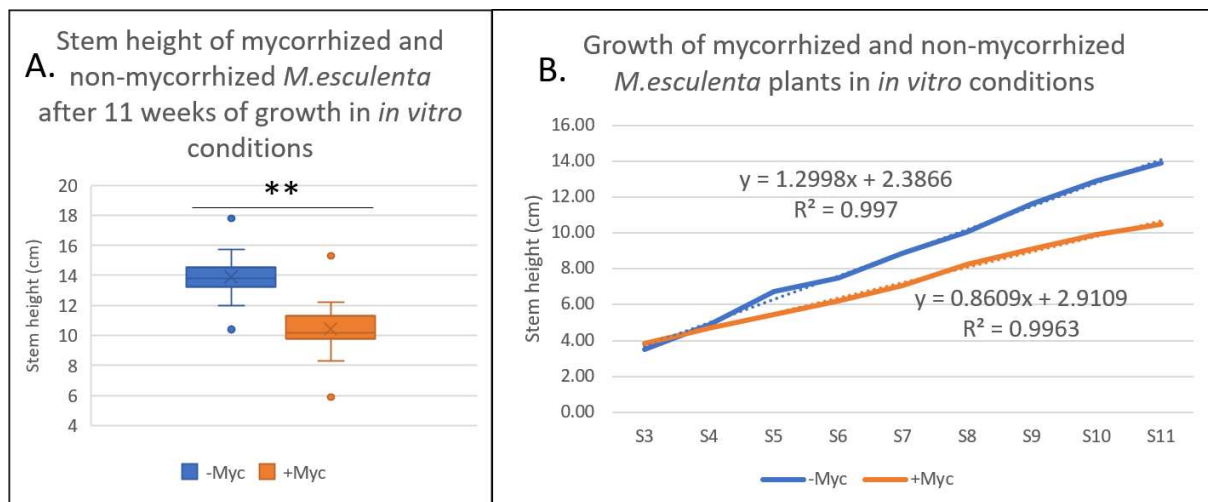


Figure 10. Growth comparison between mycorrhized and non-mycorrhized *M. esculenta* plants in *in vitro* conditions. (A) Plot of stem height of *M. esculenta* in association with *R. irregularis* and non-mycorrhized over 11 weeks. Regression lines have been added in Excel. (B) boxplots of the stem height of the same systems after 11 weeks. Data presented are the mean $\pm$ SD of 13 and 11 biological replicates. Means are significantly different (**= $P < 0.01$; Student tests).

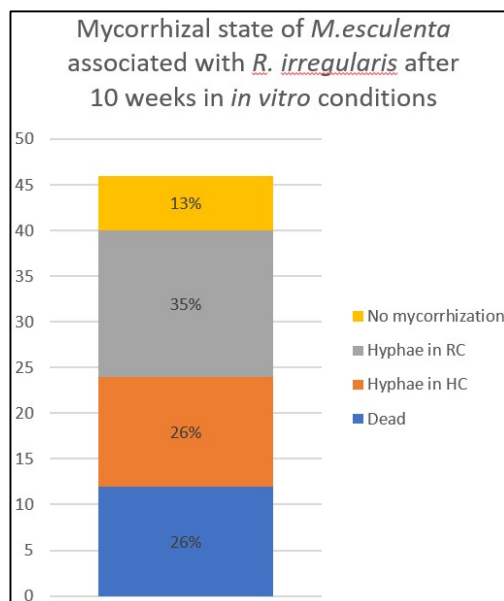


Figure 11. Level of mycorrhization observed in all mycorrhized *in vitro* systems initially designed (47), 10 weeks after spores inoculation.

All systems inoculated with *R. irregularis* led to different levels of AMF development (Fig. 11) Of the 47 *in vitro* systems that were inoculated with AMF spores, 61% established mycorrhization. Mycorrhizal development in 26% of the systems extended to HC while colonization was restricted to RC for 35% as they had not yet crossed the frontier between the HC and the RC (represented by the edge of the lid of the small Petri plate, see fig 7). 13% of the systems failed to establish mycorrhizae, and the remaining 26% died.

AMF root colonization was evaluated on 3 replicates of +Myc-ACMV and two replicates of +Myc+ACMV (Fig 12). Even though %CA, %CV and %CT means are higher in the +Myc-ACMV treatment, there were no significant differences in colonization parameters between the two

treatments, indicating that ACMV had no effect on AMF root colonization parameters.

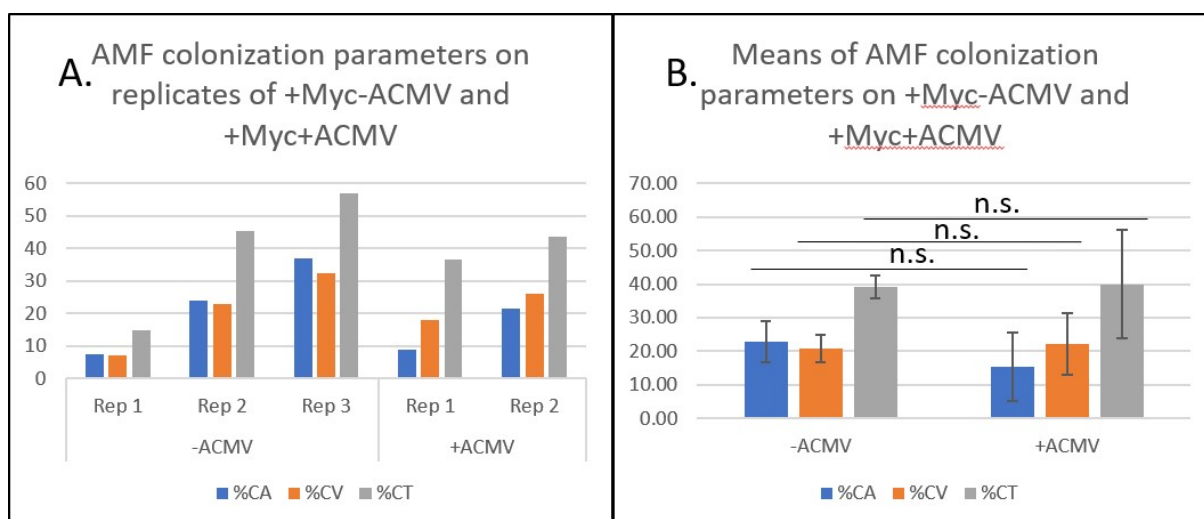


Figure 12. Evaluation of the percentage of arbuscular colonization (CA), vesicular colonization (CV) and total colonization (CT) of infected and uninfected mycorrhizal systems. (A) data of CA, CV and CT from three and two replicates were (B) averaged but are not significantly different between +Myc-ACMV and +Myc+ACMV. Data presented are the mean±SD of three and two biological replicates. No significant differences ($P < 0.05$; Student tests).

