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"Boosting the production of plant extracts from vertical farms by modulating their phytobiomes: Centella asiatica, a case study"

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1. Introduction

Vertical farming is emerging as a disruptive trend in active ingredient production [1], enabling local sourcing of foreign plants while offering sustainable and innovative solutions for the beauty industry. In these enclosed structures, technological advancements not only allow for perfect control of the climate and plant nutrition, but also enable complete management of all plant growth parameters: light intensity, modulation of photoperiods, etc.
However, the cosmetic and medicinal efficacy of plants is dependent on the production of metabolites of various chemical natures (alkaloids, polyphenols, etc.) in their organs [2]. Since the production of these metabolites is closely linked to the plant's growing conditions and environment, it is natural that the conditions imposed in vertical farms have an impact on the cosmetic efficacy of the plants.

Furthermore, like all living beings, plants host a complex community of bacterial and fungal microorganisms that constitute their microbiome, and which is also referred to as phytobiome. This phytobiome is composed of the community of bacterial and fungal microorganisms associated with plants. It includes the rhizospheric microbiome, epiphytes (external microbiome), and endophytes (internal microbiome) [3]. Microbial endophytes have attracted considerable interest and attention since they have been reported to confer a wide range of beneficial effects on host plants such as protection against herbivore [4], growth promotion [5] and elicitation of defense mechanisms [6]. During their long co-existence, microbial endophytes and their host plants have evolved together in a balanced continual process within a particular ecological niche where they can interact biochemically and/or genetically. This long process could explain

why some endophytes produce the same or similar bioactive compounds as those found in the host plants.

Given its medicinal properties, the endophytic characterization from *Centella asiatica* was well studied. Numerous studies have demonstrated the involvement of the plant's commensal microorganisms in the production of valuable secondary metabolites, primarily asiaticoside and madecassoside [7]. *Centella asiatica* (Apiaceae, Umbelliferae), is a small and annual medicinal plant, creeping herb that grows near swamps on damp ground. Wide scientific literature described the pharmacological properties of *C. asiatica*'s extracts mainly due to its saponins, among all asiaticode, asiatic and madecassic acids. These plants actives are particularly used in skin care and cosmetic formulations for their improving effect in healing processes of wound, burns and skin [8]. However, *C. asiatica* is mainly found in tropical regions of India, Madagascar, Sri Lanka, China, Indonesia, Australia or South Africa, and its culture is cited in accordance with Nagoya Protocol, making its supply difficult [7]. Indoor growing seems to be a good alternative for local and sustainable sourcing.

This study focuses on understanding how the symbiotic relationship between endophytic microorganisms and medicinal plants, such as *Centella asiatica*, affects metabolite production. By comparing the phytobiomes of plants grown in controlled indoor vertical farms with those of wild plants, the study aims at understanding the impact of culture conditions on phytobiome composition and centellosides production ultimately improving our knowledge of this significant plant species within the cosmetic field.

2. Materials and Methods

2.1. Plant sampling

One sample of commercially available dried leaves of wild-type, native *Centella asiatica* (L.) Urban from Madagascar was used as a comparative control.

Also, 10 mother plants with developed stolons, cultivated hydroponically, were provided by a vertical farm based in France. Plants from two production cycles were studied.

2.2. Isolation of endophytes from *C. asiatica*

2.2.1. Samples preparation

2.2.1.a. Surface sterilization of farm-cultivated *C. asiatica* samples

The plant samples were meticoulously cleaned with running tap water and soaked for at least 10 minutes. For surface sterilization, the samples were treated with 70% ethanol for 2 minutes, and subsequently immersed in a 1-2% sodium hypochlorite solution for 10 minutes. They were then rinsed three times with sterile distilled water and underwent a final 70% ethanol dip. After

drying with sterile paper, the sterilization efficacy was controlled by centrifuging 20 mL of the last rinse water, discarding the supernatant, and plating the remaining solution.

