Tampere University of Applied Sciences

Isolation of bacteria from leaf glands of *Dioscoreae sansibarensis*

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ABSTRACT

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Isolation of Bacteria from Leaf Glands of *Dioscoreae sansibarensis*

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This study aims to find bacteria that could colonise the leaf glands of *Dioscoreae sansibarensis*, a Yam species. *D. sansibarensis* lives in a mutualistic type of symbiosis with the bacterium *Orrella dioscoreae* and in an exclusive interaction together. *O. dioscoreae* occupies a niche in glands located at the tips of the leaves and excludes all other bacterial colonisers by a mechanism still unknown to this day. In this work the microbe-free plants were created in the laboratory and its leaf glands were naturally colonized by bacteria other than *O. dioscoreae.* Once colonised, the bacteria were extracted, plated out, isolated, and identified and the bacteria with the highest potential to colonize the leaf gland were selected. The results serve as the basis for the further study, in which the isolated bacteria will be put in competition with *O. dioscoreae* to study the latter's mechanisms of defence.

The results show that the leaf gland can host diverse kinds of bacteria and that the bacteria can fully colonise the gland, remain inside the leaf gland and survive. 13 bacteria have been identified as having the best potential to be colonisers of the leaf gland niche. Results show as well that the original symbiont and the found bacteria can be put in competition for the leaf gland niche, which will provide a better understanding of the defence mechanisms of *O. dioscoreae.*

Key words: dioscoreae sansibarensis, Orrella dioscoreae, defence mechanism, bacterium, isolation.

CONTENTS

GLOSSARY

TAMK	Tampere University of Applied Sciences
LIPM	Laboratory of Plant-Microbe Interaction (Toulouse, FR)
T6SS	Type VI Secretion System
O. dioscoreae	*Orrella dioscoreae*
D. sansibarensis	*Dioscoreae sansibarensis*
PPM	Plant Preservative Mixture
MS	Growth Medium with Sucrose
HCl	Hydrochloric acid
pH	Potential of Hydrogen
TSA	Tryptic Soy Agar
TAMK	Tampere University of Applied Sciences
CFU	Colony-Forming Unit

1 INTRODUCTION

Bacteria are ubiquitous in our global ecosystem, including air, clouds in the atmosphere, ice, and deep seas. They interact closely with their habitat and ecosystems through different forms of cooperation, like parasitism, commensalism and/or mutualism. They have evolved both active and passive modes of locomotion as well as molecular machinery that allow them to produce proteins to manipulate and/or regulate their surroundings to be able to strive and survive. (Kadner and Rogers, 2022)

Among these mechanisms, the recently discovered type 6 secretion system (T6SS) is mainly used by bacteria against bacterial competitors to gain a spatial and/or metabolic niche (Sana *et al*, 2017)

The bacterium *Orrella dioscoreae* lives exclusively in interaction with the plant *Dioscoreae sansibarensis*. It occupies a niche in glands located at the tips of the leaves (also called drip tip or leaf gland), excluding all other bacterial colonisers, by a mechanism that is still unknown to this day (Carlier *et al*. 2017). The presence of *O. dioscoreae* in the gland is essential to exclude other bacteria, which are also able to live in this niche. Furthermore, *O. dioscoreae* presents several types of defence mechanisms that have been identified and especially two Type VI secretion systems (T6SSs) that are both activated in the plant. (De Meyer *et al*., 2019)

Dioscoreae sansibarensis and its symbiont *Orrella dioscoreae* live in a mutualism type of symbiosis (Carlier *et al*. 2017). Mutualism is characterized by an association between organisms of two distinct species in which each involved organisms' benefits. *D. sansibarensis* offers a safe living and breeding ground to *O. dioscoreae*, and *O. dioscoreae* offers protection from potential pathogens to the plant (De Meyer *et al*., 2019). This study bases its hypothesis on this phenomenon.

The type VI secretion system is a molecular machinery that is widespread in Gram-Negative bacteria and targets both eukaryotic and prokaryotic cells, in a

contact dependent manner, to inject toxins (Alcoforado, *et al*. 2015). Finally, according to recent unpublished results from the Laboratory of Plant-Microbe Interactions of Toulouse Laboratory of Interactions Plant Microbe Environment (LIPME), *O. dioscoreae* uses one of these T6SSs to effectively kill other bacteria under laboratory conditions.

The phyllosphere, which refers to the leaf surface, is also a term that is frequently used to refer generically to any portions of plants that are above ground such as leaves, bark, flowers, fruits, etc. Additionally, endophytes, which are organisms located within leaf tissues, are present in addition to epiphytic colonisers, which are found on leaf surfaces. The phyllosphere has a unique and complex microbial biome, home to an active and fruitful community of bacteria, yeast and fungi that can be commensal, parasitic, and mutualistic microorganisms. (Leveau, 2019)

This study aims to characterize bacteria that spontaneously colonise the leaf gland of *D. sansibarensis*, in the absence of its cognate symbiont. Then, to put *O. dioscoreae* and these bacteria in competition to test out and better understand *O. dioscoreae* defence mechanisms.

1.0 The Host: *Dioscoreae Sansibarensis*

Dioscoreae sansibarensis, also known as Zanzibar yam, originates from Madagascar and tropical Africa (Picture 1) and entered the International Plant Names Index in 1892.

PICTURE 1. Map of the Georeferencing of *Dioscoreae sansibarensis* in the world according to the GBIF (Global Biodiversity Information Facility) website.

It is an herbaceous vine with stems that can grow up to 7m long. It has tuberous roots and grows bulbils from the axils. It propagates by the aerial bulbils falling on the ground. *D. sansibarensis* (Picture 2) is considered invasive or potentially invasive, therefore it is forbidden to plant them outdoors outside of their country of origin. (Govaerts, 2017).

