PATRÍCIA CARDOSO

Avaliação metabolômica entre interações da rizosfera de *Senna spectabilis* e micro-organismos associados.

Tese apresentada ao Instituto de Química, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutora em Química

Orientador: Prof. Dr. Ian Castro-Gamboa

ARARAQUARA – S.P.
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Metabolomic evaluation of interactions in rhizosphere between *Senna spectabilis* and associated microorganisms.

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Supervisor: Prof. Dr. Ian Castro-Gamboa

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3. Special Topics: Mass spectrometry (3 credits), grade A. Lecture: Wagner Vilegas, Lourdes Campana.
4. Special Topics: Medicinal Plants: an advanced view (3 credits), grade A. Lecture: Dr. Leslie Gunatilaka (Arizona State University).
5. Theory and method of separation, isolation and purification of organic compounds (12 credits), grade: B. Lecturer: Prof. Alberto José Cavalheiro.
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Date: 21st June 2013

Duration: 40 minutes

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1. Scientific initiation with scholarship from PIBIC-CNPq: "Verificação de capacidade produtora de ácido clavulânico de isolados de *Streptomyces clavuligerus*", under the supervision of Prof. Dr. Carlos Ossamu Hokka (Federal University of São Carlos-UFSCAR) and Prof. Dr. Maria Lucia Gonsales da Costa Araújo (Department of Biochemistry, Chemistry Institute, São Paulo Sate University - UNESP). From 1st August 2006 to 30th August 2007.
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Professional Experience

1. Teacher at EE Angelina Lia Rolfsen, Government Employee. From 18th February to 30 th August 2010.

Published papers and/or submitted to indexed journals

1. CASTRO-GAMBOA, I.; BURGOS, R.; CARDOSO, P.; CARNEVALE, F.; PILON, A.; LOPES, M. N.; SILVA, D. and BOLZANI, V. S. Dereplication of Brazilian Plants from Cerrado and Atlantic Forest NMR using virtual design and hyphenated techniques. **Planta Medica**, v. 76, DOI: 10.1055/s-0030-1264282, p. 127-128, 2010.

2. OLIVEIRA, O. M. F.; Cruz, A. F.; Miura, C. E.; Lopes, L.; Schiavetto, M.; Santos, M. A.; Cardoso, P.; Pasqualotto, S.; Rocha, Z. M. and MARQUES, R. N. O PROGRAMA CIÊNCIA VAI À ESCOLA: ENSINO DE CIÊNCIAS DE 7A. E 8A. SÉRIES. **Revista Ciência em Extensão**, v. 1, p. 135, 2004.

Abstracts of the papers presented at conference proceedings

1. CARDOSO, P.; PAVANI, V. D.; PEREIRA, D. R.; SACRAMENTO, L. V. S. and CASTRO-GAMBOA, I. Detection of metabolites present in the rhizosphere isolated fungus-growing *Senna spectabilis* seedlings using LC/MS. In: 36th Annual Meeting of the Brazilian Chemical Society, Águas de Lindoia, SP, 2013.

2. CARDOSO, P.; FREIRE, R. T.; CARNEVALE, F.; PILON, A.; SACRAMENTO, L. V. S. and CASTRO-GAMBOA, I. Study of the microorganisms associated to the rhizosphere of *Senna spectabilis* using hyphenated techniques. In: Workshop on Holistic Analytical Technologies for Biomedical Food and Plant Sciences, Athens, Greece, 2012.

3. CARDOSO, P.; SACRAMENTO, L. V. S, CASTRO-GAMBOA, I. Microorganisms associated with the rhizosphere of seedlings *Senna spectabilis*: A metabolomics approach applied to hydroponic systems. In: 35th Annual Meeting of the Brazilian Chemical Society, Águas de Lindoia, SP, 2012.

4. CARDOSO, P.; SACRAMENTO, L. V. S. and CASTRO-GAMBOA, I. Exploring the rhizosphere of the *Senna spectabilis*: Isolation of microorganisms with potential biological properties. In: 3rd BCNP Brazilian Conference on Natural Products and XXX RESEM, Ouro Preto, MG, 2011.

5. CARDOSO, P. and CASTRO-GAMBOA, I. Metabolomic analysis of rhizosphere constituints from *Senna spectabilis* (Fabaceae) and associated microorganisms. In: São Paulo School on Natural Products, Medicinal Chemistry and Organic Synthesis- integrated solution for tomorrow's world-ESPCA Chemistry, Campinas, SP, 2011.

6. CARDOSO, P.; BURGOS, R. C. R.; ALECIO, A. C.; OLIVEIRA, J. E.; BOLZANI, V.S.; ARAUJO, A. R.; CASTRO-GAMBOA, I. Detecção dos constituintes lipofícos majoritários de *Colletotrichum gloeosporioides* (Penz.) Sacc. fungo endofítico isolado de *Garcinia xanthochymus*. In: 32ª Reunião Anual da Sociedade Brasileira de Química, Fortaleza-CE. 2009.

7. CARDOSO, P.; LOPES, M.N.; ARAUJO, A. R.; SILVA, D.H.S.; BOLZANI, V.S. and CASTRO-GAMBOA, I. Caracterization of the main micromolecular constituints from *Colletotrichum gloeosporioides*, an endophyte fungi isolated from *Garcinia xanthochymus* (Clusiaceae). In: 2nd Brazilian Conference on Natural Products (2nd BCNP) and XXVIII Annual Meeting on Micromolecular Evolution, Systematics and Ecology (XXVIII RESEM), 2009, São Pedro. 2nd BCNP and XXVIII RESEM, 2009.

8. FREITAS, T. L.; CARDOSO, P.; ARAUJO, M. L. G. C. and HOKKA, C. O. Obtenção de mutantes de *Streptomyces clavuligerus* ATCC 27064 através de mutagênese classica para a otimização da produção de ácido clavulânico. In: 2 Workshop de Biologia Molecular e Identificação de Biomoléculas, 2007, São Carlos-SP. 2 Workshop de Biologia Molecular e Identificação de Biomoléculas, 2007.

9. FREITAS, T. L.; HERNANDES, I. L. C. SALVATO, F.; CARDOSO, P.; ARAUJO, M. L. G. C. and HOKKA, C. O. Produção de ácido clavulânico por *Streptomyces clavuligerus* submetido a tratamento mutagênico por metil metano sulfonato. In: Sinaferm - XVI Simpósio Nacional de Bioprocessos, 2007, Curutiba, PR. XVI Simpósio Nacional de Bioprocessos, 2007.

10. CARDOSO, P.; HOKKA, C. O.; ARAUJO, M. L. G. C. and FREITAS, T. L. Manutenção da capacidade produtiva de *Streptomyces clavuligerus*. In: 7ª Jornada Cientifica da UFSCAR, 2007, São Carlos. 7ª Jornada Cientifica da UFSCAR, 2007.

11. MILARE, T.; MOURA, A.; GIROTTO JUNIOR, G.; DA SILVA, C. S.; SCHIAVETTO, M. G.; CARDOSO, P.; ROCHA, Z. M.; LUIZ ANTONIO ANDRADE DE OLIVEIRA, L. A. A.; OLIVEIRA, O. M. M. F. and MARQUES, R. N. Educação Ambiental e Artística: Uma Parceria que deu certo. In: V Encontro de Investigações na Escola, 2004, Lajeado. Anais do V Encontro de Ivenstigações na Escola. Lajeado : Editora Unicates, 2004. p. 1-2.

12. MARQUES, R. N.; MOURA, A.; DA SILVA, C. S.; GIROTTO JUNIOR, G.; S.; SCHIAVETTO, M. G.; CARDOSO, P.; ROCHA, Z. M.; LUIZ ANTONIO ANDRADE DE OLIVEIRA, L. A. A. and OLIVEIRA, O. M. M. Sistema Solar, uma abordagem interativa para

o Ensino Fundamental. In: 7 ENAST, Encontro Nacional de Astronomia, 2004, Brotas. 7 ENAST, Encontro Nacional de Astronomia, 2004.

13. MARQUES, R. N.; MILARE, T.; MOURA, A.; GIROTTO JUNIOR, G.; DA SILVA, C. S.; SCHIAVETTO, M. G.; CARDOSO, P.; ROCHA, Z. M.; LUIZ ANTONIO ANDRADE DE OLIVEIRA, L. A. A.; OLIVEIRA, O. M. M. F. Educação Ambiental e Artística: Uma Parceria que deu certo. In: V Encontro de Investigações na Escola, 2004, Lajeado. Anais do V Encontro de Investigações na Escola, 2004.

14. CARDOSO, P.; MOURA, A.C.S.; BONI, T. C.; CAPANA, A. S.; SCHIAVETTO, M. G. S.; MEDEIROS, J. B.; OLIVEIRA, L. A. A.; MARQUES, R. N.; OLIVEIRA, O. M. M. Experimentação: uma ferramenta no ensino de ligações químicas. In: XII ENEQ- Encontro Nacional de Química, 2004, Goiânia -GO. XII ENEQ- Encontro Nacional de Química "As novas políticas educacionais e seus impactos no ensino de química", 2004.

15. MOURA, A.C.S.; SILVA, C. S.; MILARE, T.; CARDOSO, P.; SCHIAVETTO, M. G. S.; GIROTTO JUNIOR, G.; ROCHA, Z. M.; OLIVEIRA, L. A. A.; MARQUES, R. N. and OLIVEIRA, O. M. M. A reciclagem de papel como ferramenta para o ensino de química e formação de cidadania. In: XII ENEQ- Encontro Nacional de Química, 2004, Goiânia -GO. XII ENEQ- Encontro Nacional de Química, 2004.

16. MILARE, T.; MOURA, A.; C. S; CAPANA, A. S.; MEDEIROS, J. B.; CARDOSO, P.; MARQUES, R. N.; OLIVEIRA, L. A. A. and OLIVEIRA, O. M. M. A química no Ensino Fundamental como porposta do eixo temático "Tecnologia e Sociedade" dos Parâmetros Curriculares Nacionais. In: XII ENEQ- Encontro Nacional de Química, 2004, Goiânia -GO. XII ENEQ- Encontro Nacional de Química, 2004.

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2. Biota 8th Symposium, 8th Biota Program Assessment Meeting and 4th Bioproprospecta Program Assessment Meeting, 2014, São Pedro, SP.
3. São Paulo Advanced School on Bioorganic chemistry - ESPCA, 2013, Araraquara, Brazil.
4. 36th Annual Meeting of the Brazilian Chemical Society, 2013, Águas de Lindóia, SP.
5. Workshop on Holistic Analytical Technologies for Biomedical Food and Plant Sciences, 2012, Athens, Greece.

6. Biota 7th Symposium, 7th Biota Program Assessment Meeting and 4th Bioproprospecta Program Assessment Meeting, 2011, São Carlos, SP.
7. 35th Annual Meeting of the Brazilian Chemical Society, 2012, Águas de Lindoia, SP.
8. BCNP 3rd Brazilian Conference on Natural Products, 2011, Ouro Preto, MG.
9. ESPCA -São Paulo Advanced School, 2011, Campinas, SP.
10. 32ª Annual Meeting of the Brazilian Chemical Society. 2009.
11. 2nd Brazilian Conference on Natural Products (2nd BCNP) and XXVIII Annual Meeting on Micromolecular Evolution, Systematics and Ecology (XXVIII RESEM). 2009.
12. IV Workshop NuBBE. 2009.
13. 17th Encontro da SBQ -Regional Interior Paulista. 2009.
14. III Worshop do NuBBE, Araraquara. SP. 2008.
15. VI Symposium and VI Biota Program Assessment Meeting BIOTA/Fapesp, 2008.
16. XV CIC Ufscar. 2007.
17. XXXVII Semana da Quimica 2007.
18. 2º Worshop de Biologia Molecular e Identificação de Biomoléculas. 2007.
19. XXXVI Semana da Quimica. 2006.
20. Comemoração do dia do Químico. 2006.
21. III Evento de Educação em Química. 2005.
22. XXXV Semana da Química. 2005.
23. XII ENEQ- Encontro Nacional de Química, Goiânia –GO, 2004.
24. II Evento de Educação em Química- EVEQ, 2004.
25. XXXIII Semana da Química. 2003.

Scientific Events Organizations

1. Organizing Committee of the São Paulo Advanced School on Bioorganic Chemistry - ESPCA, Araraquara, SP. 2013.
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5. Organizing Committee of the "VI Simpósio & Reunião de Avaliação do Programa BIOTA/FAPESP e III Reunião do BIO*prospec*TA". 2008.
6. Organizing Committee of the XXXVII Semana da Química, Araraquara. 2007.
7. Organizing Committee of the XXXVI Semana da Química, Araraquara. 2006.

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Araraquara, 22 de maio de 2015.

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Àqueles que apesar de todas as adversidades sempre acreditaram que eu seria capaz de atingir meus objetivos.

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[…] The only true voyage of discovery, the only fountain of Eternal Youth, would be no to visit strange lands but to possess other eyes, to behold the universe through the eyes of another, of a hundred others, to behold the hundred universes that each of them beholds, that each of them is […] (Marcel Proust, 2006, p. 657)*.

*PROUST, M. The Verdurins and M. de Charlus. In:_____. Remembrances of things past. 2006. v. 2, p. 657.

RESUMO

Senna spectabilis é uma planta medicinal detentora de inumeras aplicações terapêuticas tradicionais. Como uma fonte rica de metabólitos com atividades biológicas a rizosfera de *S. spectabilis* foi o objeto de estudo neste trabalho. O conhecimento da população da microbiota da rizosfera contribuiu para a compreensão das interações que poderiam ocorrer nessa região dinâmica e rica do solo. Um número de métodos moleculares têm sido desenvolvidos nos últimos anos para o estudo da comunidade microbiana utilizando PCR no sequenciamento das regiões de rRNA 16S e ITS. Os objetivos deste estudo foram identificar a população microbiana da rizosfera de mudas de *S. spectabilis* e em sequência selecionar algumas linhagens e submetê-las a cultivos mistos em meio sólido. A identificação foi conseguida através do 454-pyrosequenciamento das raízes e sequenciamento por iluminaseq das soluções nutritivas em que as plantas foram cultivadas. Os resultados mostraram uma população rica e variada de bactérias e fungos, com destaque aos filos *Verrucomicrobia*, *Proteobacterias*, *Firmicutes* para as bactérias e para os fungos os filos *Basidiomycota* e *Ascomycota*. Espécies selecionadas de fungos foram cultivados em meio Czapek-dox líquido e uma análise do perfil metabólico foi conduzido utilizando HPLC-DAD e HPLC-DAD/MS. Entre os metabólitos detectados nessa pré-avaliação estão os derivados policetidicos produzidos por *Fusarium*: zearalenonas, conhecidas micotoxinas produzidas por uma grande quantidade de espécies deste gênero. Estes resultados induziram a seleção de cinco fungos de *Fusarium*, *Paecilomyces* e uma espécie de bactéria, *Burkholderia* sp para o cultivo misto em meio de agar sólido. O objetivo foi detectar mudanças na produção metabólica e analisar as alterações esperadas por RMN de ^{1}H associados aos métodos quimiométricos de análise PCA e PLS e tambem por LC/MS. Uma série de eniatinas pode ser detectada por LC/MS, indicando que a co-cultura induziu alterações nos metabólitos. As informações decorrentes desta pesquisa contribuirão para uma melhor compreensão da comunidade microbiana de *S. spectabilis* e ajudarão na compreensão das interações que ocorrem na rizosfera desta importante planta medicinal.

Palavras-chave: *Senna spectabilis*, rizosfera, micro-organismos, co-cultivo, desreplicação, analise multivariada, RMN de ^{1}H, LC/MS.

ABSTRACT

Senna spectabilis is a medicinal plant with innumeral traditional therapeutic applications. As a source of rich metabolites with biological activities *S. spectabilis'* rhizosphere was the object of study in this work. The knowledge of the population of the microbiota of the rhizosphere contributes to the understanding of the interactions that may occur in this dynamic and rich region of the soil. A number of molecular methods have been developed in recent years to study the microbial community using PCR associated in the sequencing of 16S and ITS regions of rRNA. The objectives of this study were to identify the microbial population of rhizosphere seedlings *of S. spectabilis* and in sequence to select a few strains and submit them to co-cultures in a solid medium. Identification was achieved with 454-pyrosequencing of roots and iluminaseq sequencing of the hydroponic solutions, in which the seedlings were cultivated. Results showed a rich and varied population of bacteria and fungi notably the bacteria phyla *Verrucromiobia*, *Proteobacteria*, *Firmicutes* and for the fungi the phyla *Basidiomycota* and *Ascomycota*. Selected species of fungi were cultivated in liquid Czapek-dox medium and a screening of the metabolic profile was conducted using HPLC-DAD and HPLC-DAD/MS. Among the metabolites detected in this previous evaluation are the polyketides derivates produced by *Fusarium*: zearalenones known as mycotoxins produced by many species of this genus. These results have induced to the selection of five fungi from *Fusarium*, *Paecilomyces* and one specie of bacteria, *Burkholderia sp* for mix cultivation in agar solid medium. The aim was to detect changes in the metabolic production and analyse the expected alterations by ^{1}H NMR associated with chemometric method PCA and PLS and LC/MS. A series of enniatin could be detected by LC/MS indicating that co-culturing induced the modifications in the metabolites. Information arising from this research will contribute to an enhanced understanding of microbial community in *S. spectabilis* and assist in the understanding of the interactions that occur in the rhizosphere of this important medicinal plant.

Keywords: *Senna spectabilis*, rhizosphere, microorganisms, co-culture, dereplication, multiway analysis, ^{1}H NMR, LC/MS.