ACMV symptoms in *In vitro* grown *M. esculenta*

Plants infected with the *A.tumefaciens* strain with plasmid lacking the ACMV genome did not develop any symptoms. Several ACMV-infected systems died, but likely due to damages induced by the inoculation procedure rather than the virus itself. 3 types of symptoms were recorded: (i) a malformation of the leaves which would make it adopt a silhouette other than that of a normal leaf, (ii) a curving of the leaves which causes them to “roll up” downwards and (iii) a discoloration of the leaves, making them appear yellowish instead of green. Examples of symptoms can be seen on figure 13. During the course of the observation, symptoms appeared only on younger leaves and leaves that were already present at the moment of the infection never develop any kind of symptoms. +Myc+ACMV plants grew leaves showing strong bending and discoloration symptoms in two replicates while symptoms were scarce and mild on the third replicate. Oppositely, -Myc+ACMV plants showed high levels of malformation and limited bending and discoloration (Fig 14).

	Bending	Malformation	Decoloration
A	Light	Medium	Medium
B	None	Light	Light
C	Light	Strong	Medium
D	Strong	None	Strong
E	None	Strong	Strong
F	None	Light	None

Figure 13. Example of symptoms of ACMV infection developed on all inoculated

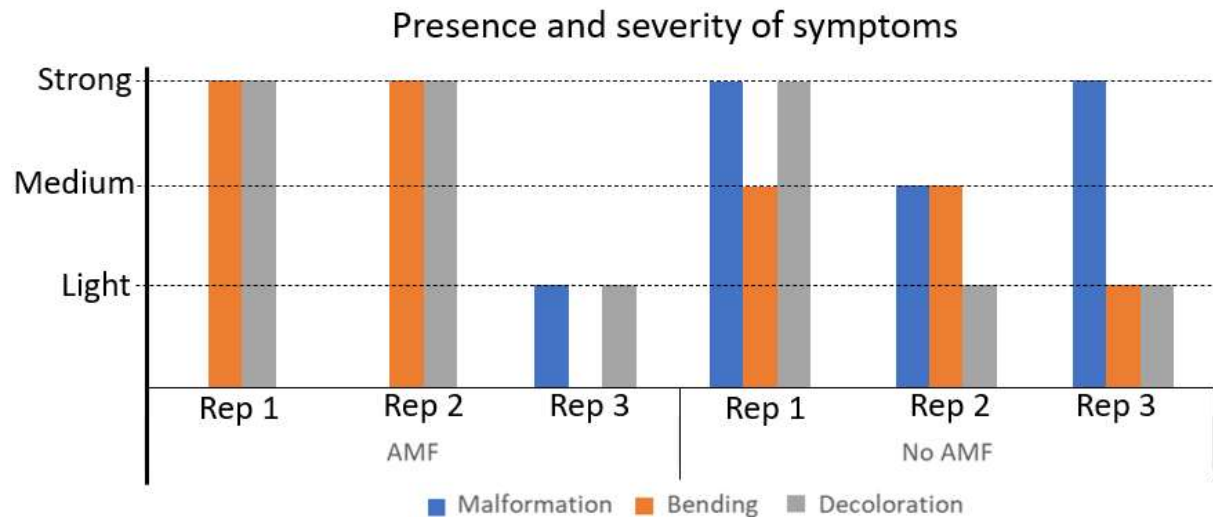


Figure 14. Presence and severity of ACMV symptoms in mycorrhized and non-mycorrhized *in vitro* grown *M. esculenta*. Symptoms were reduced to three types : leaves malformation, bending and discoloration and three severity : Light, medium and strong. Three replicates +Myc+ACMV (left) and three replicates -Myc+ACMV (right) were investigated for symptoms evaluation 3 weeks after ACMV-infection.

Thiamine biosynthesis-related genes expression

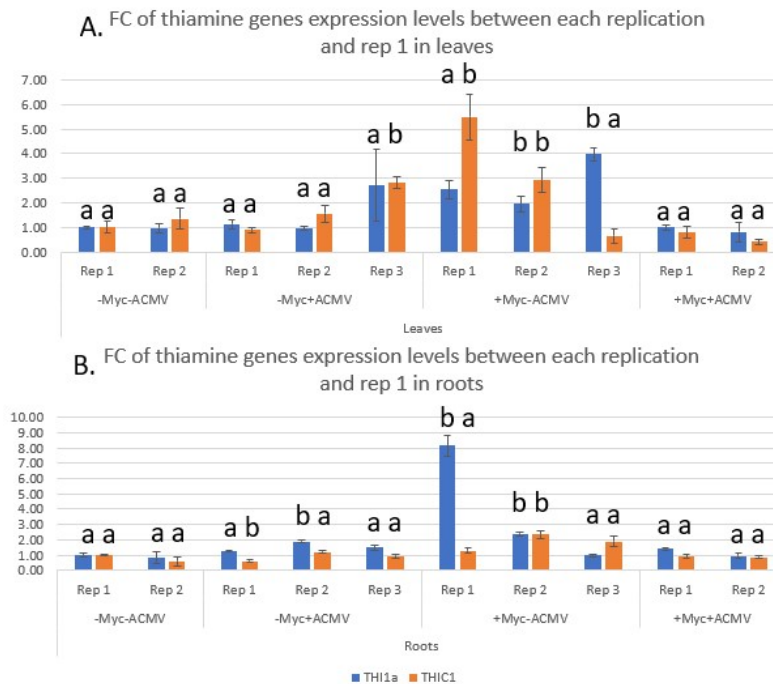


Figure 15. Expression levels of THI1a and THIC1 in leaves and roots in the different biological conditions. Expression levels of THI1a and THIC1 were assessed via qPCR analysis in leaf (A) and root (B) samples of two replicates for -Myc-ACMV and +Myc+ACMV, and three replicates for -Myc+ACMV and +Myc-ACMV. For each gene, the results are expressed in fold changes (FC) relative to the level of expression of replicate 1 of the -Myc-ACMV treatment. Data presented are the mean $\pm$ SD of three technical replicates. Significant differences ($P < 0.01$; Student tests) are indicated by different letters above the

The expression of the thiamine biosynthesis-related THI1a and THIC1 genes in leaves and roots of all biological conditions of *M. esculenta* plants under *in vitro* conditions was investigated via qPCR analysis (Fig 15). Two replicates were used for -Myc-ACMV, three for -Myc+ACMV, three for +Myc-ACMV and two for +Myc+ACMV. The fold change (FC) was calculated taking the rep 1 -Myc-ACMV as the reference. The THI1a and THIC1 genes were differentially expressed among the different biological conditions, both in roots and leaves. In leaves, the expression of THI1a and THIC1 was not statistically different in the -Myc-ACMV and +Myc+ACMV conditions. In addition, two out of three replicates of the -Myc+ACMV condition also were not statistically different, but the third replicate showed a significantly superior THIC1 expression, while the expression of THI1a was not statistically different despite a FC almost three times greater than the control replication (replicate 1 of the -Myc-ACMV condition). The second replicate of the +Myc-ACMV condition was the only one showing a statistically superior expression levels of both genes in leaves, while the first and third replicates both showed a single significantly different expressed gene, respectively THIC1 and THI1a. In roots, the same tendency as in leaves for the absence of significant differences among the replicates of the conditions -Myc-ACMV and +Myc+ACMV was observed. The third replicate of the -Myc+ACMV also showed not statistically different expression, but the first two replicates rep 1 and rep 2 both had a gene expressed

significantly differently, respectively a lower expression of THIC1 and a superior expression of THI1a. Finally, the second replicate of the +Myc-ACMV condition was again the only one to show a statistically different expression of the two genes, while the first replicate shows an outstanding expression of THI1a and the third replicate was not statistically different from the control replicate.