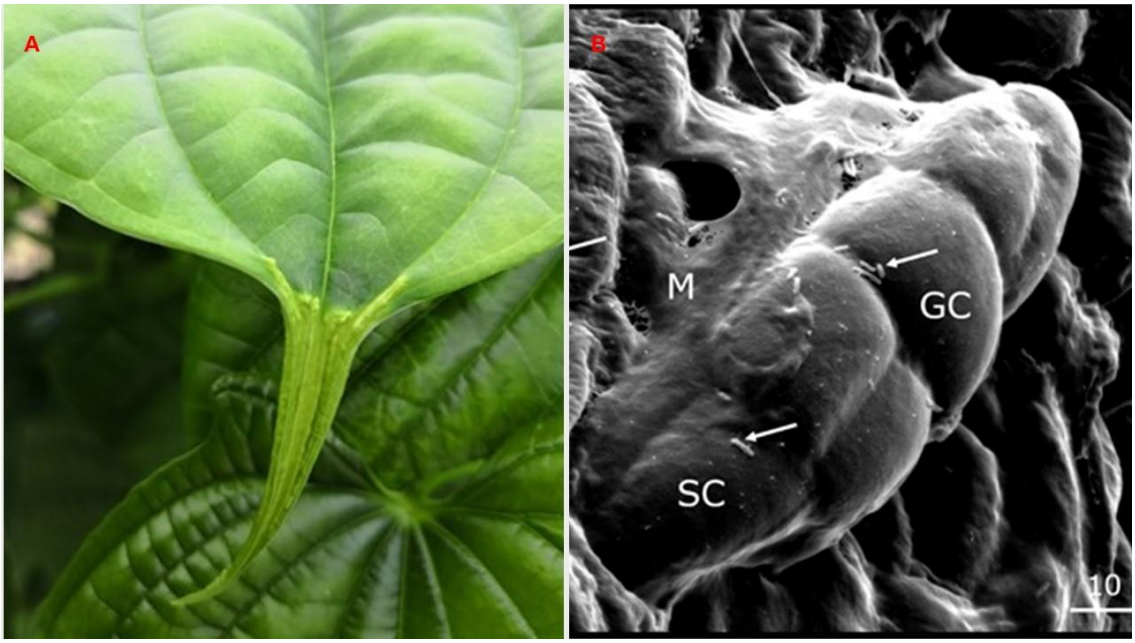
PICTURE 2. *Dioscoreae sansibarensis* parts. A. leaf size compared to an adult woman hand. B. Leaf gland. C. Bulbil. D. Adult plants in lab greenhouse. E. Tuber

As many species of Yams *D. sansibarensis* is a fast-growing plant, that can grow to be several meters long in a month, which makes it easy to study in a laboratory setup.

1.1 The Symbiont: *Orrella dioscoreae*

It is a Gram-negative bacterium of the *Alcaligenacea* family from the order *Burkholderiales*. *Alcaligenaceae* organisms have colonised an extensive range of habitats, alone in water and soil or combined with animals. Their ecological roles can be nitrogen fixation, pathogenesis to animal hosts, degradation of different organic compounds and many more. (Austin, 2014).

O. dioscoreae (picture 3) is non-spore forming and considered non-motile cocco-bacilli. Its size ranges between 0.9 and 1.1 μm. All strains so far were isolated from leaf acumens of *D. sansibarensis* shown in picture 3 (Carlier *et al*. 2017). The bacterium inhabits the gland of the leaves, in high concentration but can also colonise other parts of the plant.



PICTURE 3. A. *D. sansibarensis* leaf gland. B. Scanning electron microscopy of leaf primordia in the shoot tip shows *O. Dioscoreae* (arrow) colonizing the glandular trichomes. Stalk cell (SC), glandular cells (GC), and mucus (M). Scale 10 μm. (Published with the kind permission of Acar, *et al*. 2022)

2 SCOPE OF THE STUDY

The aim of this study was to find bacteria that could colonise the leaf glands of *D. sansibarensis.*

To find the said bacteria, microbe-free (also called apo-symbiotic) plants were produced, and other bacteria let to colonise the leaf gland. Once colonised, through the extractions, plating out, isolation, and identification the bacteria species that have the best potential to colonise the leaf gland niche can be selected. These can later be used to compete with *O. dioscoreae* to test and better understand the mechanism of defence of the symbiont bacteria, *O. dioscoreae*.

3 METHODS AND MATERIALS

3.0 Distribution of the bacterium through the plant and its identification

Understanding the distribution of *O. dioscoreae* in the plant was the first step to understand how the bacteria colonise each tissue. A quantitative analysis was done to compare results with the inoculation of genetically modified *O. dioscoreae* strains. The purpose was to measure the loads of bacteria in different tissues and to compare it with genetic mutants of genes of interest. After inoculation of genetically modified strains, e.g., disabling the motile gene or giving them glowing protein to be easily identified in the plant, it was possible to see how they evolve in the plant and in which quantity.

As presented by Acar, *et al* (2022), depending on the genetic modification done to the bacteria it was possible to see if the bacteria can colonise the plant passively or if it needs mobility capacities.