LIST OF ABREVIATIONS

ACN	Acetonitrile
AFLP	Amplified fragmente length polymorphism
AM	Arbuscular micorrhizae
ATP	Adenosintriphosphate
Blast	Basic local alignment search tool
C	Carbon
C-18	Octadecyl carbon chain bonded silica column
DAD	Diode Array Detector
DCM	Diclorometh ane
DNP	Dictionay of Natural Compounds
ESI	Electrospray Ionization
EtOAc	Ethyl Acetate
ETS	External transcribe spacer
FID	Free Induction Decay
GC-MS	Gas Chromatography- mass spectrometry
HPLC	High Performance Liquid Chromatography
^{1}H NMR	Hydrogen Nuclear Magnetic Resonance
LC-DAD/MS	Liquid Chromatography -Diod Array Detector- mass spectrometry
LC-DAD/MS-MS	Liquid Chromatography -Diod Array Detector- tandem mass spectrometry
LC-SPE-NMR	Liquid Chromatography – Solid phase extraction- Nuclear magnetic resonance
LV	Latent variable
MALDI	Matrix-assisted Laser desorption/ionization
MeOH	Methanol
MeOD	Deutered Methanol
MS	Mass spectrometry
m/z	Mass-to-charge ratio
N	Nitrogen
NCBI	National Center for Biotechnology Information
NMR	Nuclear Magnetic Resonance
OTU	Operational Taxonomic Units

PC	Principal Components
PCA	Principal Components Analysis
PCR	Polymerase Chain Reaction
PFGE	Pulse field gel electroforesis
PLS	Partial Least Squares
PLS-DA	Partial Least Squares – Decomposition Analysis
PPi	Pyrophosphate
ppm	Parts per milion
RDP	Ribosomal Database Project
RFLP	Restriction fragment length polymorphism
rRNA	Ribosomal ribonucleic acid
RT	Retention Time
SPE	Solid Phase Extraction
TIC	Total Ion Chromatogram
TMSP	Trimethylsylyl propionic acid sodium salt
UV	Ultraviolet
WHO	World Health Organization

LIST OF SYMBOLS

α	alpha
β	beta
γ	gama
δ	delta
δ	chemical shift
ε	epsilon
L	liters
λ	Wave lenght
μ	Micro
s	seconds
nm	Nanometer

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PREFACE

The search for new sources of natural products has been responsible for the increased use of analytical tools that enable a quick and efficient analysis of these complex matrices in current bioprospecting programs. Thus, methodologies for dereplication associated with the spectroscopic state of the art and science of various *in vitro* and *in vivo* bioassays have been critical in the choice of biologically promising extracts. Also in this context, the exploitation of new sources of metabolites has grown considerably, such as the rhizosphere. In this region of high biological activity, we can find different organisms sharing the same environment. These interactions induce the biosynthesis of active compounds that are beneficial or not among themselves. Studies of how these interactions occur are increasing, and showing the importance of evaluating the biological context in which microorganisms are generally involved is an accomplishment this work intends to provide. The strategy that has been used to mimic the interactions in which organisms are subjected is called “mixed-culture”. This thesis aims to understand the molecular relationships in mixed cultures of microorganisms isolated from the rhizosphere of *Senna spectabilis* generating a rational approach in the study of the interactions existing in the rhizospheric region. The NuBBE, Nucley of Bioassays, Biosynthesis and Ecophysiology of natural products, has incorporated in its bioprospecting projects, experimental strategies and agile and current methodologies for selecting belonging to its extratoteca extracts. Recently, the use of high performance liquid chromatography (HPLC) coupled with high spectral resolution mass spectra (HRMS) allows the metabolic analysis of plants and extracts of endophytic fungi accelerating the selection of promising fractions, aiding the composition of micromolecular studies prior to separation and purification of the best known bioactive chemotypes and detecting those with novel molecular characteristics. Spectroscopic techniques forefront in nuclear magnetic resonance (NMR), aimed at dynamic studies in complex matrices, are emerging as promising approaches coupled to the HPLC due mainly to its robustness, high sensitivity and unique spectroscopic versatility of NMR.

Chapter 1 provides a view of the microbiota associated to *Senna spectabilis* rhizosphere and focused on individual, cultivable bacterial and fungi species, and their integrated community associated with the seedlings rhyzosphere. The concept of metagenomics was apllied in this study. This approach provides an advance to the

field of metagenomics or genomic micro-community (refers to microbiota), specific genomic of a community of organisms that inhabit a particular ecosystem. The result is a metagenome, where individual genes are not necessarily belonging to individual organisms, but belong to the ecosystem of the community in question. Metagenomics can provide information on the phylogeny of organisms in a particular ecosystem, heterogeneity and evolution. Thus, this approach is valuable, on the above picture of the rational exploitation of the rhizosphere. Current techniques are applied in independent culture based on Polymerase Chain Reaction (PCR) and aim high yield, reproducibility of results and cost-effectiveness associated with molecular techniques. The application of these techniques provided valuable data especially for the microrganisms that are unable to grow in culture, this culture technique has as main point setting direct cloning of DNA.

In Chapter 2, randomly pre selected microorganisms species of fungi were cultivated in liquid medium and their crude extracts were evaluated by HPLC-DAD. The first objective was to select among those the ones to be used in co-culture experiments. Among those pre selected, five microorganisms from *Fusarium* species, *Paecilomyces* and *Burkholderia* were cultivated first in pairs and then all together at the same Agar plate. The aim was to detect changes in the metabolic profile of co-culturing experiments compared with the pure strains. The pack of experiments was investigated using ^{1}H NMR one-dimensional and the chemometric methods Principal components analysis (PCA) and Partial least Squares (PLS).

OBJECTIVES

- Cultivate seedlings of *Senna spectabilis* in sand and in hydroponic cultivation;
- Identify the microbiota associated to rhizosphere of the plants using classic methods of taxonomic classification and molecular methods of pyro and sequencing of the 16S and ITS regions of rRNA for the identification of bacteria and fungi;
- Select microorganisms through a metabolic evaluation using HPLC-DAD and HPLC-DAD/MS;
- Co-cultures experiments;
- Identify and discuss the possible modifications on metabolic profile that may occur using mono-dimensional ^{1}H NMR and the chemometric tools of Principal components Analysis (PCA) and Partial Least Squares (PLS).

CHAPTER 1:

Senna spectabilis: Seedlings cultivation, isolation and identification of microbiota from rhizosphere

1 INTRODUCTION

1.1 Rhizosphere: an overview

Plants are colonized by a large number of (micro) organisms that influence their whole functioning as seed germination, plant growth and development, physiology (nutrition, gas exchange of O_2 and CO_2, soil units gradients mineralization, ammonification, nitrification)[1-2], diseases, and productivity. In return, plants release their photosyntetical carbons that act as messengers and food source directly on the surrounding areas of the roots, allowing and interfering in the colonization by different organisms. These plant/environment interaction areas are denominated as: spermophere – interaction space between seeds and its communities; phyllosphere – microbial community interaction with leaves; rhizosphere - the surrounding area close to the roots which interacts with micro (organisms) and mycorrhyzosphere – roots plant and fungi.[3]

Of the entire cross talking that occurs in the plant, the rizhosphere is the most dynamic and complex interaction than in any other part of the plant[3]. Rhizosphere is the region of the soil immediately influenced by roots where exudates are leaked by the living roots to stimulate the microbial communit[3]. The area that can be influenced by the exudates or the so called rhizosphere effect extends from 1mm to no longer than a few centimeters. Some factors that influence this spacial scale include the architecture and density of the roots, the photoperiod and intensity of the light, depth, texture and moisture of the soil, just to name a few. In the rhizosphere the population of organisms that will interact with the plant is driven by the roots exudates leaking in the sorrounding area. The nature of these interactions involve not only communication with roots and soil microbes but it also works as a traffic signal between roots of competing plants and it is a one-way signal to the roots in the soil properties.[3-4]

How these processes are done is poorly understood. What is known is that root exudates play an active crucial role in the regulation of symbiotic interactions with microbes.[5-6] They also help the plant defense against pathogenic microorganisms, attract specific microbes that will help the plant with pathogens and competitor plants, keep the roots moist, obtain nutrients and change the chemical properties of the soil around roots. In other words, this variety of metabolites suggest that roots can

regulate the composition of soil and change its neighbours in its own benefit, especially in case to protect themselves from other plants nearby.[7]

Roots exudates are composed by sugars and aminoacids that work as C and N sources for fungi and bacteria; organic acids, carbohydrates, vitamins, mucilage, proteins, which make the microbial activity in rhizosphere far higher than in any other part of the soil far from the roots.[2;8] Some exudates seem to be species-specific and some of these compounds can move away fom their original sources. Others such as phytotoxins are responsible for the detritment of neighboring plants, and whilst some organic compounds reduce these negative effects in a few plants, many compounds have antimicrobial effect. The composition of exudates changes according to the development stage of the plant and the conditions of the environment surrounding. For example, roots release compounds at night, what allows the expansion of the root in the soil but when the transpiration process starts during the daylight, those exudates dry out and adhere to the roots, once the soil gets dry these exudates lose water to soil keeping the humidity necessary to plant.[9]

The rhizosphere is a center of intense activity provided by roots and it works as food supply to microbes that are atracted to this rich source of nutrition. Bacteria, actinomycetes, fungi, protozoa, algae, nematodes, enchytraeids worms, earthworms, millipedes, centipedes, insects, mites, snails, small animals and soil viroses[2;8] are a few examples of the organisms associated to the plant due to the rhizospheric effect. Of course this population is also affected by the pH and the soil chemistry.

The function of microbial interactions specially the ones that happen in the rhizosphere is to allow microorganisms to survive through the establishment of a microbial symbiosis between neighbors and diverse environments.[9] A microorganism can respond to environmental stimuli using metabolic changes, including small molecules and proteins. These microbial interactions not only influence the survival of microbes but also have an important role in morphological and developmental processes of organisms themselves and their neighbors.[10]

1.2 The plant specie: *Senna spectabilis*

Senna genus belongs to the *Fabaceae* Family in the subfamily *Caesalpinioideae*, is one of the largest genera with about 600 species distributed throughout the world.

The plants of the genus *Senna* along with *Cassia* genus are an important source of substances with varied chemical structures in the *Fabaceae* Family. Besides, in folk medicine, the properties of plants belonging these two genus are used in treatments as laxatives, purgatives, anti-inflamatory, antimicrobial, analgesic, antiparasitic, insecticidal, antitumor and hepatoprotective.[11]

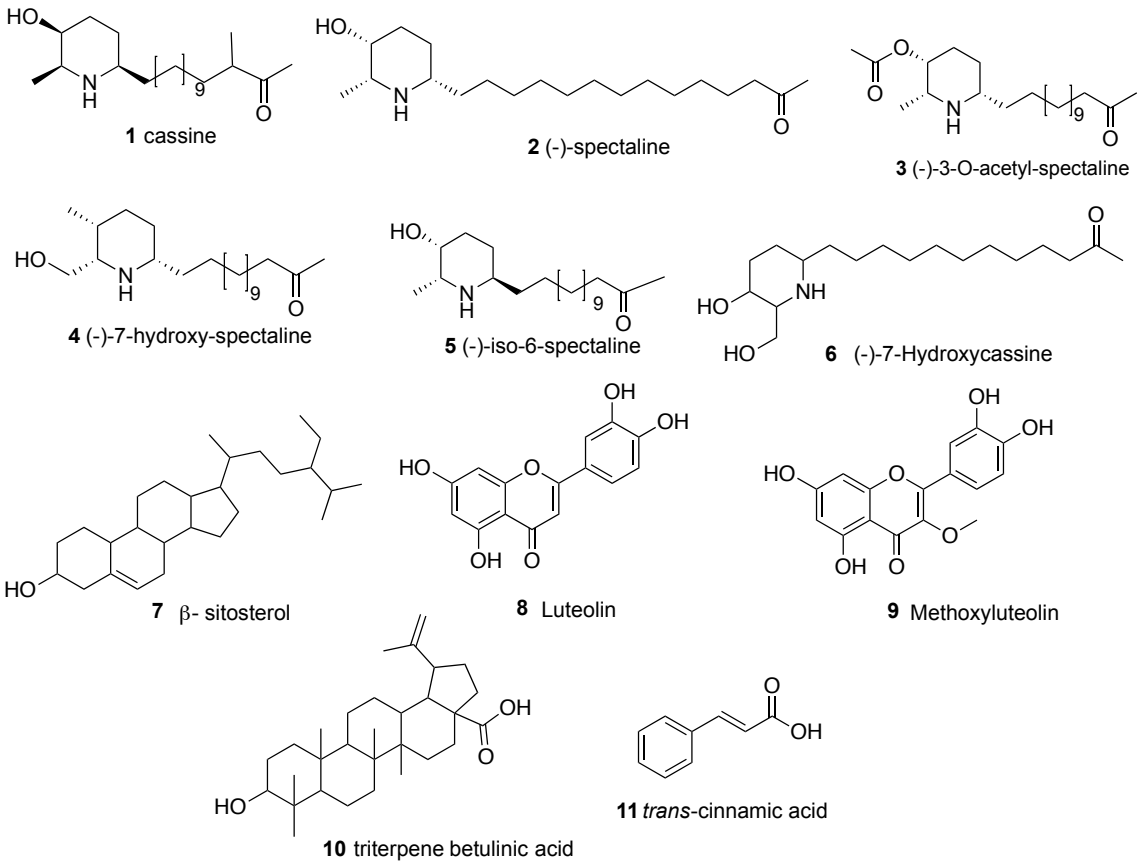
Senna spectabilis is a deciduous tree species, heliophytic pioneer; with a height of 6 to 9 m. It is a plant native to South America and can be found in Brazil especially in the northeast but also in the states of São Paulo and Minas Gerais. It is popularly known as São João, Cassia-do-nordeste, canafístula-de-besouro and pau-de-ovelha, having as a botanical synonym *Cassia spectabilis*.[10] It has dark green leaves, alternate, paripinnate and yellow and large flowers. The fruits are composed of a long pod, 25-32 cm, indehiscent with an irregular and wrinkled surface, a black color and a sweet smell, containing lots of small, flattened and brown seeds.[12-13]

In Brazil, *Senna spectabilis* specimens are employed mainly in urbanization due of the great beauty of its yellow flowers and its great potential for recovering of degraded areas. *S. spectabilis* occurs in two distinct varieties mainly by size: *Senna spectabilis* (DC) Irwin & Barneby var. spectabilis (*Cassia spectabilis* DC.), large tree - up to 17 m and *Senna spectabilis* (DC) Irwin & Barneby var. *excelsa* (Schrad.) Irwin & Barneby (*Cassia excelsa* Schrad), small tree-up to 6 m.[13]

Concerning the metabolic production, literature reviews show that 350 secondary metabolites were isolated over from different classes of substances over 30 plant species, and beyond that, the chemical study, ethnopharmacological and pharmacological properties have been validated for various activities[14-16]: Sedative and anticonvulsant, antimalarial and antimicrobial, cytotoxicity[14], central nerval system from studies of various organs of *S. spectabilis*[16 apud 11]. Among those compounds that exhibit biological acitivities are piperidinic alkaloids (–)-cassine (**1**), (–)-spectaline (**2**), (–)-3-O-acetylspectaline (**3**), (–)-7-hydroxyspectaline (**4**) and (–)-iso-6-spectaline (**5**), which had selective cytotoxic activity in strains of *S. cerevisiae* with disabilities in DNA repair and is an indicative of antitumor activity potencial[14]. Viegas Junior et al.[17] reported in flowers and green fruits of *S. spectabilis* the isolation of 2,6-dialkylpiperidin-3-ol alkaloid, named (–)-7-hydroxycassine (**6**), along with five known piperidine alkaloids (cited above) and non alkaloids: steroid β-sitosterol (**7**), the flavonoids luteolin (**8**) and 3-methoxyluteolin (**9**), the triterpene betulinic acid (**10**) and trans-cinnamic acid (**11**) (figure 1).

Recent studies have shown that piperidinic alkaloids are inhibitors of the acetylcholinesterase enzyme which have potencial aplications in the Alzeimer's desease.[17] Pivatto et al.[18] demostrated the presence of alkaloids cassine and spectaline on the ethanolic extracts of flower of *S. spectabilis*. However there is no mention on the literature about piperidinic alkaloids or their pharmacological properties on the roots or rhizosphere microorganisms of *S. spectabilis*. One endophytic fungus, *Phomopsis cassiae* isolated from the leaves of *S. spectabilis* have shown antifungal activity and are a promissing target on the development of anti-inflamatory agents.[18-20]

Figure 1: Secondary metabolites found in *Senna spectabilis*.



1.3 Hydroponic cultivation

Hydroponics is a cultivation technique alternatively protected in which the soil is replaced by an aqueous solution containing only mineral elements essential to the plant in the absence or presence of natural substrates or artificials.[21-22] The soilless culture provides better plant growth, plant health, and high yields when compared to the traditional system. This is due to the lack of root disease, once the material is free

of pathogens, and combined with better control of plant nutrition, through the use of appropriate nutrient solution to each species. The solution allows you to keep near the roots the desired concentration of nutrients, proceeding to adjustments, if necessary. The nutrient solution is the means by which nutrients previously dissolved in water are made available to the plants and is considered one of the most important parts of the hydroponic system[22-23]. Much of the existing formulations are the modification of the original solution of Hoagland[24], developed from the nutritional requirements of tomato. The latter is the most used in the investigation of nutritional problems of plants and appears as the basis for formulating various nutrient comercial solutions.[21]

The seed dormency is to prevent a premature germination in a non-favorable environment.[25] In some trees, as in *S. spectabilis* case, seed dormancy is difficult to break even when the environment is ideal. In theses cases, some treatments such as seed scarification can be performed to break dormancy and start germination. Seed scarification involves breaking, scratching or softening the seed coat so that water can enter and begin the germination process.[26] There are several methods of scarifying seeds: acid scarification - seeds are put in a glass container and covered with concentrated sulfuric acid. The seeds are gently stirred and allowed to soak from 10 minutes to several hours, depending on the hardness of the seed coat. When the seed coat has become thin, the seeds can be removed, washed, and planted. Hot water scarification – seeds are put into hot water (70 to 100°C) until water cools to room temperature. Mechanical scarification - seeds are rubbed with sandpaper or cracked with a hammer to weaken the seed coat.[25-27]

1.4 Identification by DNA of the rizosphere microbiota

Knowledge of the genetic and functional diversity of the microbiota associated with the rhizosphere is fundamental to the advance in understanding the relationship between the microorganisms and the plant.[28] Obtaining pure cultures and knowing what specie is about is important in the context of functional diversity because it allows the study of biocatalytic mechanisms, physiological requirements of the cells, the better understanding of the interactions between microorganisms and plants by inoculation of cells in plants and to explore microbial potential through technological application to obtain value-added products metabolites.[28]

The classical form of fungi identification is based on the morfological, physiological and biochemical aspects of one individual. Besides, species have been classified in sexual (teleomoph) or asexual (anamorph) according to reproductive structures that do not represent the phylogenetic relations between groups. In these classical taxonomy identification methodologies the structures are seen in the microscope and compared with the literature using keys for identification. For that, it is necessary to cultivate the isolated colony in an appropriated culture media and in some cases the sexual structures are not present.[29] Therefore, changing the cultivation conditions is mandatory. In order to induce sporulation, a poor culture media can be used as agar-water, increasing the intensity of ligth or exphosing to low doses of UV light. On the other hand, some fungi are not able to produce spores in a sinthetic media even when changing conditions; in that case, molecular techniques are required. These techniques are mainly based on the sequencing of the spacer region (ITS) of the ribossmal DNA and a database comparison.[29-30]