The ratio of gene expression in leaves over expression in roots was inferior to one in every biological condition (Fig. 16.A), indicating that thiamine biosynthesis-related genes are more expressed in the roots than in the leaves. In addition, the ratio of THI1a expression over THIC1 was also inferior to one, both in roots and leaves (Fig. 16.B), indicating that THI1a was less expressed than THIC1

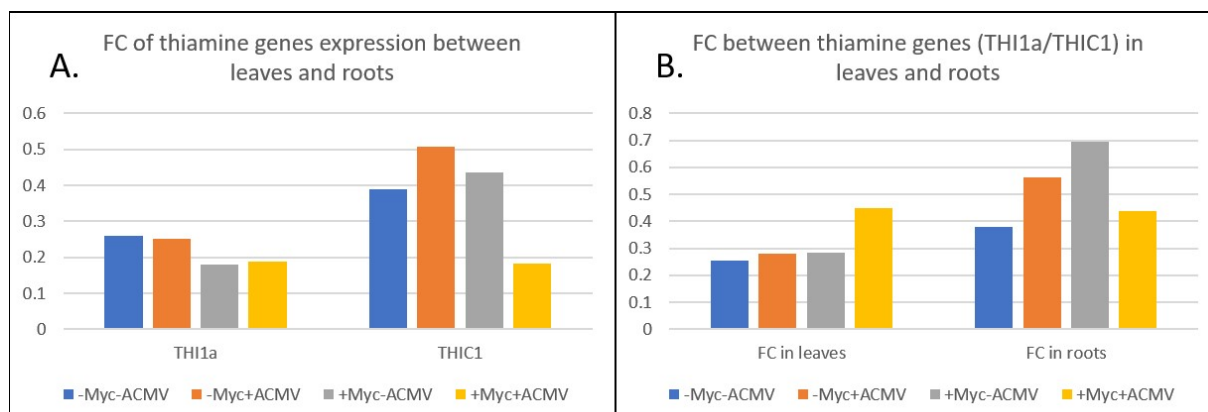


Figure 16. Thiamine biosynthesis-related genes expression. (A.) FC of thiamine biosynthesis-related genes between leaves and roots in all biological conditions. For each gene, FC have been calculated by dividing the transcript quantity in a leaf sample by the transcript quantity in the associated root sample. (B) FC between THI1a and THIC1 in leaves and roots of all biological conditions. FC have been calculated by dividing the mean transcript quantity of THI1a in a biological condition by the mean transcript quantity of THIC1 of the same biological condition.

SWEET genes quantification expression

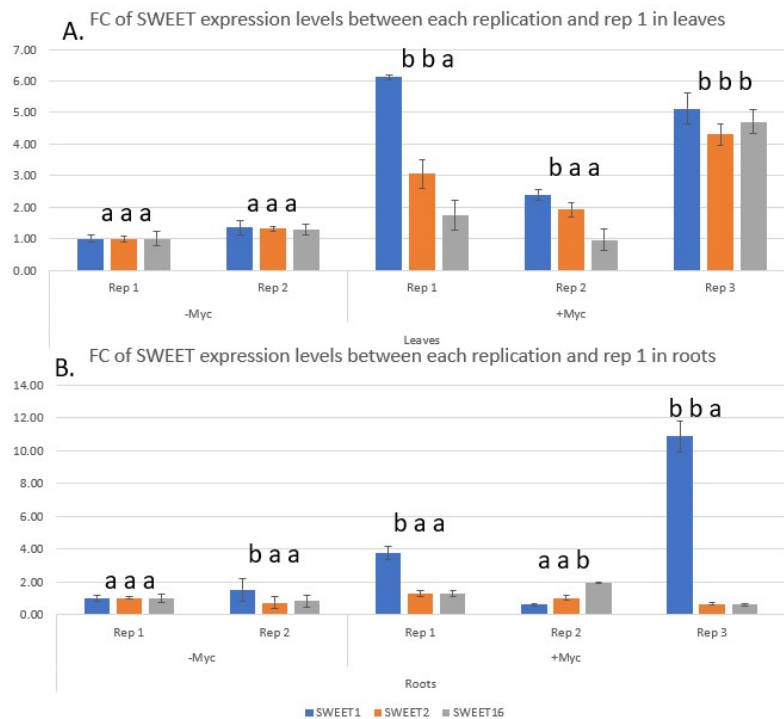
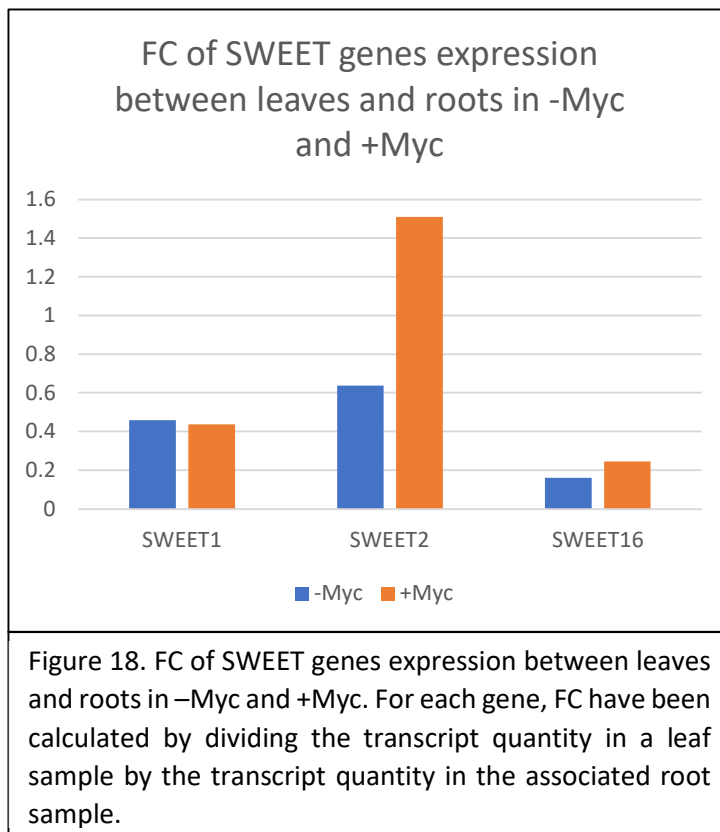


Figure 17. Expression levels of SWEET1, 2 and 16 in the different biological conditions. Expression levels of SWEET1, 2 and 16 were assessed via qPCR analysis in leaf (A) and root (B) samples of two replicates for -Myc-ACMV and three replicates +Myc+ACMV. For each gene, the results are expressed in FC relative to the level of expression of replicate 1 of the -Myc-ACMV treatment. Data presented are the mean $\pm$ SD of three technical replicates. Significant differences ($P < 0.01$; Student tests) are indicated by different letters above the concerned column.