This section explains how the quantitative analysis was conducted. First, using sterile tweezers and scalpel, the tip of the leaf was cut off, and the tissue was homogenized using 100 µl of 0.4 % NaCl and 3 sterile glass beads for 1 minute at 30 round/s in a Retsch ball mill (MM 400). The homogenized mixture was centrifuged for 7 seconds to precipitate debris. Then, 100 µL of the cleared mixture was plated out on tryptic soy agar (TSA) plates and incubated for 2 days at 28°C in the dark. If bacteria grew on the plate, the identification of the colonies was done using colony polymerase chain reaction (PCR) technique or using 16S rDNA sequencing (Acar, *et al.* 2022). Though, after some time it became easier to recognise the colonies of *O. dioscoreae* by shape, colour, and aspect and so the identification tests by PCR were not always conducted. Another technique used to identify *O. dioscoreae* was to plate a drop of the mixture on TSA plates where nalidixic acid was added. Nalidixic acid is a is an antibiotic effective against gram-negative bacteria. (Crumplin & Smith, 1975). As stated previously *O. dioscoreae* is a gram-negative bacterium. Therefore, TSA plates containing nalidixic acid are a good and straightforward way to see if the tested bacteria are *O.*

dioscoreae or not. If growth was observed, it meant that the tested bacteria was not *O. dioscoreae.* This allowed for faster work and less expenses.

The same protocol was repeated for distinct parts of the plant: leaves, stems, growth centre, nodes, and apical bud.

3.1 Apo-symbiotic culture breeding

The creation of microbe-free plantlets is usually done by sterilizing seeds. Unfortunately, so far *Dioscoreae sansibarensis* rarely produces flowers in the wild and never when produced indoors or in laboratory conditions. Instead of flowers and seeds, the plant produces bulbils that will fall on the ground and grow to become tubers. From these tubers and after a dormancy phase new stems emerge and the cycle repeats.

Wild-type plants were grown in the greenhouse of the Laboratory of Interactions Plant-Microbe-Environment (LIPME) in Castanet-Tolosan, France. Other plants were grown in climate chambers at 28°C, 70% humidity and a light cycle of 16h light, 8h dark. (Acar, *et al*. 2022)

The propagation of *Dioscoreae sansibarensis* by growing plantlets in tissue culture, also called micropropagation, was done using a protocol adapted from (Alizadeh, *et al*., 1998).

Creating apo-symbiotic plantlets happened in several stages. First, node cuttings were done using adult plants, about 3 months old, grown in the greenhouse or culture chambers of the laboratory. Node cutting means that a piece of the stem was cut at the intersection of a leaf, corresponding to a growth centre where the future bulbils will develop. The part of the stem cut during the node cutting phase is called an explant. Each round of node cutting contains about a hundred explants. Next, the obtained explants were sterilized in a solution of 5% plant preservative mixture (PPM), and set to shake for 8 hours at 100 rpm and 28°C.

Then, after 8 hours, the explants were taken out under sterile condition and the dead/bleached extremities cut off. Explants were then placed in sterile 6-well multidish (3 explants per well), see Picture 3, and with sterilized growth medium of a composition (TABLE 1).

TABLE 1. Composition of the sterilized growth medium used in 6 well-mutidish.

MS	4.4g/L, 2% sucrose
Vitamins	Glycine (2mg/L), Myo-inositol (100 mg/L), nicotinic acid (0.5mg/L), pyridoxine-HCl (0.5mg/L), Thiamine-HCl (0.1mg/L) and L-cysteine (20mg/L)
pH	5.7
Antibiotics	carbenicillin (200 µg/ml), cefotaxime (200 µg/ml)
PPM	0.2% v/v

Finally, explants were incubated at 28°C with a cycle of 16h of light and 8h of night for 10 days. The growth medium was changed after 10 days, and contaminated or dead explants were thrown away. After 21 days, the growth medium was changed using the same solution as described above but without antibiotics. If the explants had developed leaves and were viable, they were transferred in magenta boxes, see Picture 3, and incubated at 28°C, with the same light cycle as before.

Picture 3. Bottom left: 6-well multidish (VWR, Tissue Culture Plate, Non-treated, Sterilized, Non-pyrogenic). Top right: Magenta boxes (Merck, GA7).

The amount of explant needed was high due to the low success rate of this technique. It has a 58.5 % rate of success. This means that not all explants survive to grow to full plants. The original success rate was 31 % and through optimization, testing and adjusting the protocol the team succeeded in increasing the number of viable plantlets. To get to this result, it took the team two years of testing and adjusting the protocol.

3.2 Inventory of bacteria invading the bacteria-free plant

Plants used for the endophytic leaf gland bacteria samplings were kept in a 6m^2 growth chamber, with climatic conditions of 25°C and a light cycle of 16h of day and 8h of night. Inventory of the leaf gland endophytic bacteria started with taking samples of the leaf glands. Then, to eliminate all epiphytic organisms and have only endophyte bacteria left from within the leaf gland a protocol of surface sterilization was done. And finally, the samples were homogenized, plated out on microbiological agar media, and set to conditions where bacteria could develop in an optimum condition.

3.2.0 Leaf glands sampling

Adult plants that had been previously tested by the team and marked to be aposymbiotic were randomly selected. These plants had been growing in a culture chamber for 1 to 4 months and were exposed to ambient air. In total 18 different plants were selected. Two leaf glands samples per plant were collected using sterilized tweezers and scalpel to hold and cut the leaf gland. Young and old leaves were selected at random as well. A total of 36 samples were collected during the first round and 18 during the second round. The collected leaf glands were placed in sterile 2mL Eppendorf tubes previously filled with tap water, to avoid the plant tissues to dry.

3.2.1 Leaf glands Surface Sterilization

From this point onward, all experiments were carried out under sterile environment conditions. As previously mentioned, surface sterilization was a primordial step, as the endophyte bacteria were the main interest of this study.

From the previously collected sample kept in tap water the first step was to take the water out of the tubes using a pipette. Then, 1mL of ethanol 70% was added to each tube, and they were slowly shaken by hand for 30 seconds and left for 5 min. After 5 minutes, the ethanol was taken out using a pipette and the same operation was repeated with a solution of sodium hypochlorite 1.4%. Finally, the sodium hypochlorite was taken out and the samples were rinsed 3 times with distilled water using the same method as described above

3.2.2 Samples Homogenisation and plating out

In this step homogenisation was used to extract endophyte bacteria and have them in a solution of water and sodium chloride (NaCl), to be easier to plate out. NaCl was used in this media culture to allow adjustment in osmotic pressure and preserve the bacteria. (Welsh, 2000).