Before the end of 19th century most of the pathogenic bacteria known today were described. At that time the identification and classification included pathogenical potential, morphology, chemical reactions and requirements to grow and later the physiology had an important place on taxonomy.[31] All those tools are still in use nowadays. The form of classification for bacteria started to change only after 1950s when new techniques were developed such as chemotaxonomy, DNA-DNA hybridization and a partial database rRNA gene sequence compiled by Carl Woese[31] in the 1970s that allowed sequence comparitions for classification. This work expanded the techniques used in protein sequence comparison that were developed in the preceding years. After the 1980s and nowadays, nucleic acid analyses such as: 16S rRNA sequence analysis, protein-encoding gene sequence analysis, and gene profiling methods were developed and improved and are still very important on bacterial taxonomy.[31] Of course many adjustments and the integration of old and new methods are necessary for the requirements for defining new species but the molecular methods enable bacterial taxonomists to decode bacteria phylogeny and distinguish among different types of bacteria. In order to obtain pure cultures serial ten-fold dilution of the inoculum, in solid or liquid media, has been widely used for samples from different sources (rhizosphere soil, bioreactors, sediment) and the phylogenetic identification of individual cells by the total or partial sequencing of the 16s rRNA gene, a gram stain is also used for bacterial classification.[31]

In fact, for the classification of both eukaryotic and prokaryotic organisms, the advent of molecular techniques has provided a breakthrough in the understanding of genetic diversity and taxomic of microorganisms and plants.[29] Besides those techniques allow to analyze the interplay between prokaryotic and eukaryotic in a community as rhizosphere, to investigate the microbial diversity without the need of a cultivation and the identification of the phylogenetic of pure cultures. In order to enhance the classification of microorganisms and expand knowledge regarding the existing taxonomic relationship among closely related genera and species several molecular methods have been developed. Those methods are based on the comparision of nucleid acids sequences (genotypic methods) or protein (protein based methods) profile of a microorganism with a documented data on known organisms (database: Genbank- www.ncbi.nlm.nih.gov/genbank/).[29;31]

Genotypic methods include DNA-DNA hybridization[29;31] - apllied for procaryotes and eukaryotes organisms. In this aproach 80% of hybridization idicate that two isolates belong to the same specie and values under 20% indicate total lack of identity; PCR[29-32] (polymerase chain reaction) - amplifying DNA sequences *in vitro* by separating the DNA into two strands and incubating it with oligonucleotide primers and DNA polymerase. It is the most used because it can be associated with the sequencing of conservative genoma as found out in ribosomes.[29;33-34] Due to the structure (that creates bonds with itself) of the ribosomes and its function (translating mRNA into proteins), some parts of its genes have been changed a little compared with others. The ribosome is formed by two subunits: the small subunit (SSU) and the large subunit (LSU) that sandwich the mRNA as it feeds through the ribosome for translation. In general for prokaryotes 16S rRNA codes the SSU and 23S rRNA and 5S rRNA code for the LSU.[32] To the eukaryotes 18S rRNA for SSU and the LSU coded by 5.8S and 28S rRNA code these region.[29] The ribosomal RNA has highly conserved regions and it can be divided into three subunits which are internal transcribed spacer and externs (ITS and ETS) and also have intergenic spacer (IGS) or non-transcribed spacer (NTS) that separate copies in tandem in the chromosoms.[33-36]

The PCR also can be used for comparisions using: GC-content (Guanine-Cytosine content) - based on the content of 1-2% of DNA and can be used to differenciate between different species; RAPD (random amplified polymorphic DNA)[36-37] - is the amplification of genomic DNA using PCR primers with an arbitrary

sequence of 10 nucleotides. The primer binds to complementary sequences in opposite tapes of the target DNA and an in vitro amplification of the DNA segment occurs between two adjacent primers with the help of the Taq polymerase enzyme[29;31;36-37]; AFLP (amplified fragmente length polymorphism)[29-31]: is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA; RFLP (restriction fragment length polymorphism)[29-31]: is the same as RAPD and AFLP but applies to specific sequences that allow better analysis; PFGE (pulse field gel electroforesis): molecules are subjected to electric fields applied alternately in two different positions and this allows to separate DNA molecules by size.[29;31]

Futhermore, pyrosequencing is a sequence-based detection technology that enables rapid and accurate quantification of sequence variation.[38-40] Pyrosequencing is based on the detection of released pyrophosphate (PPi) during DNA synthesis. A visible ligth is produced after a complex reactions including the enzymes ATP sulfurylase, luciferase and the substrates adenosine 5 'phosphosulfate and luciferin where the pyrophosphate group released during the addition of one nucleotide and it is proportional to the number of nucleotides incorporated by DNA polymerase.[39-41] Therefore, when DNA polymerase incorporates a new nucleotide into a growing DNA chain, pyrophosphate is stoichiometrically generated resulting in the production of ATP. The ATP produced leads to the enzymatic conversion of luciferase with the emission of photons. As the reaction of the components decrease, a new cycle of reagents is introduced and then the specific nucleotide incorporation is measured sequentially.[37-41]

2 MATERIALS AND METHODS

2.1 Collection of seeds of *S. spectabilis*

S. spectabilis seeds were collected from pods of mature trees located on the premises of the Institute of Chemistry in the city of Araraquara - SP, in 2009, by Marcos Pivatto. This plant species has dried specimens deposited in the herbarium of the Botanical Institute of São Paulo under No. SP 370 917.

2.1.1 Scarification and seed germination of *S. spectabilis*

The seeds were selected according to color, size, uniformity and conservation status, eliminating broken seeds, blackened and/or injured. Before germination, the seeds need to have its dormancy broken through a scarification process[9; 11] consisted in:

- Mechanical scarification - (80 seeds) - Sanding of tegument opposite the heel up to the onset of endosperm with sandpapers n^{o} 10. It was made until affecting the very top layer, the tegument. Any cuts deeper than this could damage structure necessary for the plant to grow. Further, the seeds were soaked in sodium hypochlorite 2% for 20 min, followed by repeated washing and soaking in distilled water for 15 min.
- acid scarification - (80 seeds) - immersion in 98% sulfuric acid for 25 min, followed by successive washings with portions of distilled water and soaking for 15 min.

After the process of scraping and disinfecting the seeds were distributed in sterile petri plates containing moistened filter paper in 4 mL of distilled water. The plates were sealed with PVC and maintained in germination BOD at 30°C for 10 days. Seeds were considered germinated after the emergence of the radicle through the seed coat (approx. 3 mm).

2.1.2 Growing seedlings in rhizotron and hydroponics

The germinated seeds (30) were transferred to containers containing sand. The sand was previously treated with 0.3 M of HCl and washed with distilled water until pH=6, then it was autoclaved, 121 ˚C for 60 min for 3 times with 24h interval among the autoclave process. Such vessels received three seeds each and were kept in germination chamber for 3 days following, then introduced in the greenhouse. Before being transferred to the rhizotron and the vessels containing the nutrient solution, the seedlings had their roots washed with water, and then immersed in 2% sodium hypochlorite for 5 min. They were then washed and immersed in water for 5 min. For both crops two solutions based on nutrient compositions Clark[42] and Hoagland[24] were used in the first 15 days of cultivation, and in sequence until the end of culture, respectively (Table 1).

Table 1: Molar concentrations of Macro and Micro-nutrients of the nutritive solution in cultivations based on the composition of the solution of Clark (1975)[42] and Hoagland and Arnon (1950)[24].

Micronutrients (µmol.L^{-1})							
	NH_4^+	NO_3^-	H_2PO4^-	K^+	Ca^{2+}	Mg^{2+}	SO_4^{2-}
Clark	0,90	7,30	0,08	1,82	2,60	0,60	0,60
Hogland	15,0	-	1,0	23,46	5, 01	2,00	2,00
Micronutrients (µmol.L^{-1})							
	B	Cl^-	Cu^{2+}	Fe*	Mn^{2+}	Mo	Zn^{2+}
Clark	19,0	0,6	0,5	40,0	7,0	0,6	-
Hogland	38,0	0,6	1,0	80,0	14,0	1,2	-

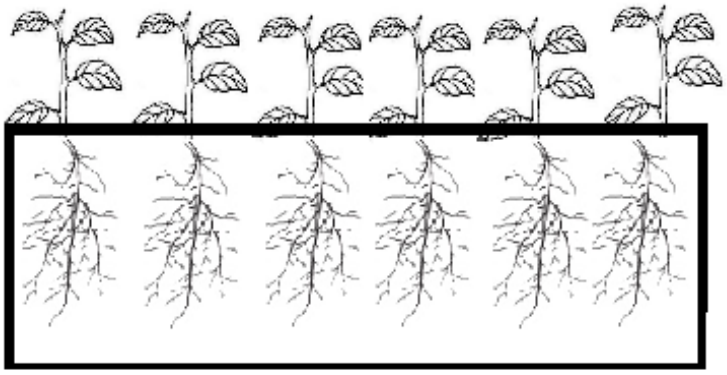
*$FeCl_2$ chelated with disodium EDTA.

Rhizotron - consists of a glass container containing sand. The container was washed with soap and water, sterilized with 2% hypochlorite solution (immersion for 30 min.), followed by washing with 70% ethanol, and distilled water. In this system, 6 plants with 17 days couting from germination were placed in the wall glass through the roots and the aerial part was allowed to shoot out of the container (Figure 2a). Then steriele sand was placed on top of the roots so that they were completely covered with approximately 5 cm thick. This system was covered with black plastic to prevent the incidence of light directly on the roots.

Hydroponic System - 20 seedlings were introduced in this system after 25 days of germination. They were grown in plastic pots (5.0 L) containing nutrient solution, constantly aired. This solution was replenished every 7 days, exchanged, stored in plastic bags type ziplock and stored in a freezer at -10 °C. The pots were painted in aluminum color and sealed so that there was no direct incidence of light on the roots. On the cover of each pot, there were two holes of 4 cm in diameter, in which two seedlings were placed in each pot using polystyrene as attachment media (Figure 2b).

Figure 2: Scheme of a) rhizotron system and b) hydroponic system.

a)



b)



2.2 Isolation of fungi and bacteria associated with *S. spectabilis* rhizosphere

- Rhizotron - After 60 days, the rhizosphere of *S. spectabilis* seedlings was collected as appropriate procedure described in ANEJA[43] .

To collect the rhizosphere region, 10 g soil adhered directly from the roots (sand) were collected in a sterile beaker, weighed and trasferred into conical flask with 90 mL of sterile 0.85% NaCl, this procedure was performed in a laminar flow cabinet. This mixture was stirred for 30 min. in a shaker at 130 rpm and 26°C. The obtained suspension was used to make serial dilutions in which was added 1 mL of the sample to 9 mL of sterile 0.85% NaCl contained in a test tube followed by vigorous stirring for 5 min. in vortex. This process was repeated until the dilution of 10^{-2} to 10^{-7} in triplicate in nutrient agar, czapek-dox agar and czapek-dox agar with gentamicin sulfate. The composition of culture media are described in Table 2.[43]

We conducted a plating of the original suspension, without dilution, in different culture media, and also rhizosphere a spatel tip, directly inoculated in the culture media.

Table 2: Composition and specifications of culture medium

Culture medium	Composition	Conc. ($g.L^{-1}$)	Purpose	Distributer
Czapek	Sucrose (30g); $NaNO_3$ (3g); $Na_2(PO_4)_3$ (1g); $MgSO_4$ (0,5g); KCl (0,5g); $FeSO_4$ (0,01g); agar (15g)	35	Base for cultivation of fungi and bacteria able to utilize inorganic nitrogen	**DFCO**
Nutrient	Peptic digest of animal tissue (5g); beef extract (1,5g); yeast extract (1.50g); NaCl (5g); agar (15g)	8	Base for cultivating microorganisms in general.	**DFCO**

- Hydroponic System - 1 mL of the nutrient solution was spread on the board with drigalsk spatel in solid culture media nutrient agar and Czapek-dox agar.

For both cultures, after the inoculum of the rhizosphere material, the plates were incubated at 28°C for 15 days and observed daily. After this period, each colony of microbes that was visible was transferred to a new agar plate and so on until obtaining a pure strain. The preservation of fungi was performed by placing chunks of solid medium in penicillin flasks containing sterile distilled water kept sealed with rubber stopper and aluminum seal, and protected from light at 28°C. For bacteria the preservation was made by adding 2 mL of the culture medium in which the organism was grown in test tubes (13 cm X 1 cm) and kept in the refrigerator sealed with rubber stoppers and parafilm and protected from light.

2.3 Molecular phylogenetic and classical taxonomic methods for identification of the rhizosphere microbiota

2.3.1 DNA extraction

Isolated bacteria: For the extraction of genetic material from the strains we boil the cellular biomass as follows: A single colony of each strain was separately resuspended in falcon tubes (15 mL) with 2 mL of culture medium (Czapek-dox broth). The cultures were incubated at 28˚C for 8 days. 1mL aliquot of cell

suspension was transferred to Eppendorf tube and centrifuged at 13,000 rpm for 2 min. at room temperature. The supernatant was discarded and the pellet washed with 700 µL of sterile Milli-Q water. The suspension was stirred vigorously in vortex for 10 seconds and centrifuged under the conditions described above. After centrifugation, the supernatant was discarded and the precipitate dried at room temperature. Then, the pellet was resuspended in 400 µL of sterile Milli-Q water and incubated in boiling water (100°C) for 15 min. to break the cell wall and release of DNA. The pellet was then cooled down at room temperature for 10 min. followed by centrifugation (13,000rpm, 15min, room temperature). Thereafter, 200µL of the supernatant was harvested, transferred to 0.5 mL tube and stored at -20°C until use that occurred within one week after extraction. The amount of DNA was determined using NanoDrop 1µL sample. This extraction was performed at Department of Applied Biology in the São Paulo State University – UNESP (Jaboticabal, SP, Brazil).

Roots biofilm: The biofilm on the roots it was washed in PBS-buffer (PBS: 0.13 M NaCl, 7 mM Na_2HPO_4, 3 mM $Na_2H_2PO_4$, pH 7.2) and subsequent centrifuged for 10 min at 6,000 rpm, at 4°C. The pellet (1g) was collected and used for DNA extraction. DNA was extracted according a phenol: chlorophorm protocol as described elsewhere.[44]

Rhizosphere from hydroponic: It was lyophilized and the genomic DNA was extracted using PowerMag® Soil DNA isolation kit by Researching an testing laboratories (Lubboch, Texas, USA) that made the procedure.

2.3.2 PCR amplification of 16S rRNA gene and ITS rRNA region

Isolated bacteria: fragments of gene 16S rRNA were amplified by polymerase chain reaction (PCR) with primers oligonucleotides fD1 (5' AGAGTTTGATCCTGGCTCAG 3') and rD1 (5' AAGGAGGTGATCCAGCC 3').[45]

After synthesis, these oligonucleotides were diluted to a concentration of 1000 pmol/µL in TE buffer, and this solution was stored at -20°C. A 1µL aliquot was diluted in 99 mL of sterile Milli-Q water to a final concentration of 10 pmol/µL, which was used in the amplification reactions. These reactions were composed of PCR buffer, 1 mM magnesium chloride, 250 µM of dNTPs solution, 10 pmol/µL of each primer, 1 µL of Taq DNA polymerase enzyme, template DNA and sterile Milli-Q water filtered (q.s. 20 µL). 3µL and 5µL aliquots of the DNA were used to verify the optimal amount of

DNA in the PCR reactions. The reactants were homogenized with the aid of a micropipettor.

The reactions were placed in a thermocycler programmed with an initial denaturation step of 95°C for 3 min. and 35 cycles consisting of: denaturation at 94°C for 1 min., the linkage 55°C for 1 min., extension at 72°C for 2 seconds, and an extra extension step at 72°C for 5 min. Reactions were kept at 4°C for preservation of the material when used in the same day or at -20°C for later use.

After the amplification process, 3 µL aliquot of loading buffer was added to 5µL of sample. The samples were analyzed by gel electrophoresis agarose[46] with a sample containing DNA fragments of known length, multiples of 1 kb (1 kb Ladder, Fermentas), used as a reference for electrophoretic migration calculating the size of fragments obtained. The horizontal electrophoresis was performed with 50V for 90 min, conducted in 1X TBE buffer and stained with ethidium bromide. The DNA fragment prepared in agarose gel was visualized by UV light (280nm) incident in a fotodocumentation system and evaluated using the negative image obtained by the software Quantity-One. This procedure was performed at Department of Applied Biology in the São Paulo State University – UNESP (Jaboticabal, SP, Brazil).[47]

Roots: the PCR was performed using barcoded primers that amplify the V4 hypervariable region of the 16S rRNA gene.[48] The primer and barcode sequences were used as follow: Forward primer 563F (5' AYTGGGYDTAAAGVG 3'), Reverse primer 577R (5' TACNVGGGTATCTAATCC 3'), PCR reactions were performed in a 454 GS FLX Titanium (Roche) at the Institute of Agrobiotechnology, Rosario (INDEAR Facility, Argentina).

Rhizosphere from hydroponic: PCR was performed using the barcodes primers Forward primer 28F (5' GAGTTTGATCNTGGCTCAG 3')[48] that amplify V1-V3 region of 16S rRNA gene and forward primer ITS1F (5' CTTGGTCATTTAGAGGAAGTAA 3') for the fungi identification that amplify ITS region of rRNA.[49] The PCR was performed according internal protocol by the Researching and Testing laboratories (Lubboch, Texas, USA).

2.3.3 Genetic sequencing for microbial identification

Isolated bacteria: The sequencing reactions were performed in microtiter plates using two replicates in a final volume of 10 µL containing: 3.0 µL of sterile ultrapure

water; 1.0 µL of Big Dye (ABI PRISM Big Dye Terminator Kit - Applied Biosystems, USA); 4.0 µL of buffer 10X; 1.0 µL oligonucleotide primer rD1 or fD1 (at concentration of 5 pmol/μL^{-1}) and 1.0 µL of DNA.

The plates were sealed with a silicone adapter and brought to the thermocycler using the following conditions: 96°C for 2 min.; 25 cycles at 96°C for 10 seconds, 52°C for 10 seconds, 60°C for 4 min. and a final cycle at 4°C. Then, the DNA fragments were precipitated and the dNTPs not incorporated labeled with fluorescently were removed by repeated washings. For the precipitation of labeled DNA amplified by PCR sequencing was added 80 µL of isopropanol 75% (v/v) to the samples; The plates were shaken thoroughly by vortexing for a few seconds, incubated at room temperature for 15 min. and centrifuged at 20°C for 45 min. at 3,220 x g. The supernatants were discarded and 150 µL of 70% ethanol (v/v) were added to the samples. The plates were centrifuged for 5 min. at the same temperature and centrifugal force described above, supernatants were discarded and the plates dried plus Concentrator (Eppendorf). For application in sequencing, samples were resuspended in 9 µL of Hi-Di Formamide (ABI PRISM), subjected to denaturation for 5 min. at 95°C. The sequencing was performed in a capillary system model ABI Prism 3100 DNA Analyzer.[47]

The sequencing of the nucleotides was done on the ABI Prism 3700 DNA in Biochemistry Laboratory of Microorganisms and plants, Dept. of Technology, Faculty of Agricultural and Veterinary Sciences (FCAV) - UNESP, Jaboticabal. A total of 36 samples were sequenced.