qPCR analysis was also used to investigate the expression of SWEET1, 2 and 16 in leaves and roots of all biological conditions of *M. esculenta* plants under *in vitro* conditions (Fig.17). two and three replicates were used for the -Myc and +Myc conditions, respectively. The FC was calculated taking the rep 1 -Myc-ACMV as the reference. SWEET1, 2 and 16 were differentially expressed among the different biological conditions, both in roots and leaves. In leaves, both replicates from the -Myc condition had statistically not different expression of all SWEET genes, while the replicates of the +Myc condition showed much more variable levels of expression and associated significance. Indeed, the first replicate expressed SWEET1 and 2 at significantly higher levels, the second replicate showed significantly higher expression level of SWEET1 and the third replicate had significantly higher expression levels for all genes. In roots, The expression level of SWEET1 of the second replicate was the only significantly higher among replicates of the -Myc condition. In comparison, the first and second replicates of -Myc had significant higher levels only of one gene, respectively SWEET1 and SWEET16, while the third replication showed two genes statistically differentially expressed, i.e. a dramatic significant 10 times higher level of SWEET1 and lower expression level of SWEET2.



The ratio of SWEET genes expression in leaves over expression in roots was inferior to one in every biological condition at the exception of the ratio of SWEET2 expression in mycorrhized plants (Fig. 18), indicating that SWEET transporters are more expressed in the root tissues.

Virus tittering

The Roche LightCycler96 qPCR system was used to perform virus tittering, which lead to incoherent results (not shown), likely caused by cross-contamination among samples. To investigate this problem, a regular PCR was performed using the GoTaq® G2 Hot Start Colorless Master Mix from Promega on every sample used for the qPCR assay (fig 19). All infected samples lead to an amplified band except samples from mycelium, while half of the uninfected and therefore supposedly negative samples showed an amplification and therefore presence of the virus.

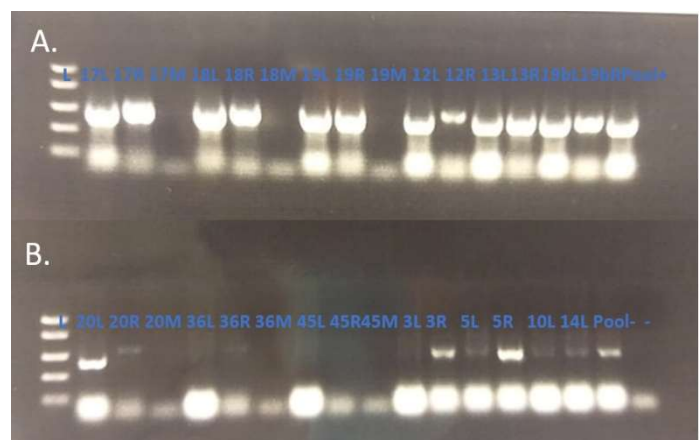


Figure 19. Electrophoresis on 1% agarose polyacrylamide gel on PCR products aiming at amplifying a section of DNA-A of the virus, in order to check for potential contamination among samples. (A) Samples of the top row derive from the ACMV-infected biological conditions -Myc+ACMV and +Myc+ACMV. (B) Samples of the lower row derive from the ACMV non-infected biological conditions -Myc-ACMV and +Myc-ACMV.

Discussion

In this work, we investigated under *in vitro* conditions, the tripartite interaction between a mycorrhizal fungus (*R. irregularis*), a virus (the African Cassava Mosaic Virus) and a plant (*M. esculenta*) which is host to both the AM fungus and virus. The impact of root colonization by the AM fungus was evaluated by evaluation of the expression of three SWEET and two thiamine biosynthesis-related genes in the plant. The impact of ACMV infection was evaluated by its impact on root colonization by the AM fungus and symptoms and severity assessment in the plant.

Firstly, one consequence for a plant, when growing *in vitro*, is to be deprived of the exogenous supply of thiamine, which is in part provided by root-associated bacterial communities. The plant has thus to fill its thiamine requirement by itself, which costs a highly significant amount of energy and could impact growth and other parameters such as defense responses. Moreover, it recently has been suggested that *R. irregularis* actively takes up thiamine from its host¹⁰⁵. We thus hypothesized that mycorrhization with *R. irregularis* will be a weight rather than a help for *M. esculenta* under *in vitro* condition. Secondly, the recently discovered SWEET genes encode for membrane proteins responsible for sugars intake or outtake. It has been shown that the SWEET genes are differentially expressed in the potato, in regards to the mycorrhizal state of the plant⁹³. Consequently, the expression of several *M. esculenta* SWEET genes will be quantified and compared between mycorrhized and non-mycorrhized treatments, in order to presumably observe a similar trend as in potato. Lastly, ACMV symptoms have never been assessed in *in vitro* grown *M. esculenta* plants. The presence and severity of symptoms on *M. esculenta* and on the AMF root colonization parameters have thus been evaluated.

Mycorrhization by R. irregularis has a negative impact on M. esculenta growth in in vitro conditions.

M. esculenta under *in vitro* conditions and without AMF grew at a height of 13.9 ± 1.91 cm after 10 weeks, while mycorrhized systems reached a height of 10.5 ± 2.17 cm. Therefore, even though AMF are well known to enhance nutritional status and offer protection against biotic and abiotic stresses^{40,41}, it seems that the establishment of a mycorrhizal association with *R. irregularis* induces a stunted growth in *M. esculenta* plants under *in vitro* conditions. There was significant difference of 3,4 cm between the average height of the two conditions after 10 weeks of growth, which signifies that mycorrhization led to a reduction of $\frac{1}{4}$ of the size of non-mycorrhized plants in mycorrhized plants.

The MSR medium used during this work was supplemented with vitamins, as transformed carrot roots do not possess stem and leaves and therefore cannot perform photosynthesis and produce their own vitamins. However, the *in vitro* systems hosted entire *M. esculenta* plants able to perform photosynthesis and therefore to biosynthesize their own vitamins. The MSR medium provided to feed the *M. esculenta* plant in the *in vitro* systems was therefore devoid of vitamins, including thiamine. By definition, in *in vitro* systems, soil bacteria and plant-roots associated bacteria are absent. Plant organisms growing under *in vitro* conditions therefore have to replace the exogenous thiamine intake brought by

beneficial bacteria by their own biosynthetic abilities. This increased need in thiamine therefore represent an additional constrain on the metabolism. In addition, thiamine represent a massive energy investment of the plant metabolism, as between 2 and 12% of plant energy is needed for the renewal of enzymes⁸³. Indeed, the thiamine biosynthesis pathway involves the enzyme THI, a particular type of protein called suicide enzymes, which catalyze only one reaction cycle, and then have to be re-synthesized^{106,107}.

A much higher amount of thiamine has been found in *R. irregularis* spores than in the roots of the mycorrhized *Lotus japonica* plant¹⁰⁵. This was unexpected, as *R. irregularis* has been shown to have lost the genes required to achieve the biosynthesis of thiamine molecules during its evolution¹⁰⁸. The authors therefore suggested that “AM fungi positively absorb thiamine from the host plant’s roots during the AM symbiosis”¹⁰⁵. Their conclusion indicates that AMF-colonization of an *in vitro* plant might exerts a worsening effect, as both the thiamine needs of the symbiont and the plant sum up and repose on the biosynthetic capacity of the plant.