2.2.1.b. Revitalization of microorganisms in Malagasy dried material

Dried leaves from Madagascar were ground in sterile mortars and resuspended in sterile phosphate-buffered saline (PBS, pH 7.4). The volume of the solution was adjusted according to the amount of tissue used.

2.2.2. Isolation of endophytic fungi

After surface sterilization, healthy leaves, leaf stalks, and roots were cut into small segments (approximately 2 × 2 mm) using a sterilized scalpel. These segments were cultured in 90 mm Petri dishes containing malt extract or potato dextrose agar medium with chloramphenicol (Chlo). Fungi emerging from the plant segments were subcultured onto 2% malt extract agar ensuring the capture of the entire endophytic community.

For wild-type dried samples from Madagascar, serial dilutions were prepared using PBS. Each dilution was plated on sterile appropriate media and incubated at 25°C for at least one week. Colonies were then stored at -130°C in a 10% glycerol solution prior to identification.

2.2.3. Isolation of endophytic bacteria

The isolation of bacteria endophytes is performed using the same method than for fungi, using tryptone soya agar (TSA) or lysogeny broth (LB) media for bacterial growth. Briefly, plant segments were cultivated in the appropriate media for 24-48 hours at 25°C, and morphologically distinct bacterial colonies were preserved at -80°C in a 25% glycerol solution for subsequent identification.

2.2.4. Molecular identification of cultivable endophytes

The main endophytic fungi and bactria encountered were identified by DNA extraction and SANGER sequencing.

Firstly, endophytic DNA was extracted from fungal and bacterial colonies using the Fast Extract DNA kit, according to manufacturer's instructions. Briefly, 50 µL of Fast Extract DNA solution was added to bacterial or fungal cells, followed by thermocycling at 65°C for 6 minutes and 98°C for 2 seconds. Subsequently, the samples were subjected to DNA amplification using Polymerase Chain Reaction (PCR). For fungi, the ITS region was amplified using primers ITS4 and ITS5, while bacterial 28S rDNA regions were amplified using 28S L0 (LR0R) and L6 (LR6) primers, and 27F and 1100R for 16S rDNA regions.

Finally, PCR products were sequenced and edited by a genetic analysis software and were compared for similarities with BLAST search.

2.3. Extraction and quantification of secondary metabolites from *C. asiatica*

2.3.1. Extraction

For secondary metabolites extraction, 900 mg of dried leaves from both origins (wild-type from Madagascar and from vertical farm) were subjected to ultrasound-assisted extraction using a 70/30 ethanol/water solution (v/v), for 1h at room temperature. Resulting extracts were subsequently filtered through a 0.2 µm syringe prior to UHPLC analysis.

2.3.2. UHPLC analysis

Secondary metabolites analysis was performed using an ultra-performance liquid chromatographic system Acquity H-Class equipped with PDA diode array detector (200-450 nm). Chromatographic separation was performed using a C8 column (5-µm size, 250 × 4.6 mm in length) with an acetonitrile solution as the mobile phase. Separation was carried out at a flow rate of 1.5 ml min^{-1}. The sample injection volume was 20 µL at 25°C.

3. Results

3.1. Comparison of endophytic diversity

Twenty-three bacterial distinctive colonies were successfully isolated and characterized from *Centella asiatica* grown in vertical farm based on their phenotypic characteristics and sequence analysis (**Table 1**). These bacteria could be classified into eleven different genera of which eight were affiliated with the phylum Proteobacteria (seven gammaproteobacteria: *Enterobacter sp., Serratia sp., Stenotrophomonas sp., Klebsiella sp., Pantoea sp. Pseudoxanthomonas sp.,* and *Pseudomonas spp.*, and two betaproteobacteria: *Burkholderia sp.* and *Achromobacter sp.*), one was assigned to the phylum Firmicutes (*Bacillus spp.*) and one belonged to the phylum Bacteroidetes (*Chryseobacterium sp.*). The predominant bacterial genera identified in *Centella* cultivated in vertical farms are *Bacillus* and *Pseudomonas*. Genera such as *Burkholderia*, *Enterobacter*, *Serratia*, *Stenotrophomonas* and *Pantoea* appear to be more specific to the aerial parts. Conversely, the root system of the plant was found to harbor *Achromobacter*, *Pseudoxanthomonas*, *Chryseobacterium* and *Klebisella* genera.