The consensus of the forward and reverse sequences obtained was performed in SeqMan program of DNASTAR software. After alignment and investigation of the presence of chimeras, the sequences of nucleotides were subjected to nucleotide similarity query through Blastn program ("Basic Local Alignment Search Tool") and compared to deposited homologous sequences in the electronic database GenBank (National Center for Biotechnology Information - NCBI)[50] for the approximation of the phylogenetic identity.

Roots biofilm: Sequences were processed on mothur 1.24.0.[51] Chimeric sequences were detected by the Chimera Slayer algorithm[52] using the Silva "Gold" aligned database as reference. Putative chimeras and sequences shorter than 160bp were excluded. The remaining sequences were aligned against the SILVA 102 reference database[53] and further clustered into operational taxonomic units (OTU) at

3% distance cutoff. In order to estimate the complexity of the microbial communities, alpha diversity indices were calculated on mothur for each sample using richness (Chao1) and diversity (Shannon) estimators, as well as by Good's diversity coverage. The Chao1 index uses the number of OTUs with one or two sequences to estimate the number of missing species. To estimate the diversity of species present in the communities analyzed was applied the Shannon index which is a general index of diversidade sensory richness and the relative abundance of species.[54] A Venn diagram was constructed to calculate the number of specific and shared OTU between each phase. Finally, sequences were assigned to taxonomic level of Genera with the Classifier tool in the Ribosomal Database Project (RDP) Naive Bayesian rRNA Classifier Version 2.10 (http://rdp.cme.msu.edu/index.jsp) using a 50% confidence threshold.[55]

Rhizosphere from hydroponic: The data was processed using the data analysis pipeline. The data analysis pipeline consists of two major stages, the denoising and chimera detection stage and the microbial diversity analysis stage. Denoising is performed using various techniques to remove short sequences, singleton sequences, and noisy reads. With the bad reads removed, chimera detection was performed to aid in the removal of chimeric sequences. Lastly, remaining sequences were then corrected base by base to help remove noise from within each sequence. During the diversity analysis stage, each sample was run through analysis pipeline to determine the taxonomic information for each constituent read. These sequences were clustered into OTUs using the UPARSE algorithm and the centroid sequence from each cluster was runned either using USEARCH alignment algorithm or the RDP Classifier against a database of high quality sequences derived from the NCBI database. The procedure above was exceuted by Researching and Testing laboratories (Lubboch, Texas, USA).

2.3.4 Classical taxonomic fungi identification

From the all filamentous fungi obtained in this work 20 fungi were randomly chosen and were identified in terms of genus and in some cases it was possible to determine the species. The isolates were grown in Malt Extract medium for promotion of sporulation during 10 days at 28˚C. The microscope structures were analyzed such as manner of arragement of spores on the fruiting bodies, color, size,

shape, hyphal caracteristics of mycelium and the fruiting bodie itself. All those caracteristics from each fungi were analyzed and identified by experienced prof. André Rodrigues at the Department of biochemistry and biology, Biosciences Institute, UNESP, Rio Claro.

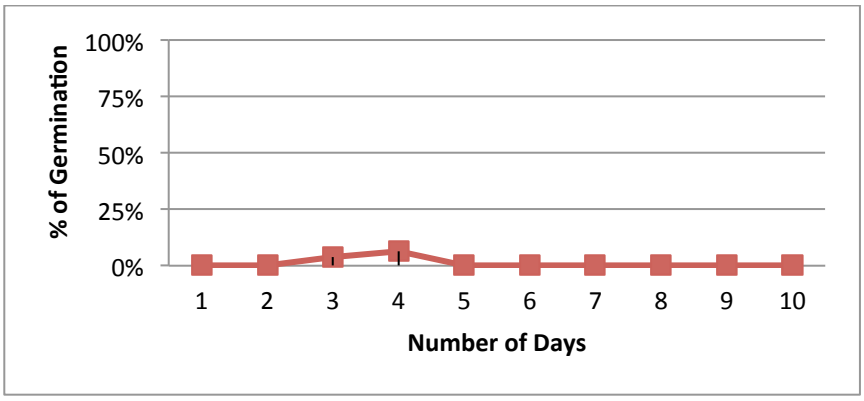
3 RESULTS

3.1 Seeds germination

The initial step to obtain the seedlings was breaking dormancy of seeds, for which of the tested methods - mechanical and chemical scarification - obtained a germination rate of 6.25% and 32%, respectively, as we see illustrated in graphic 1.

Graphic 1: Germination percentage plot of the seeds of *S. spectabilis* submitted: a) mechanical scarification with sandpaper and b) chemical scarification with sulfuric acid.

a)



b)

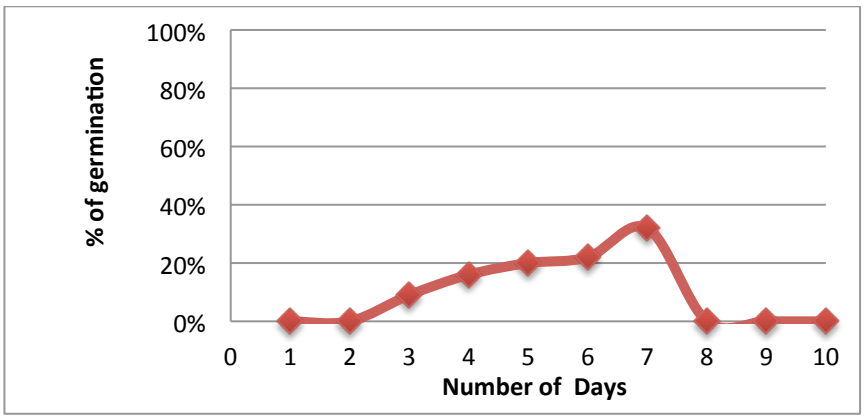
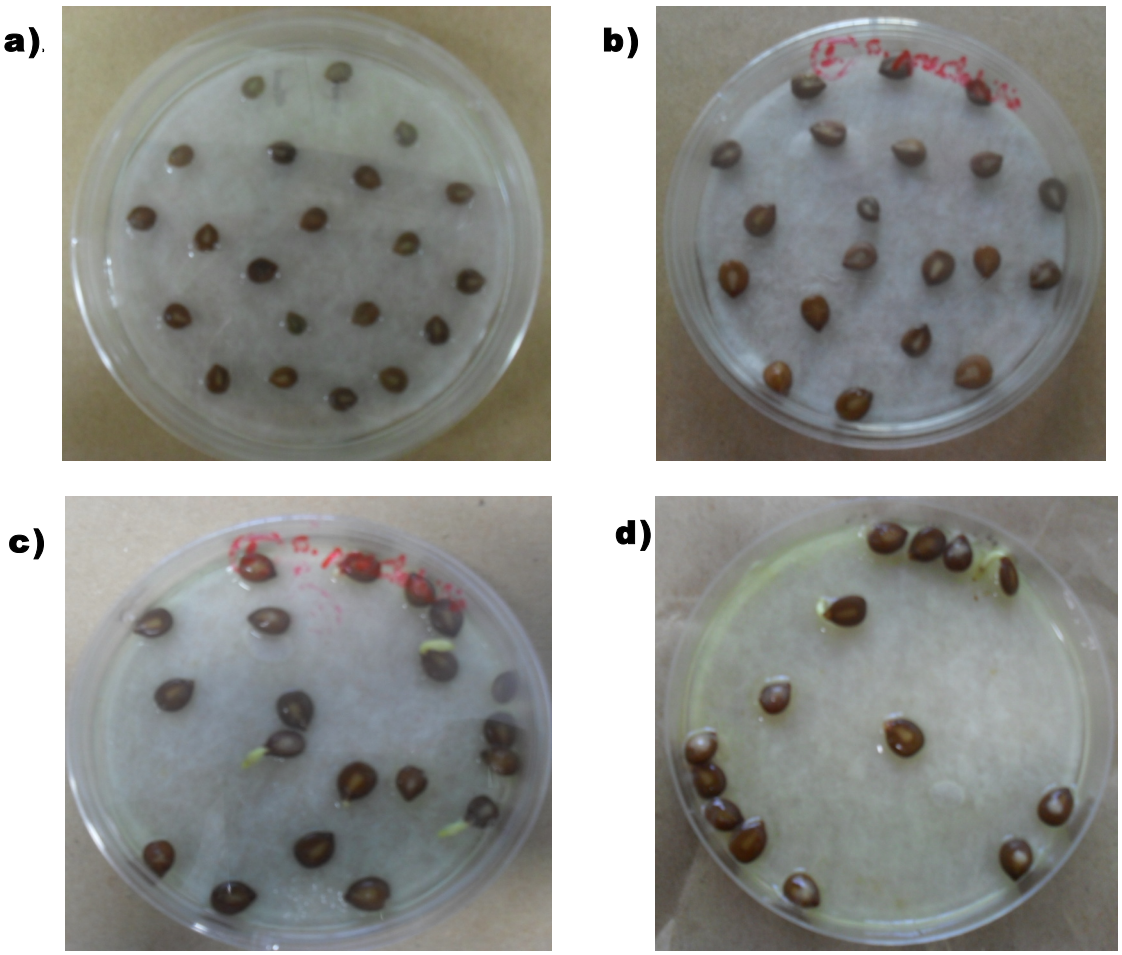
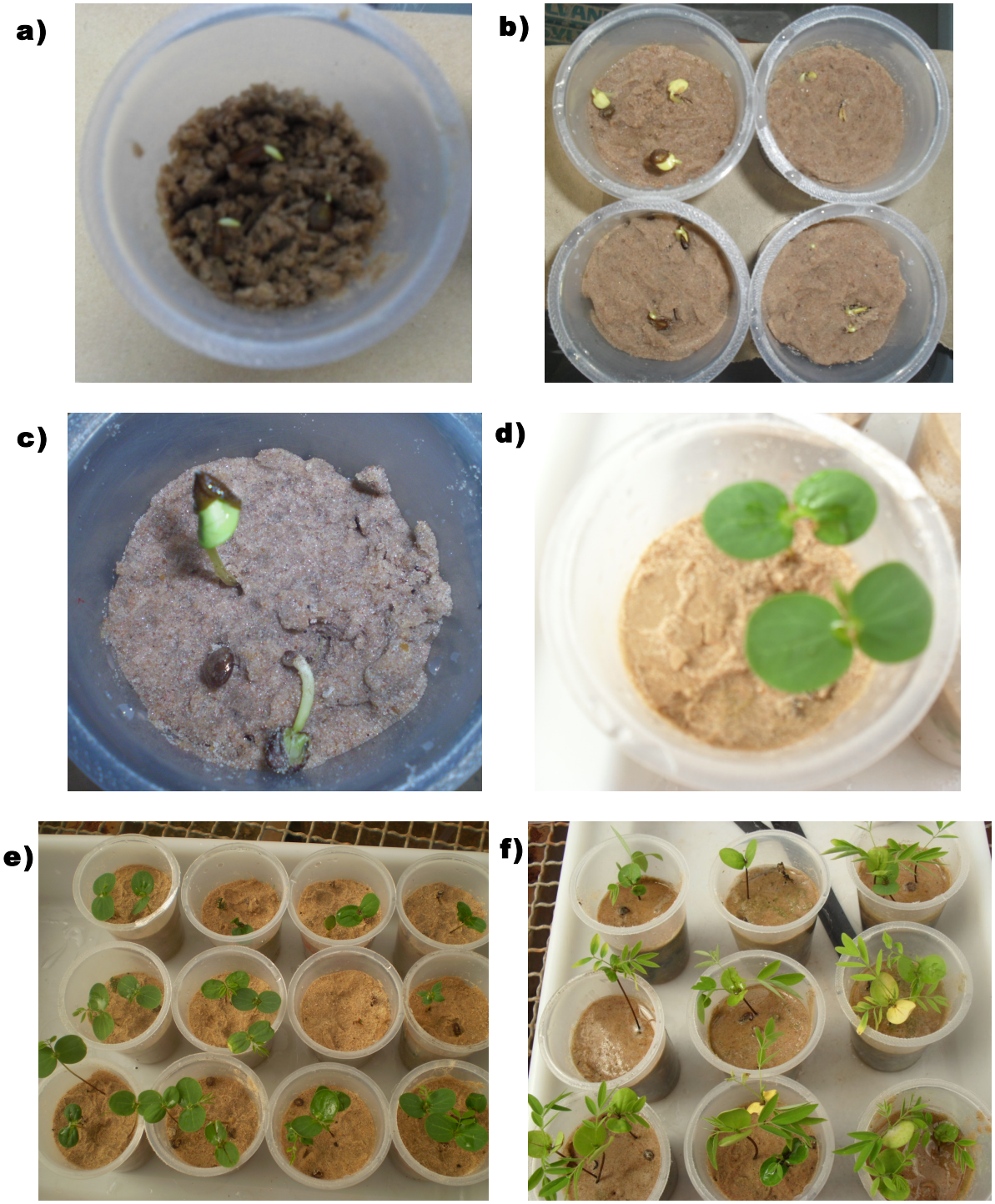


Figure 3: Germination of the seeds of *S. spectabilis* had undergone scarification with sulfuric acid: a) 24h; b) 48h; c) 72h and d) 120h.



The germinated seeds (figure 3) were transferred to pots with sand and presented a good development, with the cotyledons leaves appearing after three days on these twenty-seven seedlings. Five other plants did not develop in the sand and were discarded. We observed the development of seedlings in Figure 4.

Figure 4: Process of growth of *S. spectabilis* seeds after treatment with sulfuric acid - a) germinating seed filling sand; b) 120 hours after being placed on the vessel; c) stem growth and early appearance of the cotyledons (10 days after being placed in the vessel with sand); in the greenhouse - d) cotyledon leaves after 13 days of growth; e) appearance of the first leaves 17 days after planting in sand and f) 40 days after planting in the sand.

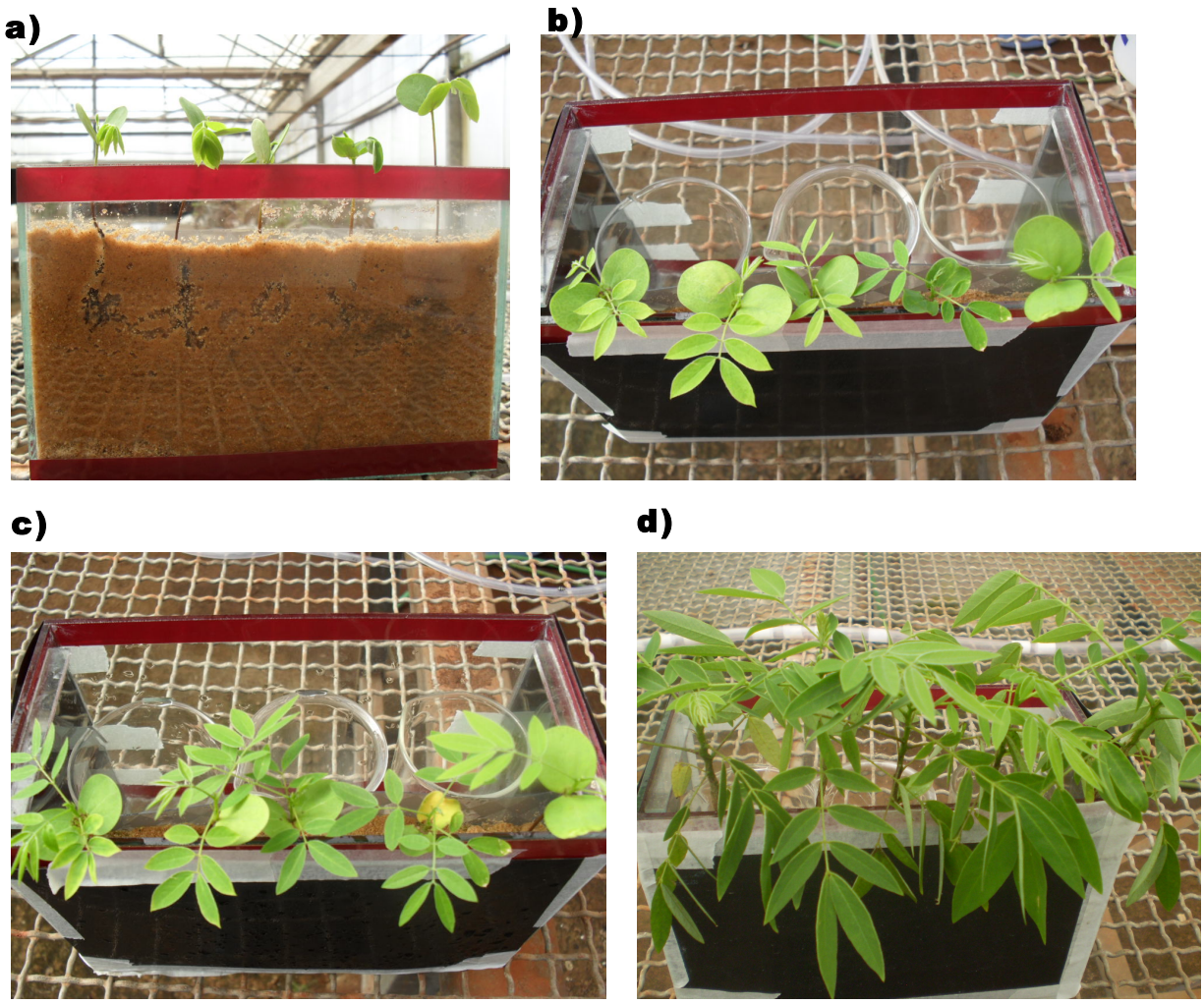


3.2 Plant cultivation and microbes extraction from rhizosphere

3.2.1 Rhizotron

Plants: Five plants after the 18-day period which were in the greenhouse, were transferred to the rhizotron, which allowed us to view the development of root and how it behaves on solid medium - in this case - sand (Figure 5). A rapid initial development of plants with root growth throughout the glass surface was observed in here. However, during 60 days the plants stagnated, that is, it was not observed a stalk growth, only small twigs and leaves.