Therefore, the reduction in growth observed in our mycorrhized systems could be due to the redirection of a part of the energy normally used for growth and development towards the synthesis of thiamine, to replace the exogenous supply which does not take place *in vitro*, and the thiamine that is absorbed by *R. irregularis*.

ACMV infection of mycorrhized and non-mycorrhized *M. esculenta* plants in *in vitro* conditions lead to different symptoms

During the 3 weeks of presence of the virus, the plants showed no symptoms during the first week, except the death of some leaves and stems for some systems, due to inoculation damages. The second week after infection was marked by the appearance of the first symptoms, without any visible preference for a particular symptom (malformation, bending, and the symptoms were accentuated during the third week. Interestingly, at the time of foliar initiation, the very young leaves had no symptoms, but developed symptoms as they grew. The leaves showing the most symptoms were therefore those that developed immediately after inoculation.

Inconveniently, multiple leaves and apexes of all three -Myc+ACMV replicates died because of the damages induced by the syringe used for inoculation. Symptoms were thus recorded on secondary stem leaves, while +Myc+ACMV replicates only underwent the death of several leaves and retained their main stem intact and growing. Therefore, the differences in symptoms presence and severity as well as the gene expression levels showed in the -Myc+ACMV condition may not necessarily be due to an impact of mycorrhization, but rather the result of developing on a secondary stem.

+Myc+ACMV plants grew leaves showing strong bending and discoloration symptoms in two replicates while symptoms were scarce and mild on the third replicate. Oppositely, -Myc+ACMV plants showed high levels of malformation and limited bending and discoloration. However inoculation damages that led to the emergence of axillary stems

might be responsible for symptoms showed on -Myc+ACMV plants. Roots did not show any kind of symptoms, which was expected as *M. esculenta* do not develop symptoms induced by the ACMV on its root other than reduction of tuber roots⁹

ACMV infection has no effect on colonization parameters

AMF root colonization parameters were not statistically different between virus-infected and non-infected plants. The AMF has been in presence of the root system of *M. esculenta* for 10 weeks. During this time, the establishment of the mycorrhiza and the subsequent development of the fungus have proceeded entirely uninfluenced by the virus, before it could even have the slightest impact. After infection, the virus only had four weeks to induce an impact, before harvesting the roots for assessment of virus impacts on colonization parameters. Given our results and the short time given for the ACMV to have an impact, it is unreasonable to consider the presence of the virus to have had a very remarkable effect on the colonization of AMF. An alternative hypothesis would consider the fact that the virus, even though present in the plant and inducing leaves symptoms, did not established in the roots. It is known however, that the ACMV normally infects the roots of its host¹⁰⁹.

Maffei *et al* (2014), in their study on tomato plants infected by the TYLCSV (Begomoviruses) showed that mycorrhization by *F. mosseae* lead to a priming effect on the mycorrhized plants that eventually alleviated symptom diseases. Such priming is mediated by typical plant defense responses against viruses that involve ABA- and SA-mediated responses and RNA-silencing pathway⁷³. ABA induces callose deposition in plasmodesmata to prevent cell to cell viral movement, while SA induces the production of Pathogenesis Related protein (PR) and Reactive Oxygen Species (ROS). The common point of these mechanisms is to create an unfavorable environment for a virus, leading to reduced symptoms severity and limiting its replication and spreading abilities^{110,111}. A hypothetical alternative strategy of defense response against viruses could be mediated via the decrease in thiamine production of infected cells, in order to slow down the metabolism of infected cells and therefore limiting virus progression.

Mycorrhization of *M. esculenta* under *in vitro* conditions might have the same priming impact, thus leading to a reduced infection by the ACMV and no effect on colonization parameters. This hypothesis is supported by the fact that TYLCSV and ACMV are members of the same viral genus.

As stated in introduction, the results brought by experiments conducted on the interplay between AM-colonized plants and viruses are not conclusive. In their review, Miozzi *et al.* (2019)⁷³ propose a model in order to explain such contradictory results. They argue that the result of such a tripartite interaction depends on several factors such as viral pathogen lifestyle, plant nutritional status and timing of interaction, and that hormones such as abscisic acid, jasmonate and ethylene may play a key role in the equilibrium reached by the system AMF-plant-virus⁷³. Therefore, AMF colonization could have a beneficial or detrimental effect, depending on the hormonal interaction induced by root colonization,

that eventually lead to a favorable or unfavorable environment for the development and spread of the virus.

Thiamine biosynthesis-related genes are more expressed in roots than leaves and THIC1 is more expressed than THI1a

The ratio of gene expression in leaves over expression in roots was inferior to one in every biological condition, indicating that thiamine biosynthesis-related genes are more expressed in the roots than in the leaves. Moreover, our results indicate that THI1a is expressed between four and five times more in roots than in leaves and that THIC1 is expressed in roots two to five more times than in leaves. In addition, our results reveal a FC between the expression of THI1a and THIC1 also inferior to one, which unexpectedly indicates an expression of THI1a lower than THIC1.

However, our results show that the difference in expression of THI1a between leaves and roots is only significant in -Myc-ACMV ($P < 0.05$) and -Myc+ACMV ($P < 0.01$), and difference of expression of THIC1 is only statistically significant in +Myc+ACMV ($P < 0.01$). Consequently, thiamine biosynthesis-related genes expression, five to 10 times higher in the roots than in the leaves, must be put into perspective because of the large variances in the data used to compare the quantity of genes and express the FCs.

These results are in contradiction with those found by Mangel *et al.* (2017)⁹⁰. In their study, they evaluated THIC1, THIC2, THI1a and THI1b expression in leaves and storage roots of different cultivars of *M. esculenta* plants after seven months of growth in *in vivo* conditions, including the cv. 60444 used in this work. In their study, THIC1 and THI1a are both expressed at significantly ($P < 0.01$) higher levels in leaves in comparison to roots. THIC1 and THI1a are expressed respectively at least five and 10 more times in leaves than in roots. In addition, the leaves of the *M. esculenta* plants grew by Mangel *et al* (2017)⁹⁰ showed an expression level of THI1a 20 times greater than that of THIC1, and a similar value in roots. Such results are expected, as THI proteins are suicide enzymes that catalyzes only one reaction, as they transfer a sulfur atom of one of their cysteine residues, and therefore have to be synthesized in much more quantities¹⁰⁷.

Several hypotheses could explain these differences in results. Firstly, Mangel *et al* (2017)⁹⁰ evaluated thiamine biosynthesis-related genes expression after seven months, while our work realized this assessment on 17 weeks old *M. esculenta* plants. The roots of *M. esculenta* only begin to develop into proper tubers after several weeks of growth. Such root conversion was not observed in our *in vitro* systems. This tissue specialization leads to changes in the pattern of gene expression and may lead to up- and downregulation of multiple genes expression. Thus, the expression of thiamine biosynthesis-related genes might be downregulated in tuber roots, which have a storage role via accumulation of starch, compared to roots not yet converted into tubers, which main activities are growth and absorption of water and nutrients. However, the molecular mechanisms of storage root development are poorly known¹¹². Moreover, no information are given about the potential presence of fungal symbiotic partners, as plants of Mangel *et al* (2017)⁹⁰ have been “transferred to soil and grown under greenhouse conditions”. The observed

differences between our works could be the results of different mycorrhizal states, or even due to the impact of multiple soil micro-organisms. But this is unlikely, as our non-mycorrhized control plants also show a superior expression in roots compared to leaves.