Regarding fungi, twenty-two cultivable species were isolated and characterized from *Centella asiatica* grown in vertical farm (**Table 1**). All isolates belonged to the Ascomycetes phylum with *Aspergillus* appearing to be the prevailing genera. *Aspergillus*, *Aureobasidium*, *Chaetomium* and *Fusarium* were found in different parts of the plant, while *Dichotomopilus* and *Sclerotinia* were only identified in roots.

Table 1. List of cultivable endophytic bacteria and fungi isolated from *C. asiatica* cultivated in vertical farm

Chlo = chloramphenicol

Tissue	Culture medium	Genus	Species
		Bacteria	
leaves	LB	*Bacillus*	*amyloliquefaciens (velezensis)*
leaves	LB	*Bacillus*	*sp.*
leaves	LB	*Bacillus*	*tequilensis*
leaves	Malt Extract Agar + Chlo	*Burkholderia*	*gladioli*
leaves	LB	*Enterobacter*	*ludwigii*
leaves	Malt Extract Agar + Chlo	*Pseudomonas*	*fluorescens*
leaves	LB	*Serratia*	*rubidaea*
leaves	LB	*Stenotrophomonas*	*sp*
roots	LB	*Achromobacter*	*sp.*
roots	LB	*Bacillus*	*amyloliquefaciens*
roots	LB	*Bacillus*	*subtilis*
roots	LB	*Bacillus*	*velezensis*
roots	LB	*Chryseobacterium*	*sp.*
roots	LB	*Klebisella*	*aerogenes*
roots	LB	*Pseudomonas*	*corrugata*
roots	Malt Extract Agar + Chlo	*Pseudomonas*	*fluorescens*
roots	Malt Extract Agar + Chlo	*Pseudomonas*	*putida*
roots	LB	*Pseudomonas*	*sp. 1*
roots	Potato Dextrose Agar+Chlo	*Pseudomonas*	*sp. 2*
roots	LB	*Pseudoxanthomonas*	*sp.*
stolon	LB	*Enterobacter*	*ludwigii*
stolon	LB	*Pantoea*	*sp*
stolon	Malt Extract Agar + Chlo	*Pseudomonas*	*sp. 1*
		Fungi	
leaves	LB	*Acremonium*	*alternatum ou sclerotigenum*
leaves	Malt Extract Agar + Chlo	*Acremonium*	*sordidulum*
leaves	Malt Extract Agar + Chlo	*Aspergillus*	*ochraceus*
leaves	Malt Extract Agar + Chlo	*Aspergillus*	*westerdijkiae*
leaves	Potato Dextrose Agar + Chlo	*Aureobasidium*	*pullulans*
leaves	LB	*Chaetomium*	*cochliodes*
leaves	Malt Extract Agar + Chlo	*Chaetomium*	*globosum*
leaves	Malt Extract Agar + Chlo	*Fusarium*	*parceramosum*
leaves	Malt Extract Agar + Chlo	*Gliomastix*	*polychroma*
leaves	Malt Extract Agar + Chlo	*Penicillium*	*sp. 1*
leaves	Malt Extract Agar + Chlo	*Penicillium*	*sp. 2*
leaves	Potato Dextrose Agar + Chlo	*Sordariomycetes*	*sp.*
roots	Potato Dextrose Agar + Chlo	*Aspergillus*	*westerdijkiae*

Table 1 (continued)