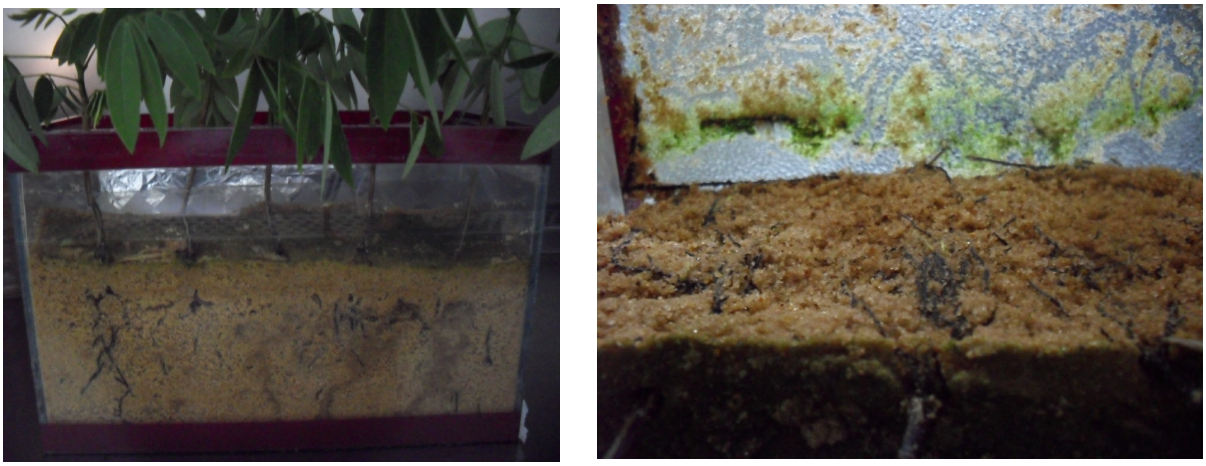
Figure 5: Cultivation in rhizotron of the seedling of *S. spectabilis* - a) start cultivation in rhizotron; b) 15 days after the start of cultivation; c) 30 days of cultivation and d) with 60 days of cultivation.



This experiment was stopped 30 days after soil sampling for isolation of microorganisms (90 days after germination) due to an increase in temperature in the greenhouse that caused the death of all individuals who were in the vessel. Therefore, it was not possible to track longer root development.

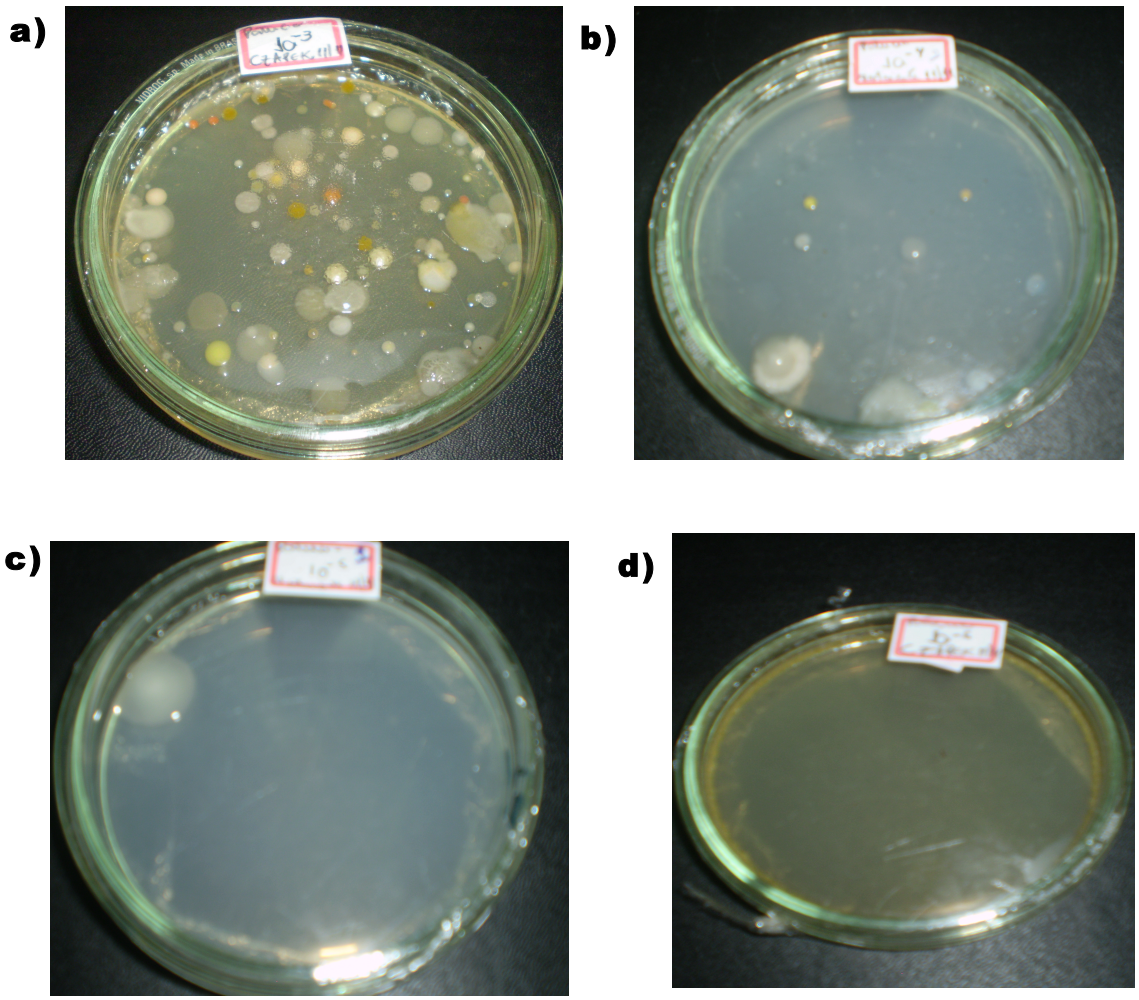
Isolation of microorganisms from the rhizosphere: The extraction of microorganisms present in the rhizosphere of plants was made with 60 days of cultivation and from the extraction process the seedlings were taken to a laminar flow camera to avoid contamination of external microorganisms (figure 6). A suspension was prepared with the material and then shaken in a shaker for maximum extraction of microorganisms. This agitation was chosen because it is a less harmful front than sonification, which can cause cell disruption of some microorganisms.[56] After extraction, the stock solution was diluted, and further, aliquots were inoculated onto solid culture medium for growth of microorganisms.

Figure 6: Rhizotron disassembly for soil collected from the rhizosphere after 60 days of cultivaton.



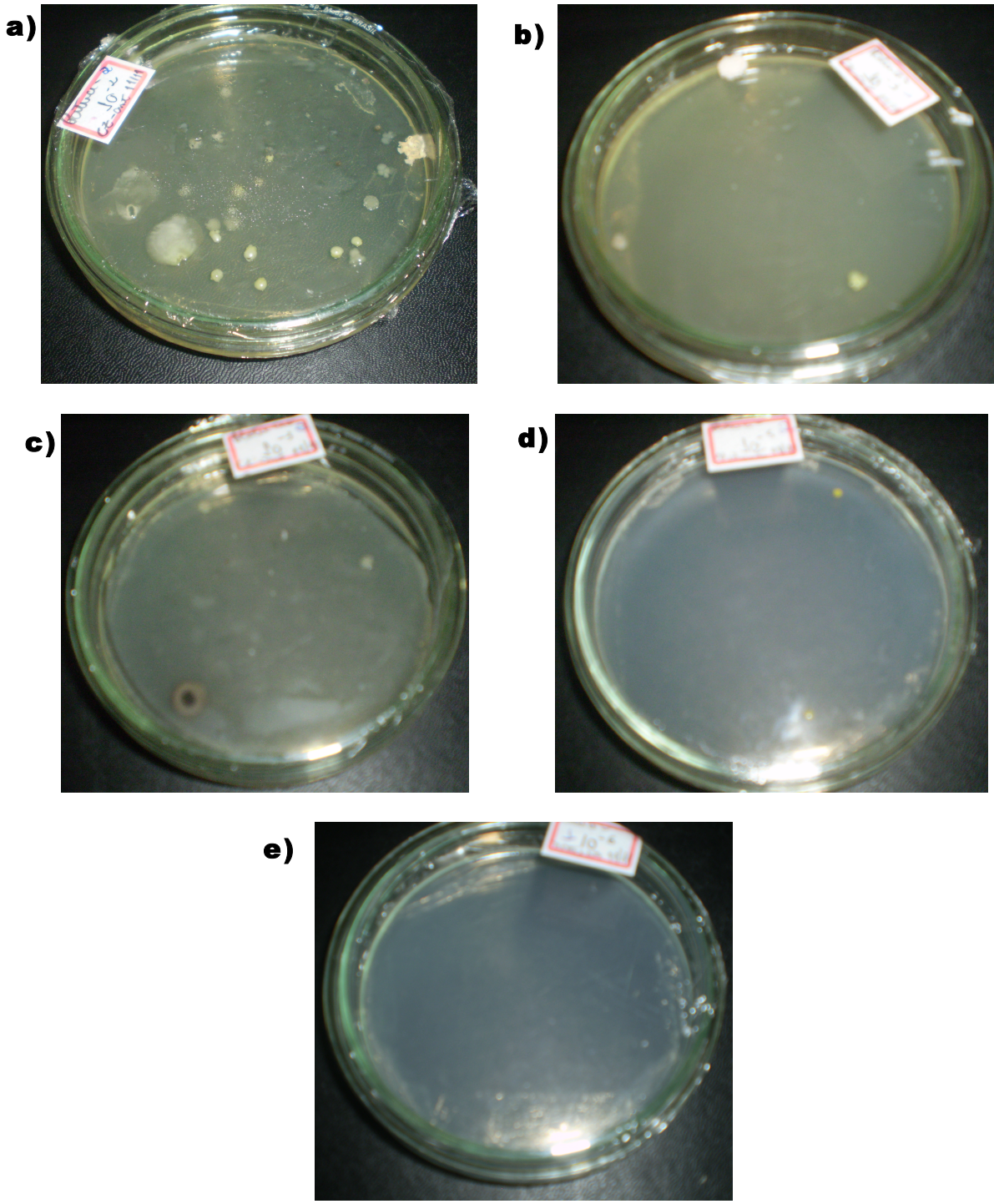
Czapek-dox agar: Dilutions 10^{-3},10^{-4} and 10^{-5} - The organisms present on this board are gelatinous, circular, with regular edges, which is characteristic of bacteria (Figure 7a, 7b and 7c). 10^{-6} dilution - Growth in a single individual plate have different aspect of the edges found in the front plate (Figure 7d).

Figure 7: Dilutions containing the rhizosphere in 0.85% NaCl in czapek-dox agar (after 10 days of inoculum) - a) 10^{-3}; b) 10^{-4}; c) 10^{-5} and d) 10^{-6}.



Czapek-dox agar + gentamicin sulfate: In dilutions 10^{-2} and 10^{-3} grew very similar organisms with the 10^{-3} medium czapek-dox, gelatinous, circular, regular (Figures 8a and 8b), except an organism that has a dry appearance, irregular border, without producing hyphae, leads us to believe to be a actinomycete (Figure 8b). The two plates are very similar except for the smaller number of microorganisms due to dilution. The plate 10^{-4} showed a microorganism fungus-like brown with transparent border that it did not grow in the other plates (figure 8c). All other further dilutions performed with this medium, there was no growth of microorganisms (figures 8d and 8e).

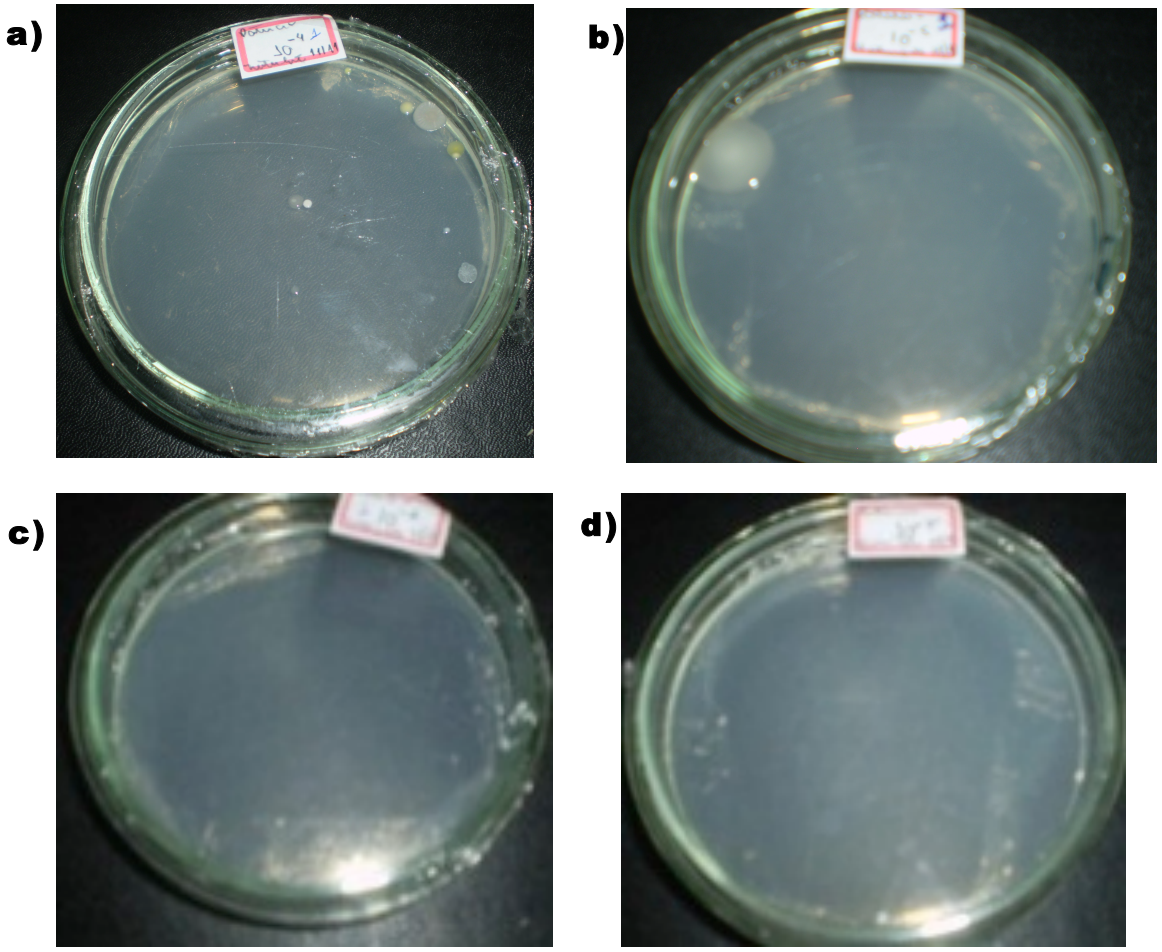
Figure 8: Dilutions containing the rhizospheric soil in 0.85% NaCl in czapek-dox agar + gentamicin sulfate (after 10 days of inoculum) - a) 10^{-2} b) 10^{-3}; c) 10^{-4}; d) 10^{-5} and e) 10^{-6}.



• Nutrient agar: The dilutions 10^{-4} and 10^{-5} contain only gelatinous microorganisms, growth regulating and circular (Figures 9a). They are similar to the plates described above. In one of the dilution plates 10^{-5} (Figure 9b), there is only one individual milky white, gelatinous, regular growth and another card of the same dilution an individual with the same characteristics of the previous strong orange

color. In the dilutions plates 10^{-6} and 10^{-7} there was no growth of microorganisms (Figures 9c and 9d).

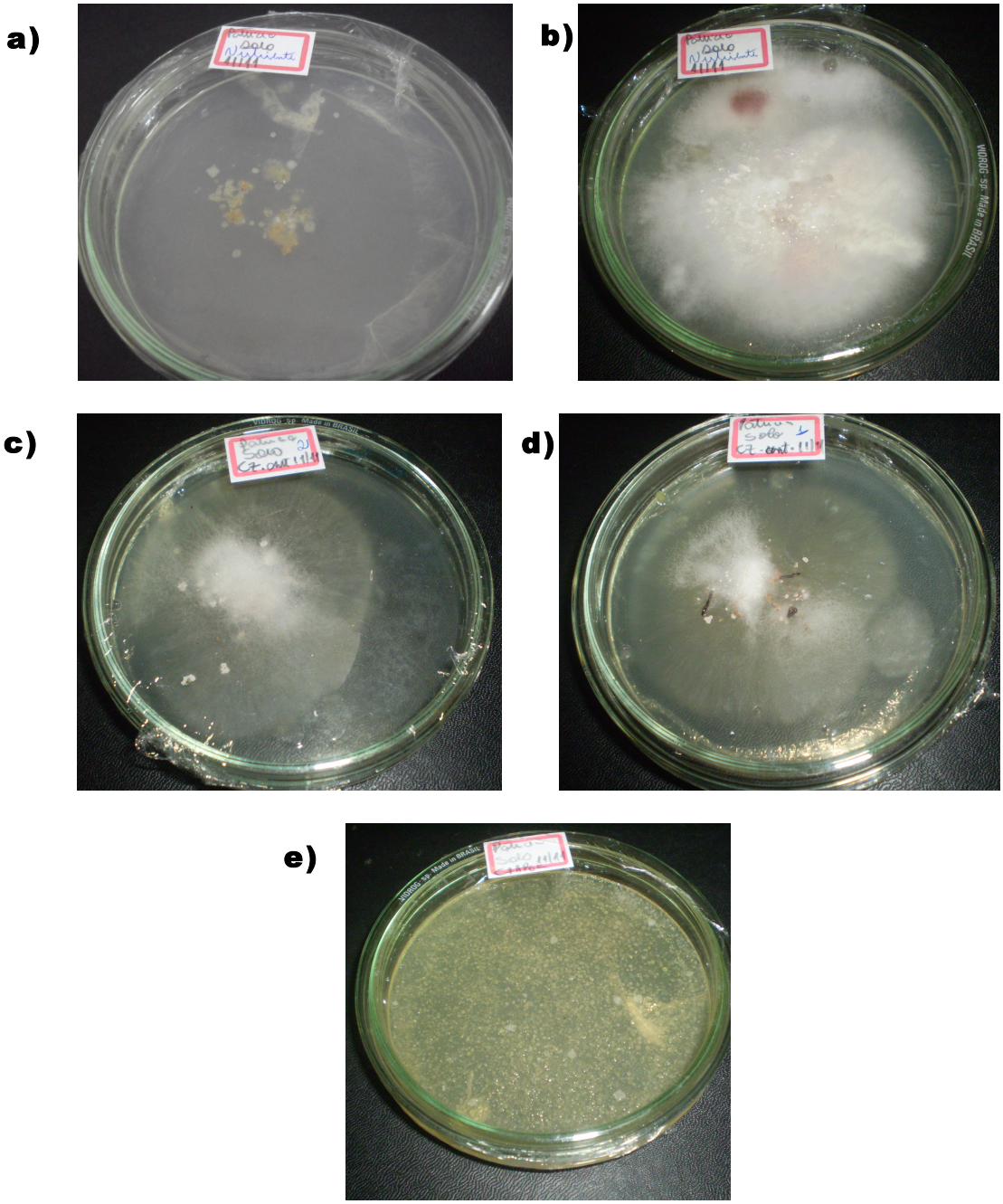
Figure 9: Dilutions containing rhizospheric soil in 0.85% NaCl in nutrient agar (after 10 days of inoculum) - a) 10^{-4}; b) 10^{-5}; c) 10^{-6} and d) 10^{-7}.