One major issue for statistical analyses of thiamine biosynthesis-related genes was the results revealed for THIC1 and THI1a expression in the roots and leaves of the three replicates of the +Myc-ACMV conditions. THI1a and THIC1 roots transcript quantity were of great variance and this consequently reperculated in statistical analyses. The same observation is valid for results in leaves and roots for THI1a and THIC1 transcript quantity. One way to deal with this problem would have been to have a larger number of replicates within each condition; some data could thus have been identified as outliers and possibly ignored to decrease the variance.

An alternative hypothesis would state that mycorrhization establishment and development induce an upregulation of thiamine biosynthesis-related genes expression patterns. This hypothesis is in agreement with the fact that thiamine has been determined as an actor of systemic acquired resistance in plants^{113–115}. For instance, the endophytic ascomycete *Hendersonula toruloidea* has been shown to upregulate thiamine biosynthesis-related genes of the oil palm tree *Elaeis guineensis* during host colonization¹¹³. Although *H. toruloidea* and *R. irregularis* are not closely phylogenetically related, this indicates that fungal organisms are able to manipulate the expression pattern of thiamine biosynthesis genes in its host plant.

SWEET genes are differentially expressed in mycorrhized and non-mycorrhized plants

In leaves, SWEET1, 2 and 16 were upregulated in +Myc plants, but only SWEET1 was upregulated in roots of +Myc plants compared to -Myc plants. Moreover, the expression ratio of SWEET genes between leaves and roots of +Myc and -Myc plants indicates that SWEET1 and SWEET16 are more expressed in roots than leaves. However, SWEET2 expression is higher in the leaves than in the roots of mycorrhized plants. Thus, mycorrhization seems to induce a reprogramming of SWEET genes expression pattern.

The analysis of SWEET genes expression in mycorrhized and non-mycorrhized *M. esculenta* seems to reveal a similar pattern as found in the potato plant for the 3 SWEET genes tested here. Indeed, in the potato plant, SWEET1 and 2 have been shown as upregulated in mycorrhized plants in comparison to non-mycorrhized plants, and SWEET16 has been shown not affected by the mycorrhizal state of the plant⁹³. Our analysis reveals a similar pattern, as the SWEET1 and SWEET2 genes of *M. esculenta* are both upregulated in mycorrhized plants. However, our results also show an upregulation of SWEET16. Therefore, the mycorrhization of *M. esculenta* by *R. irregularis* in *in vitro* conditions lead to an alteration of the profile expression of SWEET genes, and, as SWEET1 appears to be the most upregulated genes upon mycorrhization, it is more prone to be involved in C exchange between the plant and the fungal symbiont.

The biological boundary between *M. esculenta* and *R. irregularis* is represented by the apposition of the periarbuscular membrane of *M. esculenta* to the fungal wall of the fungal

arbuscule^{116,117}. This boundary represents the heart of the arbuscular mycorrhizal association and is where the nutrient exchange takes place¹¹⁸. More precisely, AM fungi take carbohydrates up from the root cortex apoplast, but the exact mechanism exerted by plants to export sugars in the apoplast is not known.

SWEET are a novel type of sugar transporter recently discovered. SWEETs have been shown to catalyze the import and export of sugars. Transported molecules include hexoses and sucrose⁹⁶⁻⁹⁸. In rice, several SWEET promoters have been shown to be targeted by the pathogenic bacteria *Xanthomonas oryzae*, which secretes Transcriptional Activator Like (TAL) effectors¹¹⁹. SWEET expression patterns are manipulated to increase sugar releasing from plant cell for the nutritional needs of the pathogens. Besides, promoters of such SWEET genes that are targeted by pathogen effectors, are therefore interesting targets for future genetic engineering in order to induce natural resistance¹²⁰.

As SWEET genes have been shown, both in previous studies and in our results to be differentially expressed in mycorrhized plants, they represent good candidates for key role players in symbiotic associations that involves nutrient exchange with a fungal symbiont.

First glimpse of the landscape of the eccDNAs of *R. irregularis* and *M. esculenta*

The eccDNAs sequenced during this work via CIDER-Seq are currently being analyzed.

The mean and median size of the eccDNAs identified here are similar, with 3293.26 and 2988 bp respectively. These mean and median values indicate that the size distribution of eccDNAs show a peak slightly below the mean and a long tail of the distribution extending into the high lengths, but representing a small number of eccDNAs. Histogram of length distribution of all sequences and of length distribution in eccDNAs both show this silhouette.

Interestingly, switching the read length distribution histogram to the eccDNAs length histogram (i.e. isolating the eccDNAs) causes a shift which decreases the peak while keeping it in the same place (Fig 9B,C). It indicates that the majority of eccDNAs have a size similar to the size of the sequences found before isolation of the eccDNAs, indeed, there is uniformity in the sizes of eccDNAs among our samples as the sequenced eccDNAs show a relatively constant size of 3000 bp (annex 4)

However, some results produced by the CIDER-Seq were biologically incoherent. For instance, several eccDNAs were shown to have dramatically small or high length (data not shown), which would not be possible here and is likely the result of a statistical incongruence.

Conclusion

The objective of this master thesis was to study the impact of Mycorrhization by *R. irregularis*, infection by the ACMV and of the *in vitro* condition on the growth of *M. esculenta*. In addition. An other objective was to sequence for the first time the eccDNAs present in *M. esculenta* and *R. irregularis* in presence and absence of the ACMV.

For this purpose, *M. esculenta* plants have been grown under *in vitro* conditions, with the presence or absence of *R. irregularis* and with the infection by ACMV or as virus-free plants. The expression levels of genes responsible of thiamine biosynthesis have been quantified, to assess the impact of mycorrhization and ACMV-infection on the metabolism of *in vitro* grown plants. And SWEET genes expression have also been quantified, as potentially differentially expressed in mycorrhized plants. Lastly, CIDER-Seq allowed us to obtain a first glimpse of the eccDNAs landscape of our organisms.

Our results show an unexpected expression of THI1a and THIC1, in fact, THI1a and THIC1, contrary to the literature, was more expressed in roots than in leaves. In addition, the expression of THI1a was lower than that of THIC1, while THI1a, as a suicide enzyme, would need to be much more expressed in order to maintain constant thiamine biosynthesis.