Tissue	Culture medium	Genus	Species
roots	Malt Extract Agar + Chlo	*Aureobasidium*	*pullulans*
roots	Malt Extract Agar + Chlo	*Chaetomium*	*globosum*
roots	Malt Extract Agar + Chlo	*Dichotomopilus*	*indicus or funicola*
roots	Malt Extract Agar + Chlo	*Fusarium*	*oxysporum*
roots	Malt Extract Agar + Chlo	*Sclerotinia*	*sclerotiorum*
stolon	Malt Extract Agar + Chlo	*Aspergillus*	*ochraceus or westerdijkiae*
stolon	Malt Extract Agar + Chlo	*Chaetomium*	*cochliodes*
stolon	Malt Extract Agar + Chlo	*Fusarium*	*parceramosum*
stolon	Malt Extract Agar + Chlo	*Penicillium*	*sp. 3*

In contrast, the microbial diversity found in the wild-type Madagascar sample resulted in obtaining seven bacterial colonies and seventeen fungal strains (**Table 2**). In comparison with *Centella* from vertical farms, other bacterial genera such as *Arthrobacter*, *Curtobacterium*, *Heyndrickyia*, *Lederbergia*, *Shingomonas* and *Stucliffiella* were identified. Fungal genera *Coniochaeta*, *Muscodor*, *Nigrospora*, *Phaeosphaeria*, *Preussia* and *Xylaria* have been detected in addition to *Chaetomium* and *Penicillium* genera, which were common to the indoor-*Centella* samples.

Table 2. List of cultivable endophytic bacteria and fungi isolated from dried leaves of *C. asiatica* collected in Madagascar

Chlo = chloramphenicol

Culture medium	Genus	Species
	Bacteria	
Potato Dextrose Agar + Chlo	*Arthrobacter*	*sp.*
LB	*Bacillus*	*subtilis*
LB	*Curtobacterium*	*luteum*
Potato Dextrose Agar + Chlo	*Heyndrickyia*	*acidicola*
LB	*Lederbergia*	*wuyishanensis*
LB	*Shingomonas*	*sp.*
LB	*Stucliffiella*	*horikoshii*
	Fungi	
Malt Extract Agar + Chlo	*Ascomycota*	*sp.*
Potato Dextrose Agar + Chlo	*Chaetomium*	*globosum*
Potato Dextrose Agar + Chlo	*Chaetomium*	*sp.*
Malt Extract Agar + Chlo	*Coniochaeta*	*angustispora*
Malt Extract Agar + Chlo	*Coniochaeta*	*cymbiformispora*
Potato Dextrose Agar + Chlo	*Fungal or Xylariales*	*sp.*
Malt Extract Agar + Chlo	*Fungal or Xylariales*	*sp.*
Malt Extract Agar + Chlo	*Muscodor*	*albus*
Malt Extract Agar + Chlo	*Nigrospora*	*sphaerica*
Malt Extract Agar + Chlo	*Penicillium*	*rolfsii*

Table 2 (continued)

Malt Extract Agar + Chlo	*Penicillium*	*sp.*
Malt Extract Agar + Chlo	*Phaeosphaeria*	*fuckelii*
LB	*Preussia*	*africana*
Potato Dextrose Agar + Chlo	*Preussia*	*sp.*
Malt Extract Agar + Chlo	*unculured coniochaetales*	
Malt Extract Agar + Chlo	*unknown*	*1-8*
Potato Dextrose Agar + Chlo	*Xylaria*	*apiculata*

At the species level, either bacterial diversity and fungal diversity showed significant variability, with only *Bacillus subtilis* and *Chaetomium globosum* being the common species identified in both samples. There results highlight distinct microbial communities, seemingly associated with distinct plant culture conditions.