In the plates inoculated with concentrated suspension, microbial growth was intense and there is no way to distinguish the bodies, however, there seems to be filamentous fungi. The plates directly inoculated with rhizosphere (figures 10a-e), there was the growth of three different filamentous fungi, and bacteria were also found in the other plates.

All organisms isolated were preserved in the test tube containing the medium itself in the refrigerator for bacteria, and bottles containing sterile distilled water for fungi. The total time of obtaining a pure line of the process was approximately 50 days.

Figure 10: Plates inoculated directly with rhizospheric soil (after 10 days incubation): a) nutrient agar medium; b) czapek-dox agar medium; c) czapek agar + antibiotics medium; undiluted suspension (solution): d) in the nutrient agar; e) czapek-dox agar f) czapek-dox agar + gentamicin sulfate.

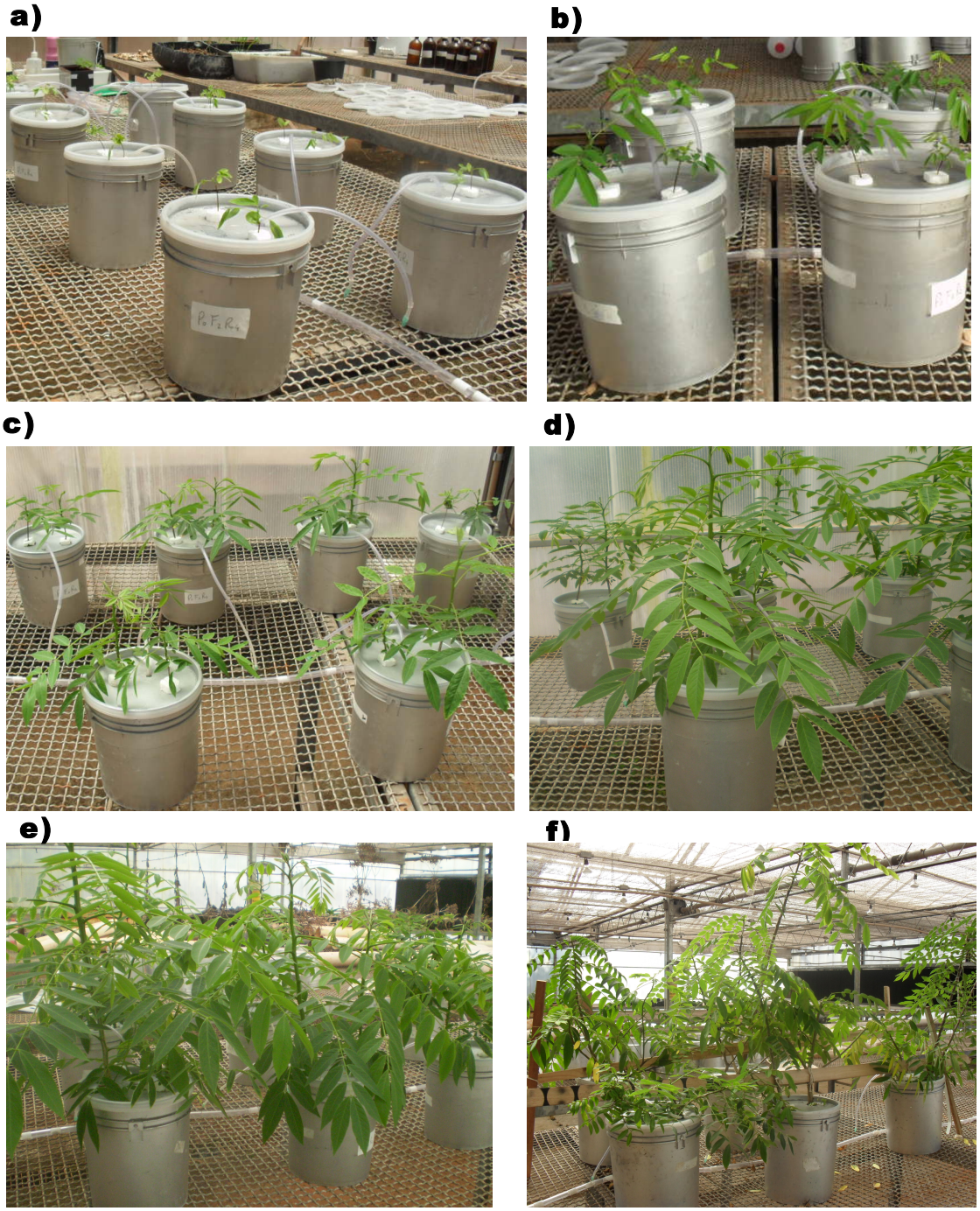


3.2.2 Hydroponic

Plants: After 37 days of cultivation in pots containing sand, eighteen plants were transferred to 3L pots containing nutrient solution Clark[42] (Figure 11), however, after 30 days of culture it was necessary to adjust the concentration of nutrients since the plants began to show yellow leaves. It was necessary to implement a solution based

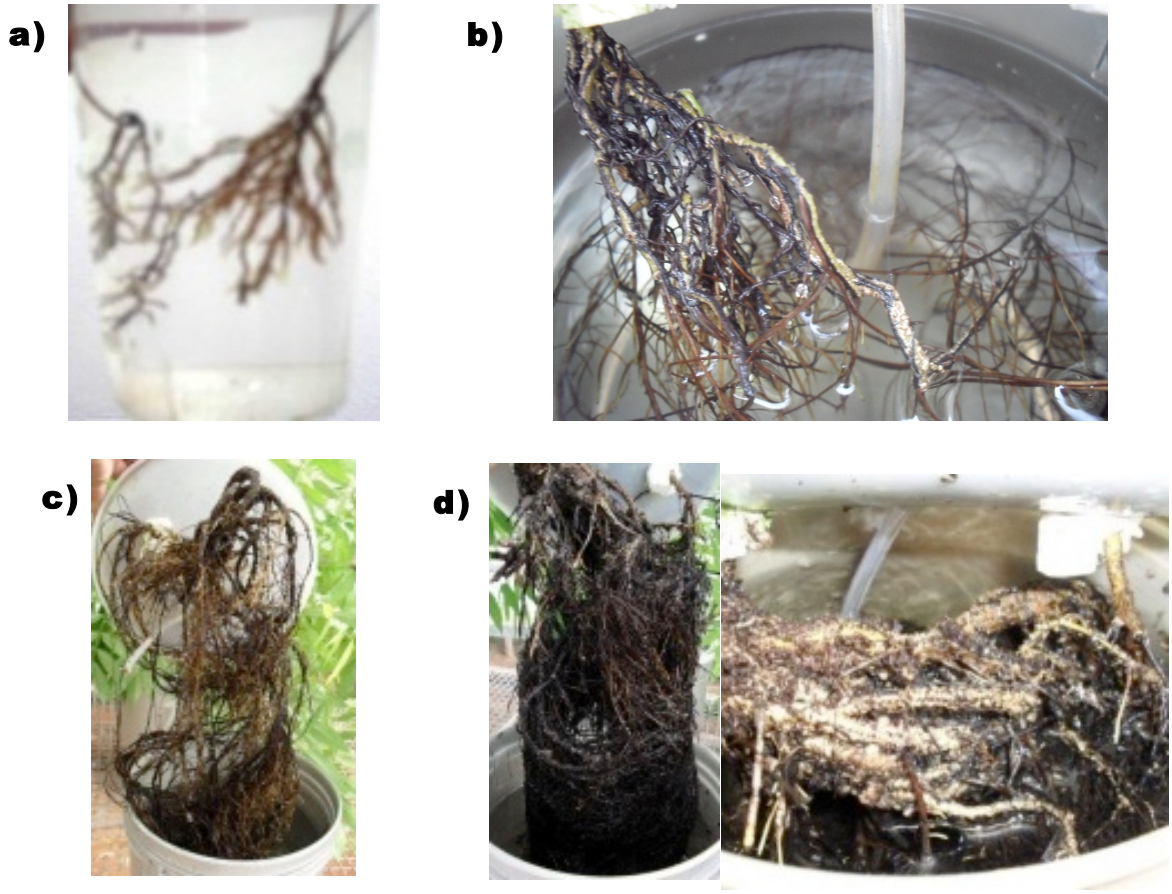
on Hoagland[24] composition (described previously at pg. 38). The plants have adapted well to this new solution, with good root development in the aqueous system. The solutions were changed completely every 15 days. With plant growth, it was necessary to transfer them to larger plastic pots, 5L of capacity and put wooden stakes for support.

Figure 11: Hydroponic cultivation of seedlings of *S. spectabilis* - a) beginning of hydroponic cultivation; b) 10 days after the start of cultivation; c) 30 days in cultivation; d) 50 days; e) 60 days and f) 320 days.



Diferently from the roots that were grown in rhizotron, these grew linearly until it reached the bottom of the vessel, only after finding that barrier, they began to intertwine the nearby plants and began to spread, forming multiple branches (Figure 12). The morphology of the roots are not different from that found in the sand system, it is a thin and brittle roots. There were no attacks of insects during the cultivation.

Figure 12: Development of the roots of *S. spectabilis* seedlings: a) at the beginning of the growing; b) after 30 days of cultivation; c) after 60 days and d) after 150 days of cultivation.



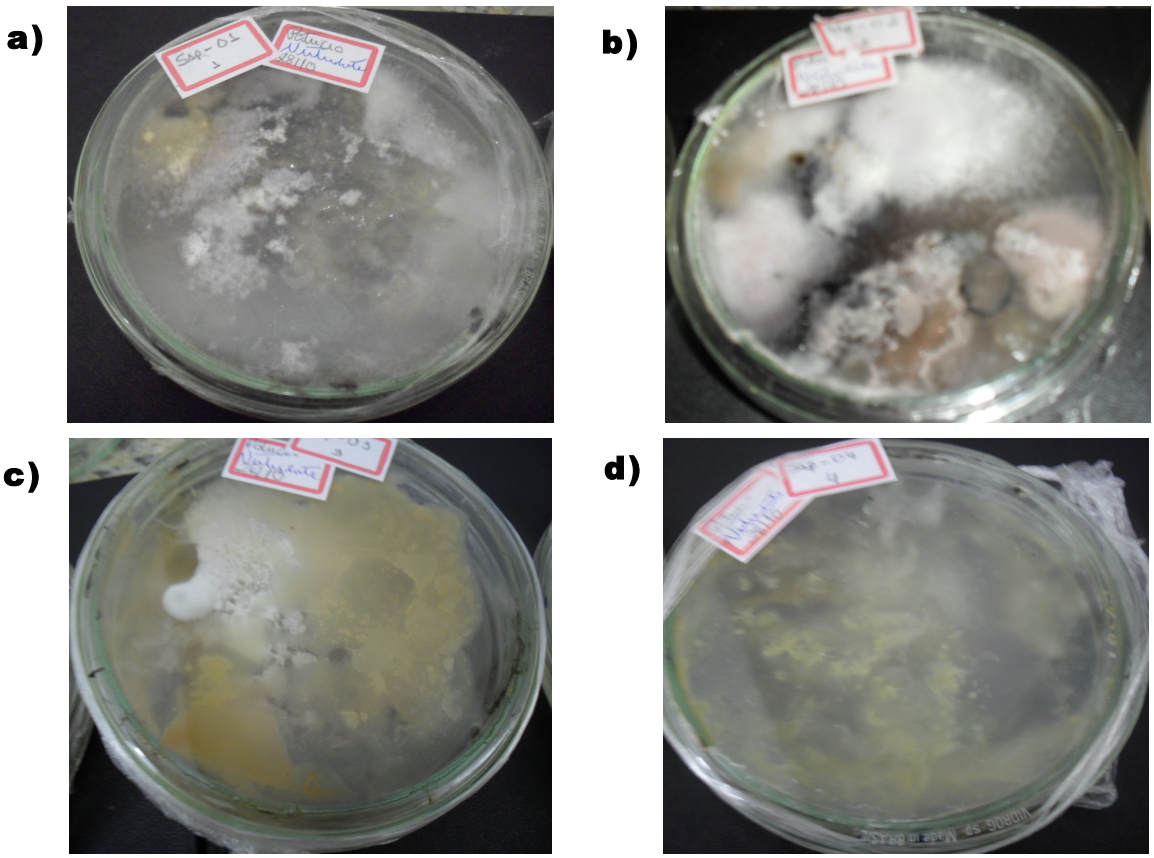
Microorganisms isolated from the rhizosphere: It was made three harvests at different periods of time and age of the seedlings. The first was carried out 15 days after the start of the hydroponics and the composition of the solution was in accordance with that proposed by Clark[42] and provided the conditions of various microorganisms associated with the roots of seedlings of *S. spectabilis* and most composite by filamentous fungi. In this experiment it can be observed that the plants, even though grown in different vessels (9 potted seedlings 2 each), are very similar

to the association of microorganisms since the profile that was seen in petri dishes after a period of incubation was very resembling.

Other two harvests were made, the second after 90 days and the third after 150 days of hydroponics cultivation. The solution here was based on the composition of Hoagland[24] therefore the nutrient concentration is different from that found in the first sample. The microorganisms that grew in this experiment were different from those at the first harvest. In both harvests, there were a high presence of bacteria over fungi. For all harvests, samples in duplicate for each culture medium used were collected: nutrient and czapek-dox agar, and in which the nutrient solution was subsequently inoculated (figure 13-18).

All microorganisms grown on the plates of two culture media were subjected to isolation process, and those in which it was possible to obtain the pure line were stored in penicillin shape bottles containing sterile distilled water and their culture media for bacteria totaling 197 individual microorganisms.

Figure 13: Plates, nutrient agar 10 days of incubation after inoculation with the collected nutrient solution *S. spectabilis* seedlings: a) first vessel; b) vessel 2; c) vessel 3; d) vessel 4; e) 6 vessel; f) vessel 7; g) vessel 8 and h) vessel 9.



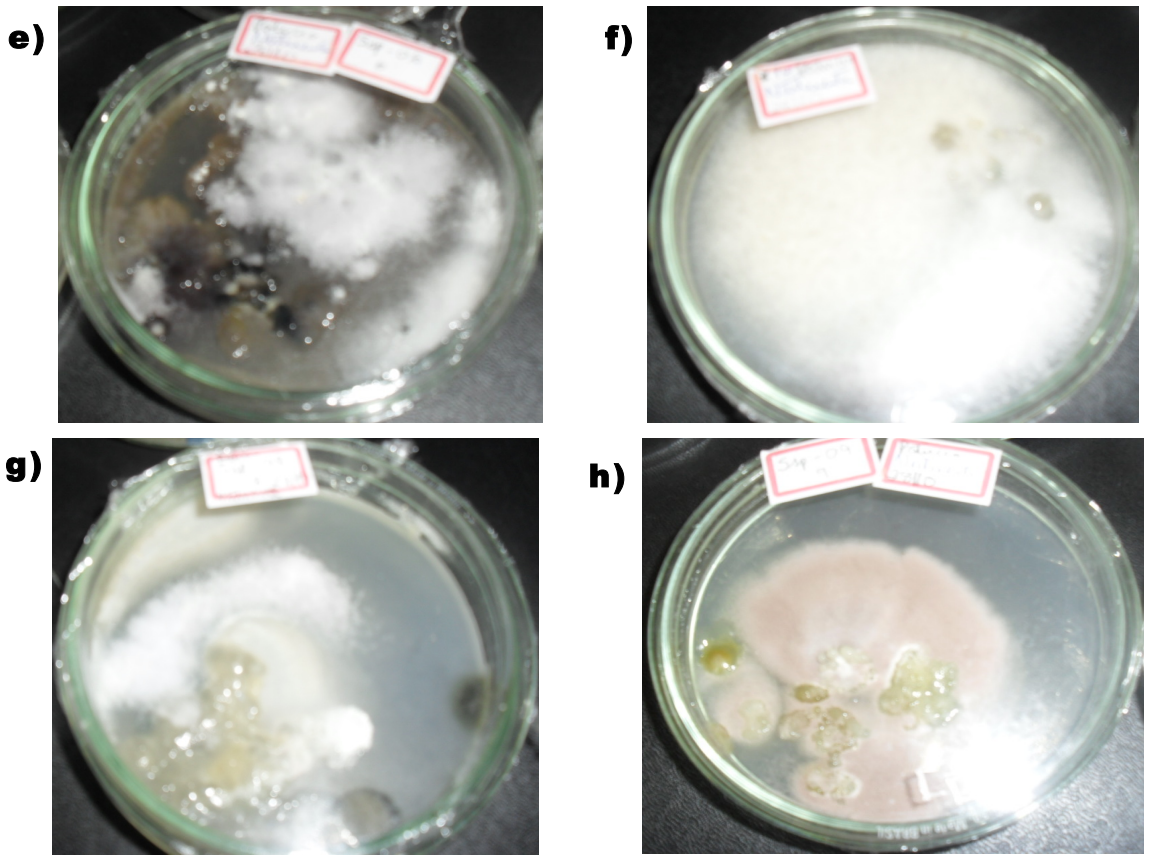
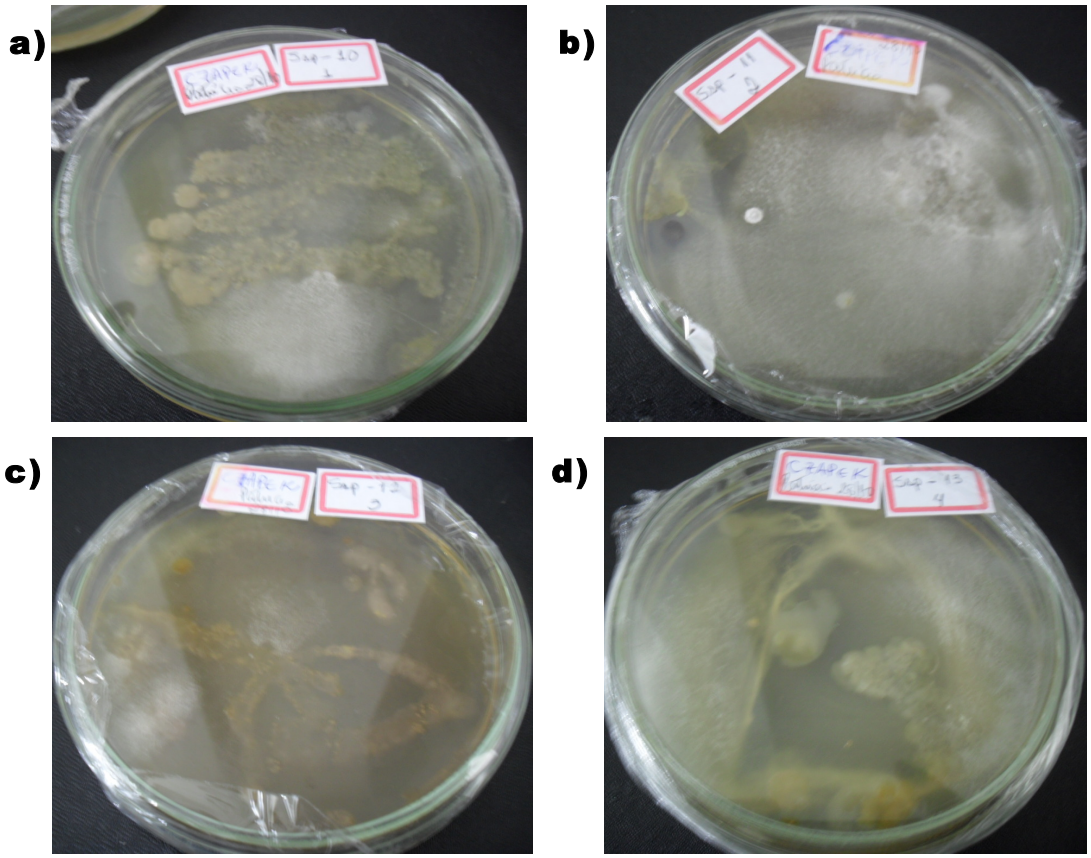


Figure 14: Plates with czapek-dox agar after 10 days of incubation with the inoculum of the nutritive solution *S. spectabilis* seedlings: a) first vessel; b) vessel 2; c) vessel 3; d) vessel 4; e) 6 vessel; f) vessel 7; g) vessel 8 and h) vessel 9.