36 sterile 2mL Eppendorf tubes with 300µL of NaCl 0.4% and 3 to 5 sterile glass beads, were prepared beforehand.

First, the sterilized leaf glands were transferred in the NaCl Eppendorf tubes, using tweezers sterilized by dipping them in alcohol 70% and heated them over a Bunsen burner flame and left to cool down for a minute. This operation was repeated between every sample. Then, the tubes were placed in a Retsch ball mill machine, model MM400, to homogenise the samples at 30 rounds per second for 30 seconds. The procedure was repeated until the samples were all well homogenized. Next, under a microbiological hood, the homogenized samples were plated out on TSA (Tryptic Soy Agar) 10% dishes, by pouring the entire solution

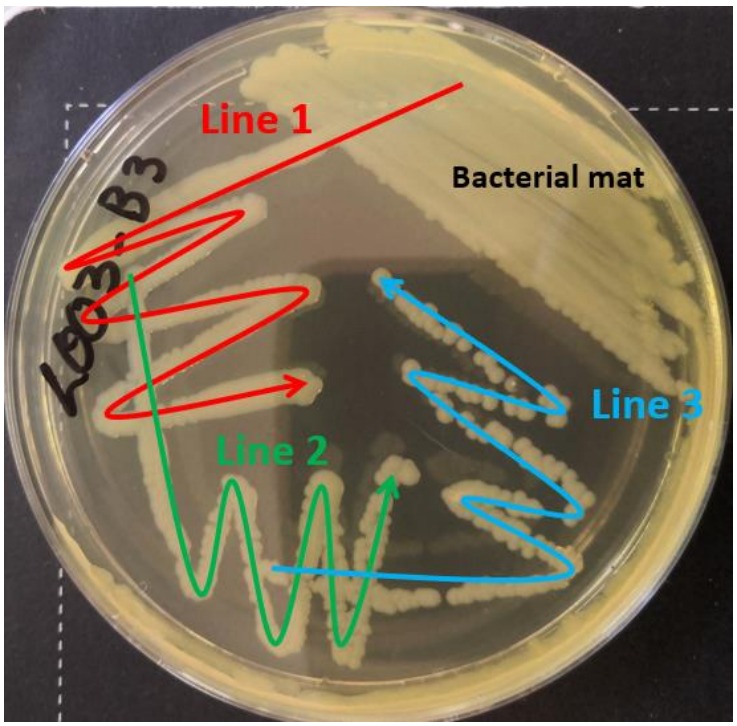
and shaking gently the plates in all directions until it is fully covered with the solution. Finally, the plates were left to dry under the hood. Once dry the glass beads were taken out and the plates were placed in a dark room at 28°C for 48h.

3.2.3 Re-isolation

Isolations of bacteria were done in a sterile environment under a Bunsen burner flame and using a stainless-steel inoculation loop sterilized by flame. See Appendix 1, 2 and 3 for the list of unidentified isolated bacteria.

Isolations were done on Tryptic Soy-Agar (TSA) 10% Petri dishes. TSA was used because it is a non-selective medium for general purposes (Sigma-Aldrich 2013). In this manipulation it was needed to see which bacteria were present regardless of their specificities and growth medium preferences. After 48h the plates containing the homogenized samples were taken out from the room at 28°C. Looking at each plate, the bacteria colonies were roughly counted, and the morphology describes, see appendix 1,2 and 3. A single colony from each type of bacteria present on the plate was selected and isolated, as shown in PICTURE 4, on a new TSA Petri dish.

First, a single colony was selected and spread to form a bacterial mat. Then, the 1st line was done, followed by the 2nd line and finally, the 3rd line was done, allowing the isolation of single colonies (Picture 4). The inoculation loop was re-sterilized by flame in between each line. The plates were placed at 28°C for 48h.



PICTURE 4. Isolation and re-isolation technique performed to obtain clean bacteria culture. 2003-B3: identification tag. Bacterial mat: 1st step. Line 1: 2nd step. Line 2: 3rd step. Line 3: 4th step.

After 48h the plates were taken out and the first round of re-isolation was done using the same technique as described previously (PICTURE 3). To ensure that there was only one species of bacterium on the medium, this procedure was performed one more time or until the bacteria culture was clean.

After the second re-isolation, a total of 58 strains of bacteria had been isolated from the first round of sampling and 31 during the second round of sampling.

3.3 PCR testing

As mentioned in chapter 2.0, the plants were not always apo-symbiotic even after the node cutting sterilization procedure. There was a non-negligible part of the plants that had a natural reoccurrence of *O. dioscoreae* during their lives and even after they have originally been tested apo-symbiotic in the first place. Therefore, all the isolations had to be tested to sort out *O. dioscoreae* from the sample that will be sent to identification by sequencing. PCR testing was then used to identify *Orrella dioscoreae* from the others. The protocol used is the following:

3.3.0 Lyse bacteria

Lysis buffer (50 mM KCl; 10 mM Tris. Cl pH8; 1 mM EDTA; 0.1% Tween 20; Sterilize through 0.2-micron filter) was previously prepared. 58 tube Eppendorf 1.5mL were prepared and marked 1 to 58 and filled with 100µL of lysis buffer. From the clean round of re-isolated bacteria and from each plate, one single colony was picked with a sterile pipette tip and suspended in the lyse buffer solution. The tubes were incubated 10 min at 95°C to free the DNA, then put immediately on ice.