3.2. Secondary metabolites quantification

Centella sample from Madagascar displayed a diverse profile, prominently featuring asiaticoside, madecassoside, dicaffeoylquinic acid derivatives (including irbic acid and 1,5-dicaffeoylquinic acid), quercetin, and chlorogenic acid. On the other hand, two distinct batches of *Centella* from vertical farm, originating from two separate production cycles, were analysed and exhibited similar profiles (**Figure 1**). Quercetin emerged as the predominant peak in both. Unlike wild-type *Centella*, the vertical farm samples lacked specific biomarkers such as asiaticoside, madecassoside triterpenes, and various dicaffeoylquinic acid derivatives.

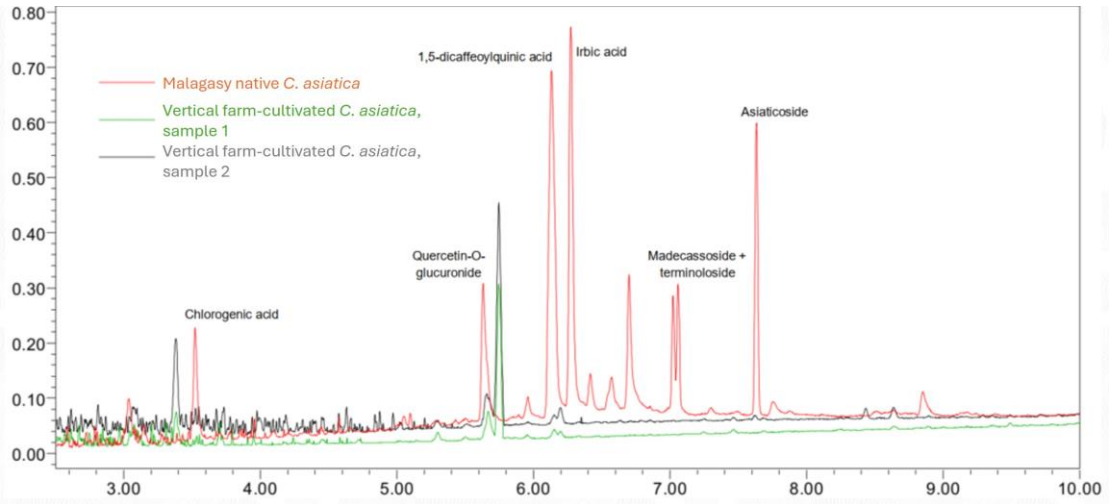

Figure 1. UHPLC chromatograms displaying the primary secondary metabolites present in *Centella* extracts

4. Discussion

This study focused on a comparison of endophytic bacterial and fungal communities between the *Centella asiatica* plant, either grown in fields or in vertical farming. Given the difficulties associated with procuring fresh Malagasy plants, particularly *C. asiatica*, we chose to utilize a commercially available dried specimen instead, for control comparison. This decision allowed us to circumvent the logistical challenges of sourcing fresh botanicals, ensuring a consistent and reliable product for our purposes. However, considering the reduced diversity observed in the dried material from Madagascar, it is possible that the drying process may have affected the endophytic population. According to Rakotoniriana (2012) [7], common major fungal endophytes identified in *C. asiatica* were from Xylariaceous taxa (with an isolation frequency of 19.2%), *Colletotrichum sp*. (13.2%), *Guignardia sp*.(8.5%) and *Glomerella sp.* (7.7%) [7]. None of these fungi were present in samples from vertical farming or in the dried *Centella* from Madagascar except for *Xylariaceae* species. Another study on the isolation frequency of endophytic fungi indicated that *Colletotrichum sp.* was present at 34%, followed by *Fusarium sp.* at 16.8%, *Curvularia sp.* at 15.7%, *Aspergillus sp.* at 15%, *Alternaria sp.* at 12%, and *Nigrospora sp.* at 4% [8]. Some *Fusarium* and *Aspergillus* species were identified in vertical farm samples and, *Nigrospora* species were found in Malagasy *C. asiatica*. Comparing these two publications revealed the emergence of *Colletotrichum sp.* as the predominant fungal species, a fungus that causes anthracnose [7]. However, this genera was not found in any of our analyzed samples. Differentiating cultivable microorganisms is a crucial first step in our study and will be followed by an isolation frequency analysis and high-throughput sequencing. This approach aims to accurately identify all species and draw further conclusions. Additionally, considering that *Colletotrichum sp*. is a well-known fungi, a differential analysis of culture media and conditions might be of help to better understand the absence of this germ within our samples. Nevertheless, it is possible that the absence of this fungi is related to plant cultivation condition, hence its absence is of interest given the variability observed in phytochemical profiles of indoor-grown *C. asiatica* compared to the native one.