The analysis of SWEET genes between mycorrhized and non-mycorrhized plants showed an alteration of the expression pattern of SWEET genes in mycorrhized plants. This was particularly striking for SWEET1 which would therefore be a good candidate for a protein involved in the transfer of sugar toward a symbiotic fungal partner

The mycorrhization of *M. esculenta* under *in vitro* condition represented a burden rather than a support. This was expected, because of the potential strain represented by the absence of thiamine in the medium, and the pressure represented by the need for thiamine from the fungal partner which sums up with the own needs of the plant. However, such complex hypotheses that itself refers to complex concepts (Plant-plant communication at distance by mean of MF-forming CMNs) cannot be tested straightforwardly, because of multiple confounding factors. To investigate and eventually elucidated these questions, performing experiments under *in vitro* conditions was a significative advantage for ruling out those factors. Although difficult to maintain and requiring a lot of work, using *in vitro* systems allows a perfect control over the environmental conditions, as the culture medium composition can be manipulated, and as the vicinity of the plant and AMF was entirely devoid of other organisms. In addition, compared to plants cultivated *in vivo*, the *in vitro* condition allows to avoid the influence of several exterior parameters, such as light, temperature and nutrients, and attacks by other pathogens. The *in vitro* culture technique therefore facilitate studies where stresses such as virus infection are applied to plants¹²¹⁻¹²³. For all these reasons, the experiment conducted by Alaux *et al.* (2020) on the CMN-mediated interplant communication under *in vitro* conditions is the most irrefutable proof that CMNs are implied in this signal

transfer, though their exact implication as well as the practical means by which it occurs remain speculative.

Perspectives

In *M. esculenta*, the SWEET genes family have been shown to contains 28 members⁹⁵. Further studies could aim at quantifying the expression levels of mycorrhized and non-mycorrhized *M. esculenta* plants in order to establish the complete modification profile of the expression of the family of genes SWEET upon mycorrhization.

Once the blast annotation of eccDNAs will be completed, the results of the CIDER-Seq will be able to be analyzed in more details. For example, the percentage of eccDNAs correspondence with the virus, fungal or plant sequences could be compared across samples

The presence of eccDNA from one organism in another might raise some interesting questions. For example, finding eccDNAs corresponding to the virus in the mycelium would be an indication for the transfer of viral nucleic acid particles from the plant to the fungus. This transferred genetic material could act as a signal that the fungus could transfer to other plants connected by the CMN. Therefore, this might represent a potential hypothesis to explain the putative signal that is transferred between challenged and non-challenged plant, which induces defense elicitation in non-challenged plants.

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Annexes

Plant (family)	Fungus	Virus (genus, family)	Virus type (baltimore classification)	Effect of AMF on virus infection	Plant tissues considered	Effect of virus infection on AMF-colonized plant	References
Tomato (Solanaceae)	<i>Funneliformis mosseae</i> (formerly <i>Glomus mosseae</i>)	Tomato yellow leaf curl Sardinia virus (Begomovirus, geminiviridae)	ssDNA (Group II)	Decreased virus titre, milder symptoms	Leaves, roots	Reduction of roots fresh weight	Maffei et al., 2014
Potato (Solanaceae)	<i>Rhizophagus irregularis</i> (formerly <i>Glomus intraradices</i>)	Potato virus Y (Potyvirus, potyviridae)	Positive ssRNA (Gruppo IV)	Milder symptoms	Leaves, stems, roots	Increase of leaves and stems dry weight	Thiem et al., 2014
Tobacco (Solanaceae)	<i>Rhizophagus irregularis</i> (formerly <i>Glomus intraradices</i>)	Tobacco mosaic virus (Tobamovirus, virgaviridae)	Positive ssRNA (Gruppo IV)	No symptoms, decreased virus titre	Leaves	Not reported	Stolyarchuk et al., 2009
Cucumber (Cucurbitaceae)	<i>Rhizophagus irregularis</i> (formerly <i>Glomus intraradices</i>)	Cucumber green mottle mosaic virus (Tobamovirus, virgaviridae)	Positive ssRNA (Gruppo IV)	Milder symptoms, reduced virus titre	Leaves	Not reported	Stolyarchuk et al., 2009
Tomato (Solanaceae)	<i>Funneliformis macrocarpa</i> (formerly <i>Endogone macrocarpa</i>)	Tomato aucuba mosaic virus* (Tobamovirus, Virgaviridae)	Positive ssRNA (Gruppo IV)	Increased virus titre	Leaves, roots	Not reported	Daft and Okusanya, 1973
Tomato (Solanaceae)	<i>Funneliformis macrocarpa</i> (formerly <i>Endogone macrocarpa</i>)	Potato virus X (Potexvirus, Alphaflexiviridae)	Positive ssRNA (Gruppo IV)	Increased virus titre	Leaves, roots	Not reported	Daft and Okusanya, 1973
Petunia (Solanaceae)	<i>Funneliformis macrocarpa</i> (formerly <i>Endogone macrocarpa</i>)	Arabis mosaic virus (Nepovirus, secoviridae)	Positive ssRNA (Gruppo IV)	Increased virus titre	leaves, roots	Not reported	Daft and Okusanya, 1973
Strawberry (Rosaceae)	<i>Funneliformis macrocarpa</i> (formerly <i>Endogone macrocarpa</i>)	Arabis mosaic virus (Nepovirus, secoviridae)	Positive ssRNA (Gruppo IV)	Increased virus titre	Leaves, roots	Not reported	Daft and Okusanya, 1973
Tomato (Solanaceae)	<i>Glomus</i> sp.	Tobacco mosaic virus (Tobamovirus, virgaviridae)	Positive ssRNA (Gruppo IV)	Increased virus titre, more severe symptoms	Roots, whole plant (for symptoms evaluation)	Increase of roots fresh weight	Jabaji-Hare and Stobbs, 1984
Sour orange (Rutaceae)	<i>Claroideoglomus etunicatum</i> (formerly <i>Glomus etunicatum</i>)	Citrus tristeza virus (Closterovirus, closteroviridae)	Positive ssRNA (Gruppo IV)	No difference	whole plant	Reduction of roots fresh weight and plant growth	Nemec and Myhre, 1984
Duncan grapefruit (Rutaceae)	<i>Claroideoglomus etunicatum</i> (formerly <i>Glomus etunicatum</i>)	Citrus leaf rugose virus (Iarvirus, bromoviridae)	Positive ssRNA (Gruppo IV)	No difference	whole plant	Reduction of roots fresh weight and plant growth	Nemec and Myhre, 1984
Tobacco (Solanaceae)	<i>Rhizophagus irregularis</i> (formerly <i>Glomus intraradices</i>)	Tobacco mosaic virus (Tobamovirus, virgaviridae)	Positive ssRNA (Gruppo IV)	more severe symptoms	leaves	Not reported	Shaul et al., 1999
Potato (Solanaceae)	<i>Rhizophagus irregularis</i> (formerly <i>Glomus intraradices</i>)	Potato virus Y (Potyvirus, potyviridae)	Positive ssRNA (Gruppo IV)	Increased virus titre, more severe symptoms	leaves, whole plant (for symptoms evaluation)	Reduction of shoots length, fresh and dry weight, and tuber weight. Slight reduction of chlorophyll content	Sipahioğlu et al., 2009
Tomato (Solanaceae)	<i>Rhizophagus irregularis</i> (formerly <i>Glomus intraradices</i>)	Tobacco mosaic virus (Tobamovirus, virgaviridae)	Positive ssRNA (Gruppo IV)	Increased virus titre	Leaves	Not reported	Stolyarchuk et al., 2009
Tomato (Solanaceae)	<i>Funneliformis mosseae</i> (formerly <i>Glomus mosseae</i>)	Tomato spotted wilt virus (Orthotospovirus, tospoviridae)	Negative ssRNA (Group V)	Increased virus titre, more severe symptoms (lower recovery)	leaves, roots	Reduction of fresh weight of epigeal and hypogean parts	Miozzi et al., 2011
Bromus hordeaceus L. (Poaceae)	<i>Rhizophagus irregularis</i> (formerly <i>Glomus intraradices</i>)	Barley yellow dwarf virus (Luteovirus, luteoviridae)	Positive ssRNA (Gruppo IV)	Increased virus titre (only with elevated CO2 concentration)	leaves	Not reported	Rüa et al., 2013
Bromus hordeaceus L. (Poaceae)	<i>Rhizophagus irregularis</i> (formerly <i>Glomus intraradices</i>)	Cereal yellow dwarf virus (Polarovirus, luteoviridae)	Positive ssRNA (Gruppo IV)	Increased virus titre (only with elevated CO2 concentration)	leaves	Not reported	Rüa et al., 2013
Avena fatua L. (Poaceae)	<i>Rhizophagus irregularis</i> (formerly <i>Glomus intraradices</i>)	Barley yellow dwarf virus (Luteovirus, luteoviridae)	Positive ssRNA (Gruppo IV)	Increased virus titre (only with elevated CO2 concentration)	leaves	Not reported	Rüa et al., 2013
Avena fatua L. (Poaceae)	<i>Rhizophagus irregularis</i> (formerly <i>Glomus intraradices</i>)	Cereal yellow dwarf virus (Polarovirus, luteoviridae)	Positive ssRNA (Gruppo IV)	Increased virus titre (only with elevated CO2 concentration)	leaves	Not reported	Rüa et al., 2013