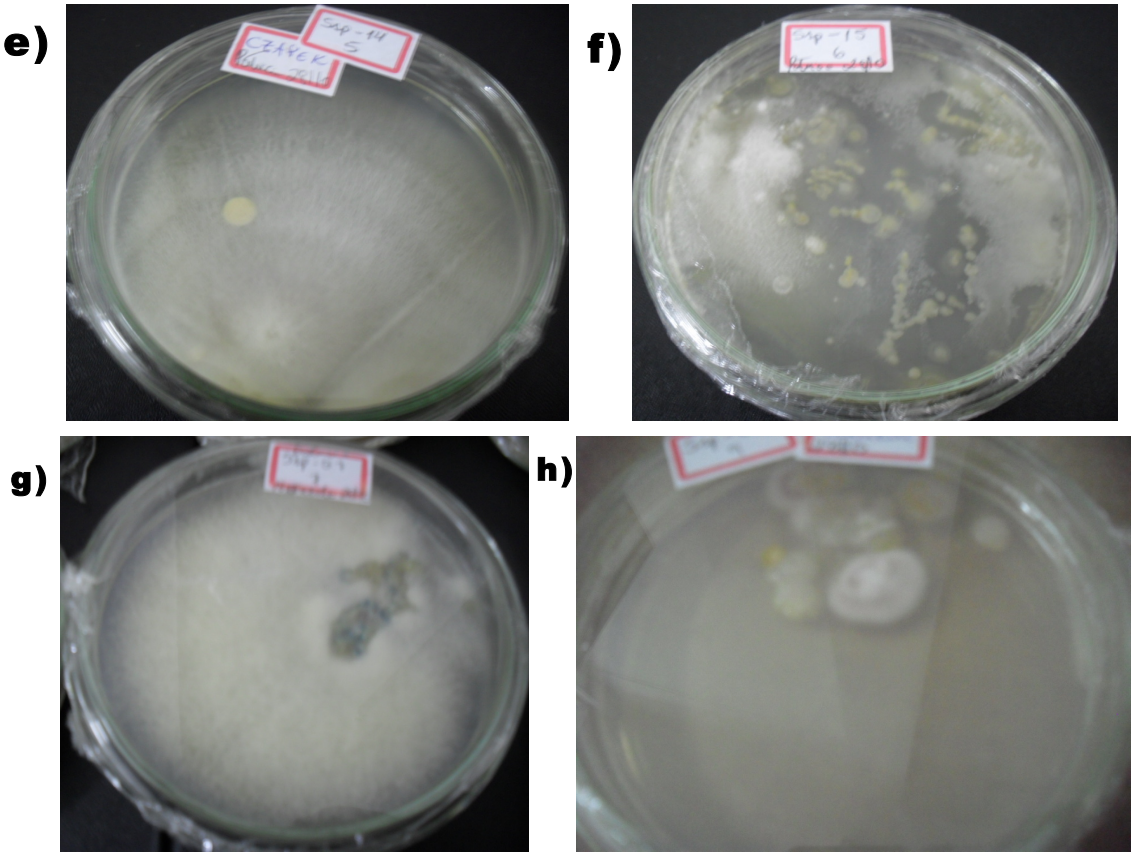
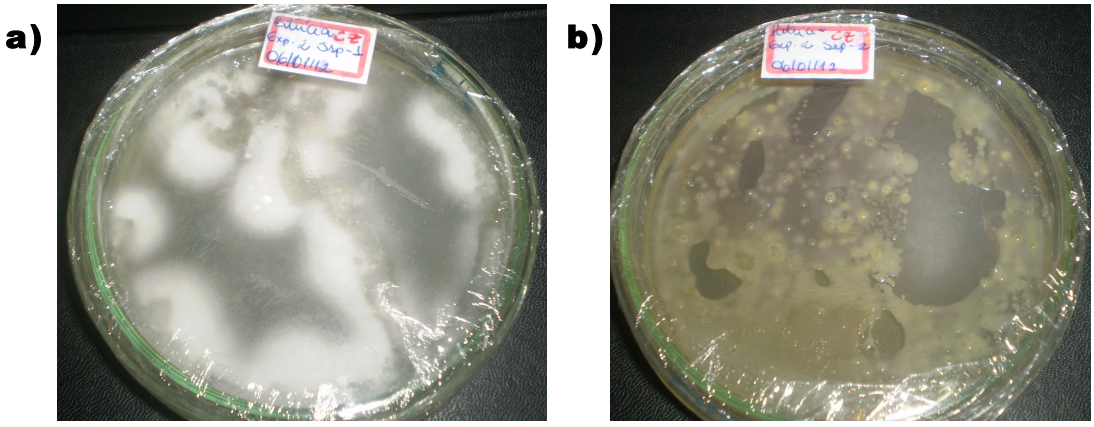


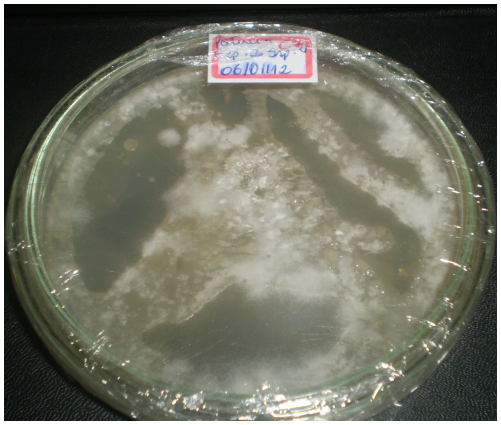
Figure 15: Plates with czapek-dox agar with 10 days of incubation after inoculation with the collected nutrient solution of *S. spectabilis* seedlings: a) first vessel; b) vessel 2; c) vessel 3; d) vessel 4; e) 5 vessel; f) vessel 6; g) vessel 7 h) vessel 8 and i) vessel 9.



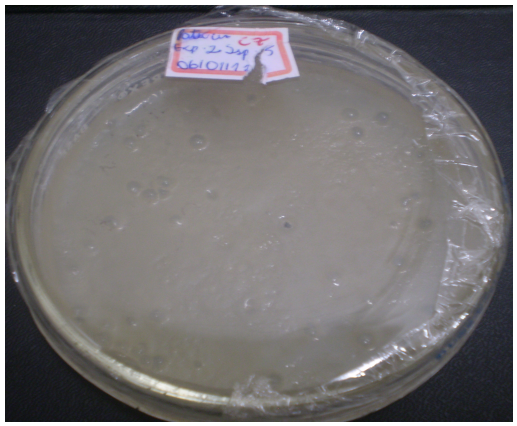
c)



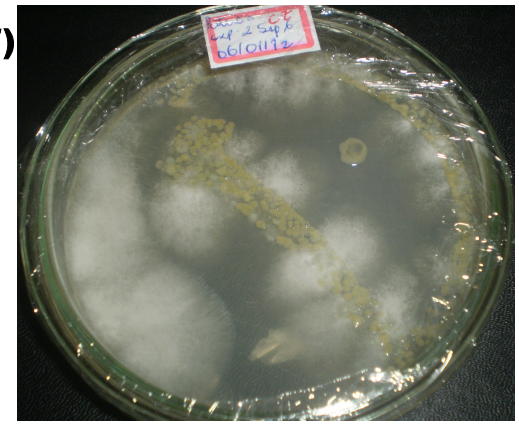
d)



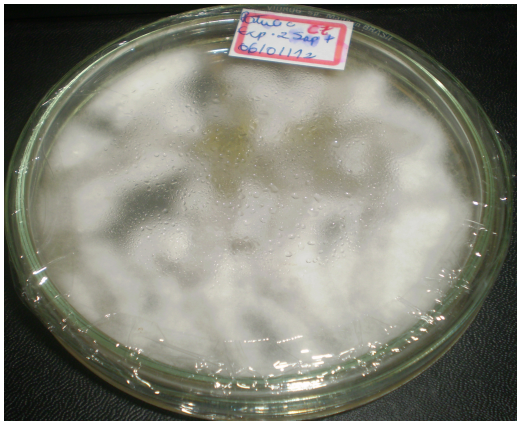
e)



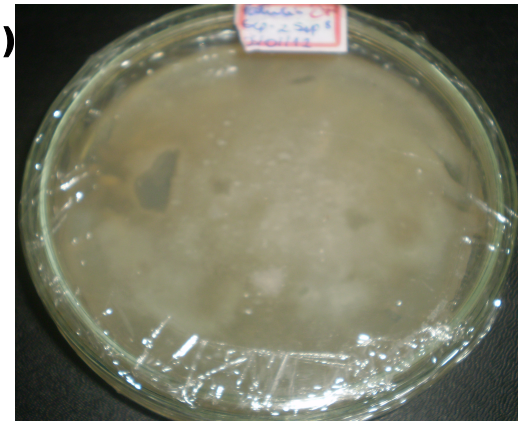
f)



g)



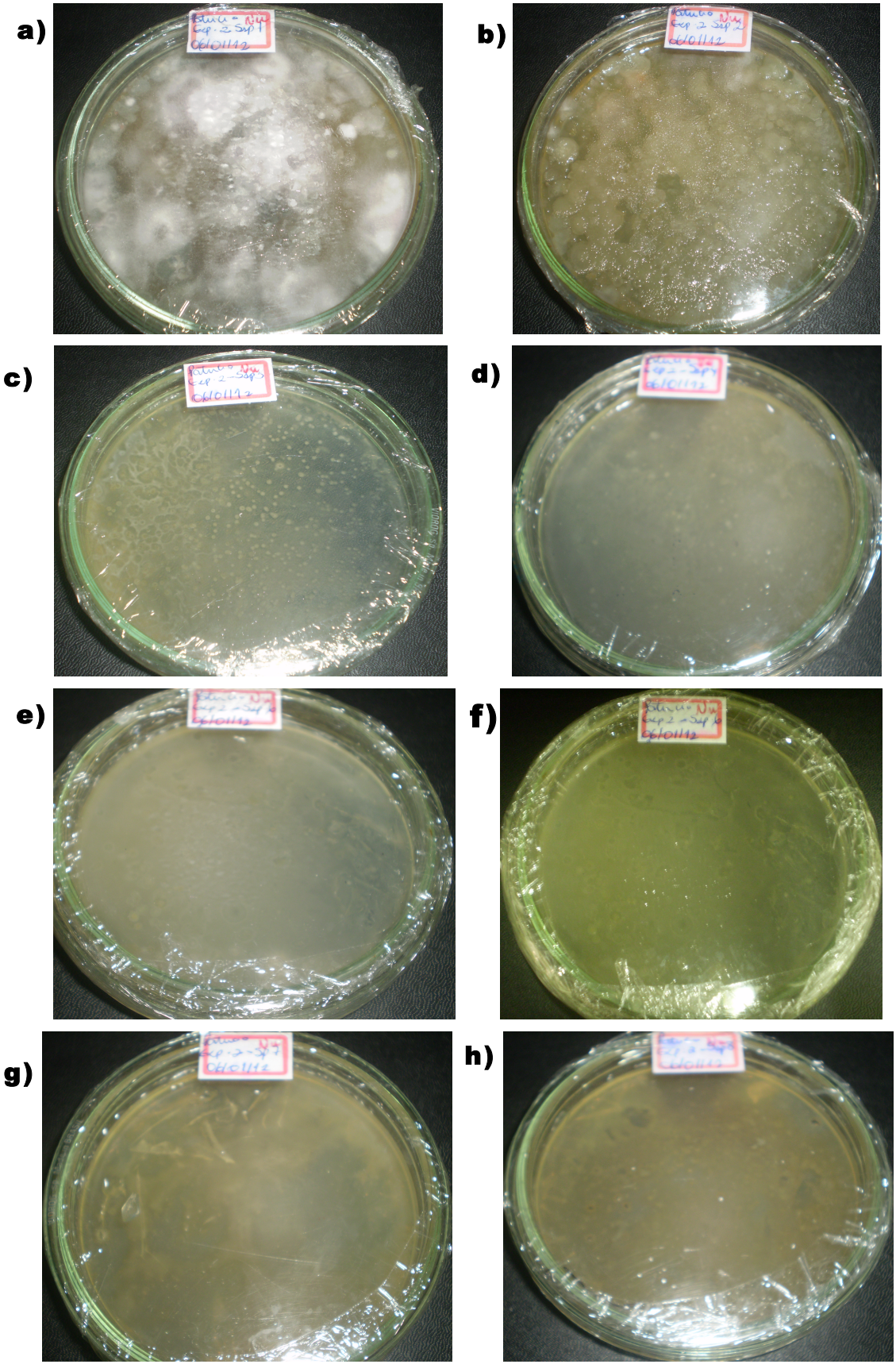
h)



i)



Figure 16: Nutrient agar plates with 10 days of incubation after inoculation with the collected nutrient solution seedling *S. spectabilis*: a) first vessel; b) vessel 2; c) vessel 3; d) vessel 4; e) 5 vessel; f) vessel 6; g) vessel 7 h) vessel 8 and i) vessel 9.



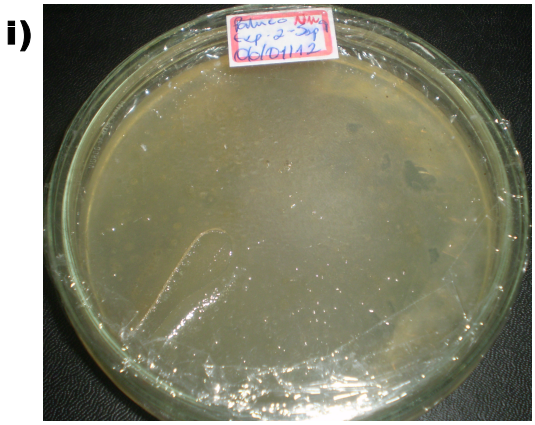


Figure 17: Plates with czapek-dox agar with 10 days of incubation after inoculation with the collected nutrient solution of *S. spectabilis* seedlings: a) first vessel; b) vessel 2; c) vessel 5; d) vessel 6 and e) 8 vessel.