3.3.1 PCR amplification solution preparation

This protocol used the polymerase Go-Taq. A volume of 20µL per sample was needed for a PCR reaction. The list of materials and quantities needed is presented in TABLE 2.

TABLE 2. List of material and quantities needed in the PCR reaction.

Material	Volume
Sample DNA	1µL
Primers at 10µM, forward (Fwd.) and Reverse (Rev)	1µL each, so, 2µL total.
Buffer Go-Taq	4µL
dNTPs (nucleotides = dATP, dTTP, dCTP and dGTP) at 10µM	0.4µL
Enzyme Go-Taq	0.2µL
Ultrapure H_2O	12.4µL

To the 58 original samples, 3 control samples and 1 extra sample (for pipetting errors) were added. It gives a preparation for 62 samples of PCR mix using table 2.

First a master mix was made by adding 62µL of each primer: Fwd. and Rev, then 248µL of buffer Go-taq, 24.8µL or ~25µL of dNTPs, 744µL of ultrapure H_2O and finally 12.4 of Go-Taq Enzymes. The solution is gently shaken to avoid destroying the enzyme.

3.3.2 PCR amplification

In specific PCR microtubes marked 1 to 61, 19µL of PCR master mix was added to each tube. Then, 1µL of Lyse bacteria DNA per tube was added, see appendix 4 for PCR number and bacteria number. Then, tube 59 and tube 61 which were the negative tests were filled with 20µL ultrapure water and with apo-symbiotic plant DNA, respectively. Tube 60 was the positive test and contained *O. dioscoreae* genomic DNA. Finally, the PCR microtubes were set in the PCR device, with 30 cycles of 30s at 95°C, 30s at 58°C and 1min at 72°C.

Through this action, nrdA gene is amplified with primers specific to *O. dioscoreae*. Therefore, only *O. dioscoreae* will be amplified and shown in the results of the PCR.

3.3.3 Gel staining for PCR results

First, a 1% agarose gel was made with: 3g of agarose powder was added to 300mL of Tris acetate EDTA buffer 1X (TAE). The solution is then poured and left to dry until the gel solidifies. Then, the gel is transferred to the electrophoresis bath, where each well is loaded with 8µL of the PCR reaction solutions from the microtubes. On each side of the gel, one well needs to be left empty of PCR reaction solution to add 3µL of smart ladder indicator. Finally, the gel is set at 100V for 30 min, to allow the PRC reaction product to migrate within the gel.

After 30 min, the gel is placed in an Ethidium Bromide (EtBr) bath. EtBr is a carcinogenic and mutagenic chemical. The gel is left in the bath for 30 min, then taken out and placed in the UV machine equipped with cameras to get the results.

3.4 Bacteria cryogenic conservation

After all plates containing *O. dioscoreae* were identified and removed, the rest of the bacteria were put into liquid culture to be stored in glycerol stock at -80°C for long conservation and to be used later if needed.

Liquid cultures are done in Trypticase Soy Broth (TSB). In a 20mL sterile glass tube 2mL of TSB was added and with a sterile pipette cone a single bacteria colony was picked, then the pipette cone was dropped in the TSB and left in the tube. This process is repeated for each strain of isolated bacteria and put to 28°C for 24h with agitation.

After 24h the liquid cultures are put in glycerol stock. In specific sterile cryogenic tubes 500µL of glycerol 60% and 500 µL of liquid culture is added and mixed well. The cryogenic tubes are put in a labelled box to – 80°C for long conservation and to be added to the bacteria library of the laboratory.

4 RESULTS AND DISCUSSION

4.1 Distribution of the bacterium through the plant

The results obtained are presented in Table 3. Since it was already known that *O. dioscoreae* was present in the leaf glands in high loads, they were not tested during this experiment.

TABLE 3. Quantitative analysis of *O. dioscoreae* in the host plant.

Location on the plant	Orrella Concentration
Apical bud	$6.27*10^4$ CFU/apical bud
Growth centre of bulbils	$7.83*10^4$ CFU/growth centre
Leaf surface	0 CFU/cm^2
Node	$3.53*10^4$ CFU/node
Stem	$1.21*10^2$ CFU/cm

The apical bud and growth centre have a significantly higher concentration than other parts of the plant (Acar, *et al*. 2022).

We can see that the concentrations are much higher in the meristematic tissues of the plants (apical bud, growth centre of bulbils and nodes). This indicates that the bacterium colonises the leaf glands in a passive way following the growth of the plant (Acar, *et al*. 2022).

Knowing this is important for the follow up of the experiment. As it has been presented by Acar (2022), *O. dioscoreae* lives inside the shoot meristems and shares a symbiotic relationship with the plant, which might be of importance during the rest of the experiment and especially when bacteria are put in competition for the leaf gland niche.

4.2 Apo-symbiotic culture propagation

Contamination is a major issue in in vitro propagation (Khan *et al*. 2018), and there has been a lot of contamination in our samples. Most common type of contamination observed during this study was fungi, followed by non-viable explants, which refused to grow or were too damaged by the phytotoxicity of PPM which caused necrosis of the tissues and died, and finally plants that tested positive to *O. dioscoreae* presence, due to natural reoccurrence. This method of culture propagation did not guarantee that *O. dioscoreae* was eliminated, it can spontaneously resurface, which was one of the biggest challenges so far. Even after years of testing and optimization contamination and natural reoccurrence is still responsible for a loss up to 41.5 % of the created plantlets. (Acar, *et al.* 2022).

One of the main reasons for contamination could be coming from the shared sterile spaces and especially shared sterile hoods. Different teams are working on different organisms (plants, bacteria, fungi) and there are many people sharing these spaces and as well a big turnover of trainees who might not respect safety procedures thoroughly. It also has been noticed that during Covid time, contaminations in plantlets seemed to drop. More sanitary measures were implemented and therefore, less paths for contamination were available and there were less people at the same time working in shared areas.