Centella asiatica harbors a rich taxonomic diversity of endophytes; however, many studies have focused on fungal endophytes, with limited exploration of endophytic bacteria. In contrast to fungi, several bacterial species highlighted in this study have also been recorded in existing literature. For example, *Pseudomonas sp., Enterobacter sp., Chryseobacterium sp.* and *Pantoe sp.* were isolated from *C. asiatica* grown in vertical farm, and were also found in leaves from Cape Town, as reported in 2022 [9]. Meanwhile, *Achromobacter sp*. and *Klebsiella sp*. were also identified in *C. asiatica* leaves collected from Madagascar in 2013 [7]. The differences in diversity are therefore more pronounced for fungi.

Numerous studies have demonstrated that fungal endophytes can act as elicitors, offering an effective and beneficial approach to enhance the production of pharmacologically important compounds from medicinal plants. In this context, potential endophytes that could enhance centelloside production through biotization have been identified in the literature and are detailed in **Table 3**. The results of the centelloside extraction revealed a nearly complete lack of biomarkers in the plants from the vertical farm. This difference may be attributed to the absence of the phytopathogenic fungus *Colletotrichum sp.*, which is typically found in wild *Centella* and has been documented in several studies for its role in enhancing asiaticoside production [10,12]. This absence underscores the potential for modulation with *Colletotrichum*. Additionally, three endophytic bacteria, *Bacillus subtilis*, *Bacillus amyloliquefaciens* and *Pseudomonas fluorescens*, were detected in indoor plants and are known to inhibit *Colletotrichum*'s virulence [7]. This suggests that these beneficial microorganisms could enhance asiaticoside production through biostimulation with *Colletotrichum* while preserving a healthy plant phenotype.

Other candidates, such as *Trichoderma harzianum* and *Piriformospora indica*, have also been recognized for their ability to enhance secondary metabolite production and could be considered for biotization assays (**Table 3**).

Table 3. List of candidate endophytes described in the literature to increase centellosides production

Endophytes	Strategies	Metabolites	References
Colletotrichum gloeosporioide	Co-culture with *C. asiatica*	Asiaticoside	**[10]**
	Fungi culture extract		**[12]**
Piriformospora indica	Co-culture with *C. asiatica*	Asiaticoside	**[13-14]**
	Autoclaved fungi culture extract		**[15]**
Trichoderma harzianum	Fungi culture extract	Asiaticoside	**[16]**

5. Conclusion

The symbiotic relationship between endophytic fungi and medicinal plants significantly impacts secondary metabolism, influencing metabolite production and the quality of crude drugs. In this context, we examined the diversity of the microbiome associated with *Centella* plants cultivated in vertical farm. Preliminary research revealed that *Colletotrichum sp.*, a fungus known for boosting asiaticoside production, is absent in indoor-grown *C. asiatica* when compared to wild-type plant. However, beneficial bacteria such as *Bacillus amyloliquefaciens, Bacillus subtilis* and *Pseudomonas fluorescens*, which suppress *Colletotrichum*'s virulence, were identified and may boost metabolite production through biostimulation. This innovative strategy, integrating phytobiome modulation with vertical farming, has the potential to revolutionize local sourcing and encourages further exploration of fungal endophytes as elicitors to enhance a more sustainable production of plant-based cosmetic ingredients.

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