*a strain of tobacco mosaic virus.

Table 2 . AMF-plant-virus biological systems investigated; in the upper section are listed the case studies reporting a protective effect of AMF against viral infection while in the lower section are listed those reporting a detrimental effect. From Miozzi *et al.* (2019)

Gene ID	Primer ID	Sequence
RNA qPCR		
MeUBQ10	MeUBQ10_F	TGCATCTCGTTCTCCGATTG
	MeUBQ10_R	GCGAAGATCAGTCGTTGTTGG
SWEET1	SWEET1_F	GTGGCTTTGCTGCCACCATT
	SWEET1_R	AACCAGGAAGTGCCACACAGG
SWEET2	SWEET2_F	TCAGAAACCGGTCAACGGAGC
	SWEET2_R	AGGGGTGTGCCATACCACAAG
SWEET 5	SWEET5_Fb	TGCAGCTCCATTAAGCATTGTG
	SWEET5_Rb	AGGCCATAGGCAAGCCACAT
SWEET16	SWEET16_F	CCAAGAATGAGGGCTAAGACAGCTA
	SWEET16_R	AGCAGCCATGCTGAAGCAAA
SWEET10	SWEET10_F	TTGCGGTTCCACAGCTGAACA
	SWEET10_R	CCAGGTTGGTTTTCTCGGTCTCT
MeTHIC1	MeTHIC1_F	ATGAGTGCTGAGTTCCTGGCTG
	MeTHIC1_R	GCTTTTTTGACCAAATCCCTGG
MeTHI1a	MeTHI1a_F	CATGGAGGTTGCGGAGATTG
	MeTHI1a_R	AAACTCTGGGCGAAGGGTAA
DNA qPCR		
MePP2A_gnmc_qPCR	MePP2A_gnmc_qPCR_F	CGCTGTGGAAATATGGCATCA
	MePP2A_gnmc_qPCR_R	CTGGCTCAAACGCAGGATCAA
ACMV	ACMV_qPCR_F	GGTCCTGGATTGCAGAGGAAGATAGTGGG
	ACMV_qPCR_R	GGTACAACGTCATTGATGACGTCGATCCC
DNA PCR		
PCR DNA-A ACMV	ACMV-DNA-A_F	GGGAAAATCACGATTACGAACAATC
	ACMV-DNA-A_R	GTATACACGCCATTCATTGCTTG
PP2A	PP2A_gene_1300_F	GGTATGGCAATGCTAATGTTTGG
	PP2A_gene_1300_R	CTGGCTCAAACGCAGGATCAA
Table 3 : Primers used for qPCR analysis, qPCR virus titrating and DNA viral PCR		

Sample		Pac Bio Reads	number of eccDNAs	% of eccDNAs in raw reads	Mean eccDNAs lengtht	median eccDNAs lengtht
+Myc+Agro	Mycelium	58332	11143	19.10	4337.42	3990
	Roots	9375	2033	21.69	3006.81	2780
	Leaves	23164	1416	6.11	2615.77	2404
+Myc+ACMV	Mycelium	8747	2880	32.93	2178.24	1590
	Roots	17736	6369	35.91	3124.05	2780
	Leaves	12798	4388	34.29	2718.61	2780
+Myc-ACMV	Mycelium	831582	10094	1.21	4021.56	3597
	Roots	20967	1457	6.95	4274.35	3840
	Leaves	33232	5043	15.18	4185.98	3270
-Myc-ACMV	Mycelium	22951	11811	51.46	2963.48	2790
	Roots	25761	6861	26.63	3692.30	3330
mean		96786	5845	23.63	3293.26	2988

Table 4. CIDER-Seq results

M. esculenta is a crop culture, particularly resistant to drought and mainly cultivated in tropical regions, where it is the third calory source, after rice and corn. Cassava culture, however, is threatened by the cassava mosaic disease, caused by viruses from the begomovirus genus, including the *African Cassava Mosaic Virus*.

Arbuscular mycorrhizal fungi are soil micro-organism that are known to establish an association with the roots of more than 80% of land plants, including numerous important crop species including cassava. This association has been extensively studied and demonstrated to promote plant nutrition and growth and to enhance tolerance against biotic and abiotic stresses.

In this master thesis, the impact of inoculation of the ACMV, mycorrhization by *Rhizophagus irregularis* and the *in vitro* conditions on the growth of *M. esculenta* is studied. The *in vitro* condition, even though hard-work demanding, allows to study the impact of several parameters on a system, and the exclusion of the impact of other potential cofounding factors.

For this purpose, the ACMV impact on the root colonization by the AMF and leaves symptoms have been investigated, and the expression of two types of genes, SWEETs genes and thiamine biosynthesis-related genes have been quantified. Besides, an evaluation of the eccDNA landscape in *R. irregularis* and *M. esculenta* have been realized.

We showed that ACMV, when inoculated after mycorrhization establishment, does not affect the AMF root colonization, as mycorrhized and non-mycorrhized plants showed equivalent colonization parameters. We also showed that mycorrhization in *in vitro* conditions lead to a stunted growth and to an alteration of the expression pattern of SWEET genes of *M. esculenta*. In addition, the plants in our *in vitro* systems had an unexpected expression of thiamine biosynthesis-related genes.