As presented in the research from Khan *et al*., 2018, the same protocol could be used to identify what kind of organisms are contaminating the plantlets. Making an inventory of all the contaminants and using various kinds of antibiotics to eliminate the contamination could be a solution. However, as discussed by Quambusch & Winkelmann (2018), the use of antibiotics can prove troublesome and involves a lot of different parameters to consider. Tests and studies have already been conducted on Yam species as presented in the research from Mbah & Wakil (2012) and show that the results obtained in our study about growth media improvement are already quite good. Another observation was recently made about the natural loss of *D. sansibarensis* symbiont. Plants coming from the second generation of bulbils are apo-symbiotic. This means that plants grown from bulbils which themselves already came from bulbils would be apo-symbiotic. Basic tests have been conducted recently, where bulbils coming from bulbils (called BB)

were grown in opposition to bulbils coming from tubercules (called BT) and were then tested to see if *O. dioscoreae* was present or not in the leaf glands. Three leaf glands per plant were tested as soon as three viable leaves were present. The first results showed that on 15 BB plants, 2 died, 3 still contained *O. dioscoreae* but not on all the tested leaf glands and 10 were apo-symbiotic or got colonised by other bacteria. For BT plants all 10 of them survived and were all containing *O. dioscoreae.* This confirms the observations and hypothesis that *D. sansibarensis* plants are losing their symbiont through generations of cultivation from bulbils. This hypothesis needs more research and experimentation. Is it the simple fact of cultivating the plants by bulbils only or are other factors also affecting the results? Could it be a problem of storage of the bulbils? Or a stress induced by climate conditions in the greenhouse? There are still many questions to answer but this is worth investigating as a way of obtaining symbiont free plants without the need for node cutting. If this hypothesis turns out to be true, this technic could save time and money, as well as solve the problem of contaminated plants.

4.3 Inventory of bacteria invading the apo-symbiotic plants' leaf glands

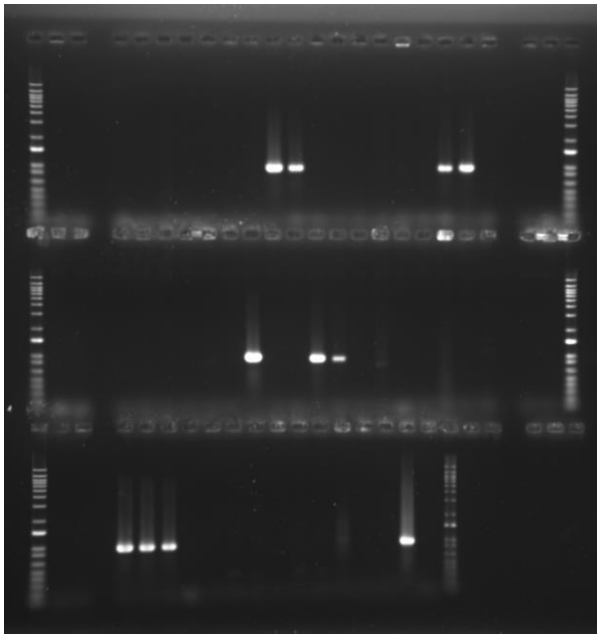
Two rounds of leaf glands bacteria isolation were done during this project, to have enough quality data and samples to work with. A complete list of results can be found in Annexes 1, 2 and 3. A very rough morphology description and scale of the growth medium spatial occupation of colonies is recorded. All this information was recorded for a tracking purpose.

In total 98 colonies were isolated. Many were similar morphologically. And some exhibited characteristic aspects which gave a first idea about what type of bacteria were present. For example, various species belonging to the genus *Pseudomonas* create a variety of colours, ranging from yellow green to blue, some of which spread into the medium (Palleroni and Doudoroff 1972), and are visible through UV light or with the naked eye, which can give a strong indication about the type of bacterium present on the plate.

To get a bigger range of potential colonisers it could be interesting to also cultivate plants in different environments. Running the same protocol in the greenhouse where diverse types of plants are grown together could bring a higher variety of bacteria and potential competitors to *O. dioscoreae*.

4.4 PCR results

For the first round of samples, *O. dioscoreae* has been identified here on well 10, 11, 18, 19, 31, 34, 35, 47, 48, 49, as shown in PICTURE 5. The white highlights in the picture correspond to O. dioscoreae DNA amplification. All samples identified as *O. dioscoreae* are removed and discarded as they are no interest in this study. For round 2 of sampling no *O. dioscoreae* had been identified.



PICTURE 5. PCR gel staining results for the 1st round of samples

4.5 Selected bacteria identification process

Out of all the isolated bacteria only 13 had been selected to be identified by PCR amplification of the 16S rRNA gene and Sanger sequencing at Eurofins, Germany. Sequences were then analysed using the EZ taxon web service and results of the identification are shown in Table 4.

TABLE 4. Selected Bacteria with their identification.

Name	Top-hit taxon	Top-hit strain	Similarity (%)	Top-hit taxonomy	Completeness (%)
M32BB1	CP027773_s	PMC12	100.00	Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;Variovorax	97.5
M32BA1	JTJJ_s	ND03	99.86	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales;Erwiniaceae;Pantoea	97.6
2231 full	Pseudomonas atacamensis	M7D1	99.79	Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;Pseudomonas	97.8
2160B1	Enterobacter soli	ATCC BAA-2102	99.71	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales;Enterobacteriaceae;Enterobacter	96.2
2160A1	CP019702_s	K599	100.00	Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Rhizobiaceae;Agrobacterium	96.3
2100B1.2	Stenotrophomonas indicatrix	WS40	100.00	Bacteria;Proteobacteria;Gammaproteobacteria;Lysobacterales;Lysobacteraceae;Stenotrophomonas	97.6
2100B1.1	Pseudomonas lactis	DSM 29167	100.00	Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;Pseudomonas	97.1
2100A1.2	Stenotrophomonas indicatrix	WS40	100.00	Bacteria;Proteobacteria;Gammaproteobacteria;Lysobacterales;Lysobacteraceae;Stenotrophomonas	97.5
2100A1.1	Pseudomonas putida	NBRC 14164	99.93	Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;Pseudomonas	97.7
2047A1	Pseudomonas citronellolis	NBRC 103043	100.00	Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;Pseudomonas	97.3
1914B2	Pseudomonas lactis	DSM 29167	100.00	Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;Pseudomonas	97.5
1914A1	Pseudomonas neuropathica	P155	99.92	Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;Pseudomonas	97.2
1914B1	Cupriavidus campinensis	WS2	99.71	Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Ralstonia_f;Cupriavidus	96.4
1814B1	Pseudomonas paralactis	DSM 29164	99.51	Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;Pseudomonas	72.7

These 13 bacteria were selected because they presented the best potential to outcompete *O. dioscoreae* as they could fully occupy the acumen. They were the bacteria that originally had created a bacterial mat or had a high coverage of the TSA plates. They are presented in annexes 1,2 and 3, in the column “Full”.

Identification was made by means of 16S ribosomal RNA, which is a hypervariable area found in gene sequences that can produce species-specific signature sequences useful for identifying bacteria (Pereira, *et al*. 2010). As presented by Mignard and Flandrois (2006) this identification technique is widely used, reliable and cost effective. The 16S rRNA technique is reliable because of the slow evolution rate of this region of the gene (Woese and Fox 1977).

Out of the 13 bacteria, the genome of the strain of *Pseudomonas putida* has been sequenced and will be the object of a genome announcement and will be added to the common database used worldwide by researchers. This strain is now used being used by the team to continue its research. The genome announcement paper is to be published during December 2022 or early 2023.

4.6 Environmental impact of research

Waste reduction is driven by the need to cut down on cost of equipment from the allocated budget. Experiments are carefully planned to avoid waste of consumable material. Research teams are encouraged to use reusable glassware when possible. Reducing the amount of plastic used in research is a challenging task due to many factors. It is often a gain of time, as sterilizing equipment in between each plant or task takes a lot of time. It is also cheaper from the point of view of the direction of the research centre, as the cost of taking care of the glassware is removed. It represents less personnel to clean, less space to store and therefore is overall cheaper than hiring extra personnel. Plastic consumables also have practical aspects: they are easy to handle and not fragile, some plastics used in research have specific properties (low DNA binding, DNA free, etc) that are essential for some experiments.

Wastes are managed as best as they can. Sorting waste is not always possible as contaminated equipment, liquids and solids need to be sterilized at elevated temperature in an autoclave before being sent to general waste collection.

It is certainly possible to reduce plastic waste by using more metal and glass ware, when possible, but it will cost an increase in time spent on a task and would require more personnel to handle washing and sterilization. Which does not go with the underfunding of the research sector for the past years.

4.7 Impact of unpredictable factors

Latest weather conditions, especially in summer in southern France, have had an impact on the plants growing in the greenhouses of the research centre. Higher temperatures are inducing stress in the plants. According to the Meteorological Institute of France website (Meteo France) normal maximum temperature between May 2022 and August 2022 were on average 3.5 degrees higher than usual. This can impact results of ongoing experiments, and especially in the

greenhouse. Some of the plants suffered from sunburn even with proper shading in place. *D. sansibarensis* is originally a tropical plant and needs a certain level of hygrometry and shade to be able to develop properly. A stress in the plant could be the cause of the loss of the symbiont that has been observed and mentioned previously. Higher temperature in the greenhouse also brings more insects and pathogens invasion that the culture staff has trouble to fight. A closer check of the culture conditions is needed and the lack of financing/funding in the public research area is making it hard to be as proficient as needed. According to a recent publication from the French Senat (2020), the funding of French public research has not changed since the 1990's and represents about 2.25% of the GDP.

The latest inflation and the war in Ukraine have affected deliveries of needed consumable goods, as well as replacement parts for machines. Research is relying heavily on disposable plastics and small equipment such as pipette tips, micro tubes, Petri dishes, etc. Higher prices and longer delivery times hinder the good development of a much-needed research.

5 CONCLUSION

This research aimed to identify potential colonisers of the leaf gland and create a bacteria bank which could be used throughout the next steps of the research project. At the beginning of this study, we did not know if the leaf gland could be colonised by other bacteria, because so far *O. dioscoreae* has been the only organism found in this niche.

While plantlet cultivation remains a challenge and still must be optimized, this study was successful in uncovering bacteria that can colonise *D. sansibarensis* leaf gland, providing that *O. dioscoreae* is not present in said leaf gland beforehand. This study results are particularly good and show that the leaf gland can not only host diverse kinds of bacteria but that some bacteria can fully colonise it, remain inside the leaf gland and survive. This study shows as well that the original symbiont and the found bacteria can be put in competition for the leaf gland niche, which will provide a better understanding of the defence mechanisms of *O. dioscoreae*.

Further research is needed to get a better success rate in the culture of plantlets. Some ideas have already been talked about and are currently being explored. Overall, the isolation process of bacteria was as efficient as it could be. However, more sampling could be done with apo-symbiotic plants grown in different environments, such as the general greenhouse or even plants grown in different laboratories. This could bring a broader variety of sampled bacteria and a wider choice, if needed, for later stages of the research project.

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APPENDICES

Appendix 1. Leaf gland bacteria isolation round 1, page 1.

Plante #	Bacteria sample #	Noth-ing	Few	Many	Uncountable, not covered	Full	Morphology
1978	B1		X				Small yellow dots
	B2				X		Yellowish small dots
	B3		X				White
	A1		X				Yellow/orange tiny dots
	A2				X		Yellowish small dots
	A3		X				White large
2003	B1		X				Yellow/orange tiny dots
	B2		X				Yellow dots
	B3		X				Egg shell white large
	A1		X				Egg shell white large
	A2		X				Yellow/orange small dots
1917	A1					X	Bacterial mat transparent
	B1					X	Egg shell small white dots
	B2					X	Yellow/orange tiny dots
2016	A1			X			Egg shell white large
	A2			X			Yellowish small dots
	B1		X				White dots
	B2				X		Egg shell white dots
	B3			X			Orange dots tiny
	B4		X				Yellow/orange big dots
1828	B1					X	Yellow bacterial mat
	B2				X		Egg shell small white dots
	A1					X	Yellow bacterial mat
1772	B1	X					
	A1	X					
1801	A1			X			Egg shell white dots
	A2		X				Yellowish small dots
	A3		X				Orange big dots
	A4		X				White big dots
	B1		X				White dots
1860	A1	X					
	B1	X					
2061	A1	X					
	B1	X					

Appendix 2. Leaf gland bacteria isolation round 1, page2.

Plante #	Bacteria sample #	Nothing	Few	Many	Uncountable, not covered	Full	Morphology
2072	A1		X				Egg shell white big, fluorescent yellow
	A2		X				Egg shell small
	A3		X				Yellow/orange tiny
	B1			X			Egg shell white big, fluorescent yellow
	B2		X				Egg shell small
	B3		X				Yellow/orange tiny
1914	A1					X	Bacterial mat yellow/orange
	B1					X	Egg shell white dots + Bacterial mat yellow/orange
	B2					X	Egg shell white dot + bacterial mat bacterial mat y/o
1814	A1				X		Egg shell small dots
	B1					X	Bacterial mat
1803	A1		X				Orange big dots
	A2		X				Yellow dots
	B1		X				Egg shell white big
	B2			X			Yellow small dots
	B3		X				Egg shell white dot
2007	A1	X					
	B1				X		Orange tiny dots
	B2				X		White tiny dots
1965	A1		X				Egg shell white dots
	A2		X				Yellow dots
	B1	X					
1734	A1		X				Yellow dot
	B1		X				Yellow dots
1823	A1		X				Egg shell big white dot
	A2		X				White dot
	B1		X				Yellow dots
	B2		X				White dot
	B3		X				Orange dots
1824	A1		X				Yellow dots
	B1		X				Yellow dots
	B2		X				White dot

Appendix 3. Leaf gland bacteria isolation round 2

Plante #	Bacteria sample #	Few	Many	Uncountable, not covered	Full	Morphology
2047	A1			X		Small eggshell white dot
	A2	>50				Tiny white dot
	A3	3				Small yellowish dot
	B1				X	Bacterial mat transparent/white
	B2	2				Tiny orange dot
2133	A1				X	Bacterial mat eggshell white/transparent
	B1				X	Bacterial mat eggshell white/transparent
1939	A1	1				Medium size eggshell white dot
	B1	6				Small orange dot
2022	A1					Small yellowish dot
	B1	>50				tiny white dot
	B2	~40				small eggshell white dot
	B3		<100			small yellowish dot
2160	A1				X	Bacterial mat eggshell white/transparent
	A2			X		Tiny white dot
	B1				X	Bacterial mat eggshell white/transparent
2158	A1			X		Tiny white dot
2130	A1	6				Big eggshell white spot
	A2			X		Small yellowish dot
	B1	~50				Big eggshell white spot
	B2	~20				Small eggshell white dot
	B3		>100			Small orange/yellow dot
M32B	A1				X	Bacterial mat eggshell white/transparent
	B1				X	Bacterial mat eggshell white/transparent
	B2			X		Small white dot
2011	A1			X		Tiny transparent/white dot
	A2			X		Small yellowish dot
	A3	1				Medium size orange dot
	A4	1				Small white dot
2100	A1				X	Bacterial mat yellow
	B1				X	Bacterial mat yellow

Appendix 4. PCR number list from the 1st round of samples.

PCR #	Bacteria #
1	2007-B2
2	1965-A1

PCR #	Bacteria #
21	1828-A1
22	1801-A2

PCR #	Bacteria #
41	1978-A3
42	2016-A2

3	1965-A2
4	1734-A1
5	1734-B1
6	1803-B1
7	1803-B2
8	1803-B3
9	1823-A1
10	2072-A1
11	2072-B1
12	1917-B1
13	2072-A3
14	2007-B1
15	2016-B4
16	2072-B3
17	2072-B2
18	1914-A1
19	1914-B1
20	1978-A2

23	1828-B2
24	1801-A1
25	1801-A4
26	1801-B1
27	1824-B2
28	1824-A1
29	1823-A2
30	2016-B1
31	2072-A2
32	2003-A2
33	1978-B2
34	2003-B3
35	2003-A1
36	1823-B3
37	1978-B3
38	1823-B1
39	1823-B2
40	1801-A3

43	1828-B1
44	1978-B1
45	1978-A1
46	1917-B2
47	1914-B2
48	1814-A1
49	1814-B1
50	1803-A1
51	1803-A2
52	2016-B2
53	2016-B3
54	1824-B1
55	1917-A1
56	2016-A1
57	2003-B1
58	2003-B2
59	MilliQ, negative test
60	Genomic DNA, positive test
61	Symbiont free plant DNA, negative test