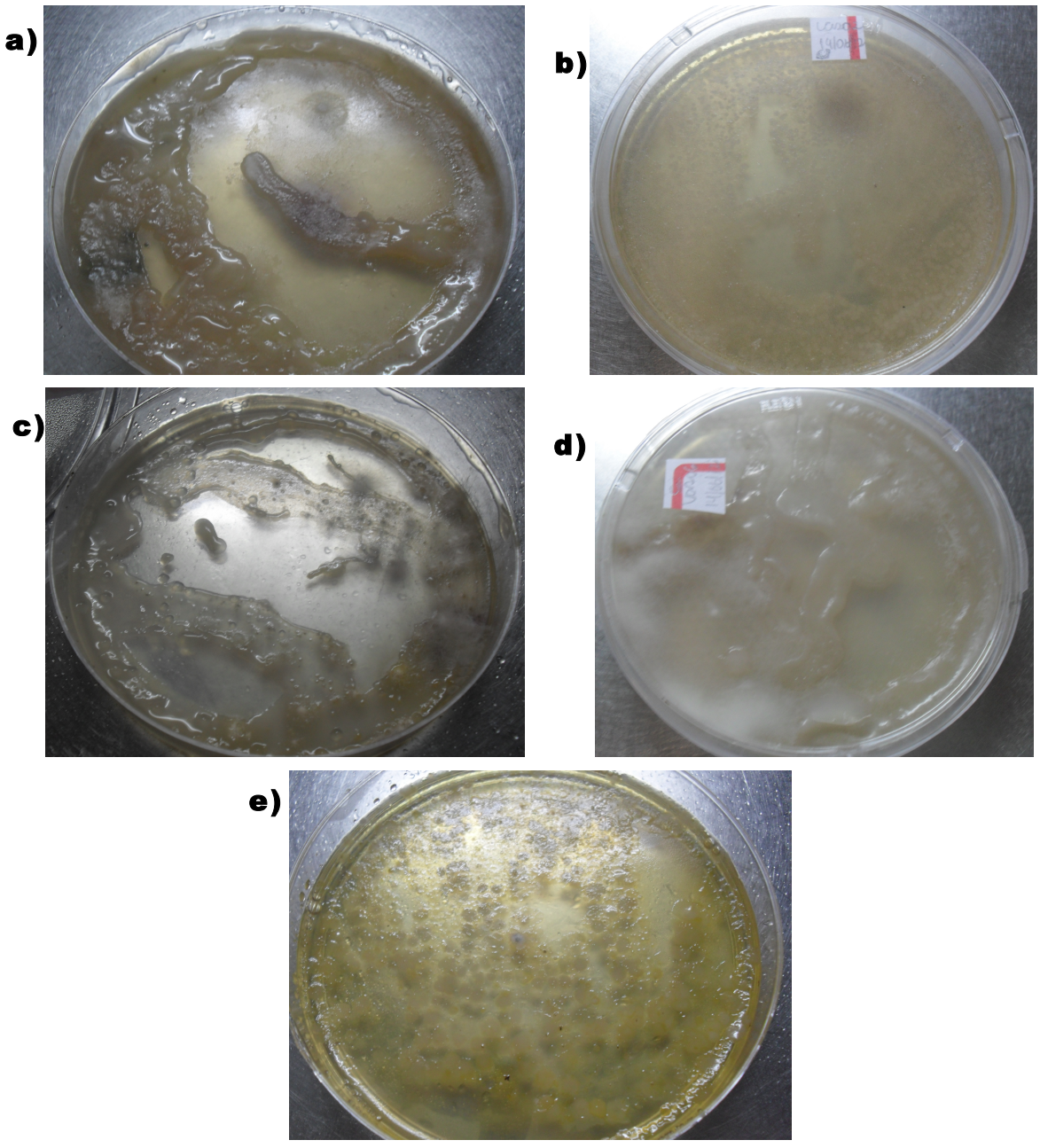
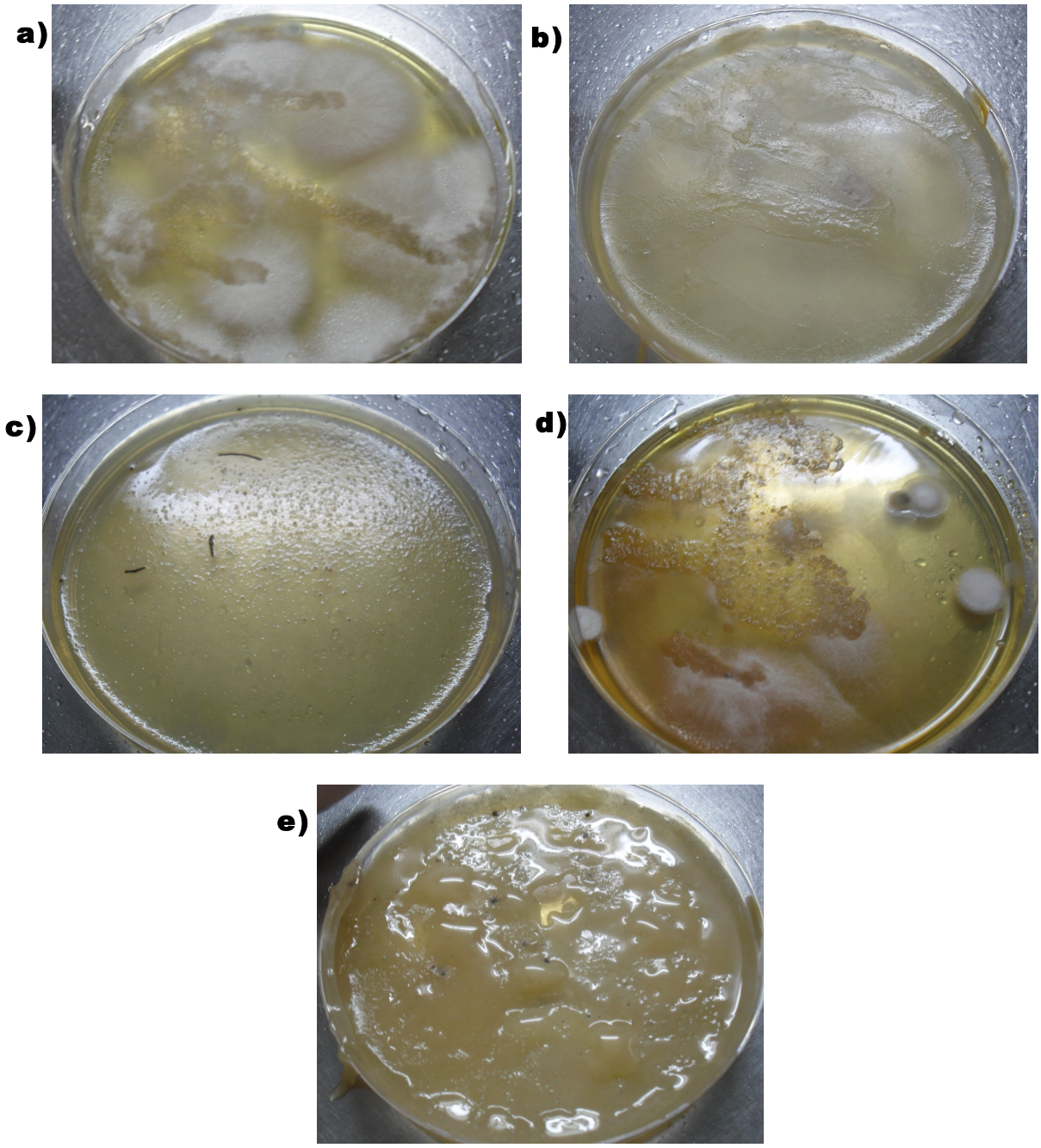


Figure 18: Nutrient agar plates with 10 days of incubation after inoculation with the collected nutrient solution seedling *S. spectabilis*: a) first vessel; b) vessel 2; c) vessel 5; d) vessel 6 and e) 8 vessel.



3.3 Phylogenetic identification of the rhizosphere microbiota

3.3.1 DNA extraction and amplification by PCR

Isolated colonies of bacteria: Table 3 shows that boiling biomass was suitable for DNA extraction of most samples with optimal quantity and quality, except for samples 4, 20, 52 and 58 where low concentrations of DNA were obtained. The quantity and

quality of DNA were determined in accordance with the Nanodrop equipment using the ratio 260/280 between 1.8-2.0 to verify the purity of genetic material. Values of this ratio above 2.0 indicate the presence of contaminants (proteins, etc.). Except for samples 2, 11, 13, 26, 38, 41,44 and 59 the 260/280 ratio was always higher than 1.8 (Table 3) indicating a quality suitable for DNA PCR reactions without the need of purification.

Table 3: Quantity and quality of extracted DNA from the isolated strains of bacteria.

Sample	Identification	DNA (ng.µL^{-1})	280/260 ratio	Sample	Identification	DNA (ng.µL^{-1})	280/260 ratio
2	CSP-4	134.8	1.28	**36**	CSP-49b	25.40	3.06
3	CSP-53a	63.2	2.19	**37**	CSP-19/2a/b/22a	28.8	2.08
4	CSP-11a	11.7	1.75	**38**	CSP-44b/31b	4.61	2.28
5	CSP-R49	94.7	2.28	**39**	CSP-50c	1.6	2.77
6	CSP-R22	43.3	2.13	**40**	CSP-9b	18.8	2.11
7	CSP-43	202.0	2.28	**41**	CSP-30a/29a	104.9	2.27
8	CSP-52b	96.8	3.05	**42**	CSP-39/26b/34	6.9	6.43
9	CSP-1	117.3	1.90	**43**	CSP-52b	51.1	3.40
10	CSP-50a	201.8	3.33	**44**	CSP-50a	44.3	3.90
11	CSP-40	46.1	1.63	**45**	CSP-53b	17.1	9.21
12	CSP-53b	119.7	2.18	**46**	CSP-7	88.3	2.46
13	CSP-4b	127.6	1.29	**47**	CSP-32c/44b	-21.1	2.02
14	CSP-36b	22.4	2.04	**48**	CSP-43	102.6	2.10
15	CSP-13ba	91.8	2.23	**49**	CSP-6a	-33.2	1.84
16	CSP-13b	45.3	1.95	**50**	CSP-15	90.3	2.26
17	CSP-R28	80.1	2.20	**51**	CSP-18	61.7	2.15
18	CSP-R25/26	101.5	2.32	**52**	CSP-12	49.3	2.31
19	CSP-43	254.5	2.17	**53**	CSP-26a/27c	24.7	2.25
20	CSP-6	13.7	2.01	**54**	CSP-49c	252.2	1.78
21	CSP-21	82.6	2.00	**55**	CSP-49/48	281.2	1.74
25	CSP-51	68.7	2.27	**56**	CSP-26	313.7	1.95
26	CSP-54	129.1	2.43	**57**	CSP-R49b	372.3	2.06
27	CSP-53c	52.5	2.72	**58**	CSP-9a	97.9	2.27
28	CSP-R28/53c	34.9	3.00	**59**	CSP-31a	17.6	2.61
29	CSP-55	-37.7	1.88	**60**	CSP-15b	70.1	2.14
30	CSP-53a	90.9	2.41	**61**	CSP-43	-37.9	1.85
31	CSP-36b	281.0	1.94	**62**	CSP-R27	-36.2	1.90
32	CSP-34	518.9	1.97	**63**	CSP-48	30.8	1.79
33	CSP-49a	54.1	2.54	**64**	CSP-56	-38.3	1.91
34	CSP-52a	92.0	2.35	**65**	CSP-R32/31	-38.9	1.87
35	CSP-12b	103.9	2.24	**66**	CSP-6a	-32.8	1.99

The Figures 19 and 20 show the amplified products in PCR reactions using the pair of primers fD1 and rD1 (Domain Bacteria) and 3µL (Fig. 17a) and 5µL (Fig. 17b)

DNA. From Figure 19 it is observed that the PCR reactions performed with DNA 3µL provided better results when compared to DNA 5µL. This shows that under the conditions of PCR and for 1-21 samples, the greater amount of DNA hindered the reaction. As for the samples 25-66, there were no amplification results when using the smaller volume of DNA, the best results being obtained with the greatest amount of DNA. It is also noticed that the amplified products were in adequate size and quality for the detection of a distinct band at the height corresponding to a molecular marker 1500pb. Nonspecific less intense bands in position beneath the 1500pb may be due, for example, to an error in the extension of primers, which can be adjusted by varying the reaction conditions, such as magnesium chloride concentration, increasing temperature and time denaturation, annealing temperature increased, the time span, as well as the use of more specific primers. For sequencing, cutting and purifying the band of interest would be another alternative. However, as there was the appearance of a single band nonspecific and of low intensity, it was decided to perform the sequencing with the product obtained directly from the PCR performed with DNA 3µL for samples of 1-21 and DNA 5µL for samples of 25-66. For the samples that there were no amplification of the 16S rRNA gene fragment, PCR was repeated, the results were positive for samples 3; 5; 11; 13:17 (data not shown) and the same submitted to the partial sequencing of the 16S rRNA gene. The other samples were optimized conditions on a case until the appearance of the expected band but new amplification experiments were performed without success.

Figure 19: Products of amplified by PCR with the set ofprimers fD1 and rD1 for samples 1-23 containing 3µL (a) e 5µL (b) of DNA concentration. C- (negative control). M (molecular marker). *(1500pb).

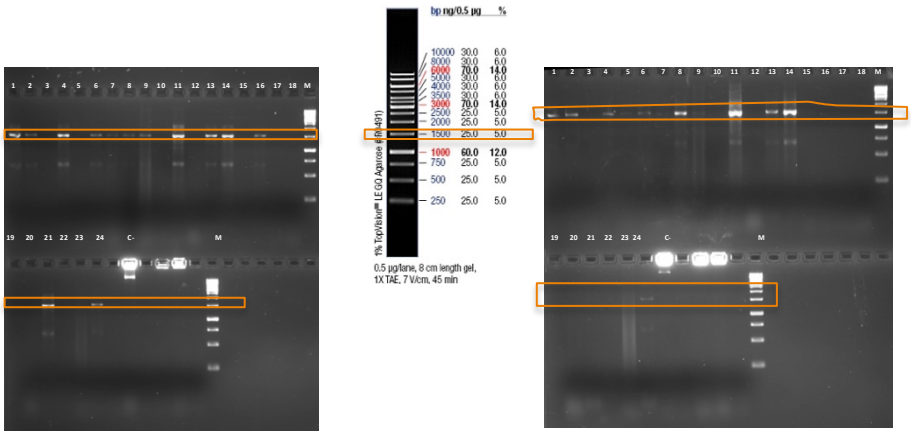
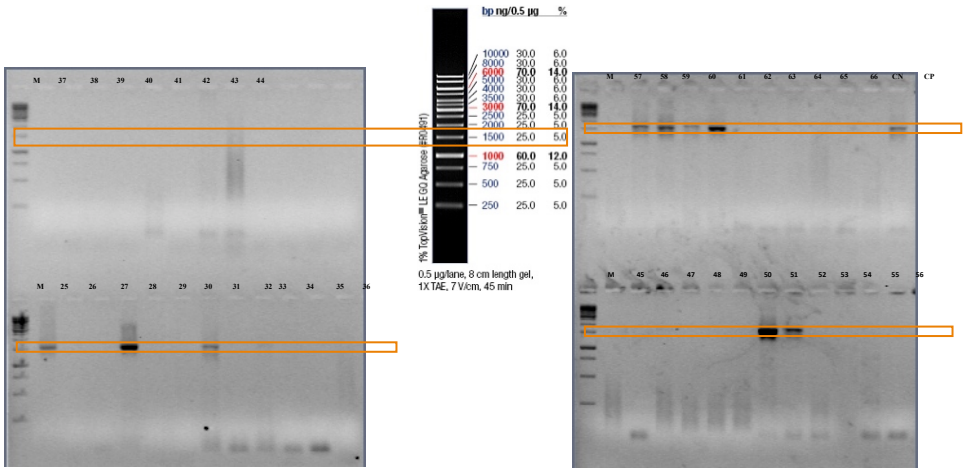
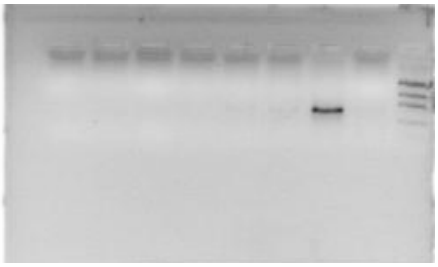


Figure 20: Products of amplified by PCR with the set ofprimers fD1 and rD1 for samples a) 25-44 and b) 45-CN containing 5µL of DNA concentration. CN (negative control). CP (positive control), M (molecular marker). *(1500pb).



Roots biofilm: The amout of DNA obtained from biofilm from the roots was 32.9-ng/µL with 260/280 ratio of 1.98, which means a pure DNA and good quality for analysis. The PCR gel for the biofilm sample is shown in figure 21.

Figure 21: Products of amplified by PCR with the set of primers 563F and 577R for the biofilm roots sample. (molecular marker - 1500pb).



3.3.2 DNA sequencing and sequence analysis

Isolated colonies of bacteria: The quality of the sequencing of PCR products both in forward and in the reverse direction was adequate (Table 4). By comparing the sequences, for both forward and reverse directions, with deposited the database, phylogenetic affiliation was the same. Samples in bold (Table 4) showed a low number of threads base pairs in the forward direction and therefore were only analyzed in reverse direction. Samples 10; 18-39; 44-49; 54-59 and 62-66 were not sequenced, because it was not possible to obtain an amplification in the appropriate PCR.

Table 4: Results from the identification of the isolated strains by partial sequencing of 16S rRNA gene.

Sample	Isolated bacteria	Hydroponic cultivation	Sand cultivation	Sequence lenght (pb)	Description on Gen Bank	Similar. (%)	Gen Bank id.
1	CSP-53b	X		499	*Unc. Shigella* sp. clone Acro289	99	KC110986.1
2	CSP-4	X		590	*Bacillus subtilis*	99	JN835494.1
3	CSP-53a	X		374	*Agrobacterium tumefaciens*	98	JQ659922.1
4	**CSP-11a**	X		214	*Burkholderia* sp	98	JN975060.1
5	**CSP-R49**	X	X	171	*Pseudoxanthomonas indica*	92	JQ660316.1
6	CSP-R22	X	X	374	*Agrobacterium tumefaciens*	98	JQ659922.1
7	**CSP-R43**	X		231	*Arthrobacter* sp	96	FJ189784.1
8	CSP-52b	X		427	*Unc. Shigella* sp. clone Acro289	99	KC110964.1
9	CSP-1	X		460	*Burkholderia* sp	99	JN975200.1
11	CSP-40	X		501	*Enterobacter* sp	99	KC205170.1
12	CSP-53b	X		501	*Enterobacter* sp	99	KC205170.1
13	CSP-4b	X		499	*Shigella* sp. clone Acro289	99	KC110986.1
14	**CSP-36b**	X		262	*Pseudomonas* sp	98	X155416.1
15	**CSP-13a**	X		289	*Stenotrophomonas* sp	95	JX842809.1
16	**CSP-13b**	X		108	*Achromobacter* sp	100	KC151254.1
17	CSP-R28	X	X	374	*Agrobacterium tumefaciens*	98	JQ659922.1
37	CSP-19v6	X			*Achromobacter* sp.	96	
41	CSP-30a/CSP-29	X			*Pseudomonas rhodesiae*	99	
40	CSP- 9b	X			*Pseudomonas* sp	100	
42	CSP-39	X	X		*Pseudomonas* sp	99	
43	CSP-52bI	X	X		*Shigella* sp.	99	
50	CSP-15	X			*Bacillus* sp	95	
51	CSP-18	X			*Achromobacter* sp	99	
52	CSP-12	X			*Bacillus* sp	99	
53	CSP-26/CSP-27	X			*Pseudomonas extremorientalis*	100	
60	CSP-15b	X			*Achromobacter marplatensis*	99	

Roots: Phylogenetic analysis of gene 16S rRNA using 454-pyrosequencing revealed a diversity of the bacterial community on the biofilm of the roots of the seedlings. Numbers of OTUs were estimated using different estimate methods as shown on table 6. Those methods are parametric (based on the rarefaction curve) and the others are nonparametric (CHAO, ACE, Simpson and Shannon). Estimate numbers of OTUs increase with number of sequences that were obtained and a plot of rarefaction curve (OTUs versus the number of sequences) approaches a maximum (Graphic 2).[57] From this curve it is possible to extrapole the true maximum value at any phylogenetic level. At 3% dissimilarity, the rarefaction estimator underestimates number of species. At 20% dissimilarity, the rarefaction estimator predicted the number of phylos. The number of OTUs was determined after defining a cutoff of four levels of sequence similarity (Table 5).

Graphic 2: Rarefaction curve of 16S rRNA sequencing of the roots of *S. spectabilis*.

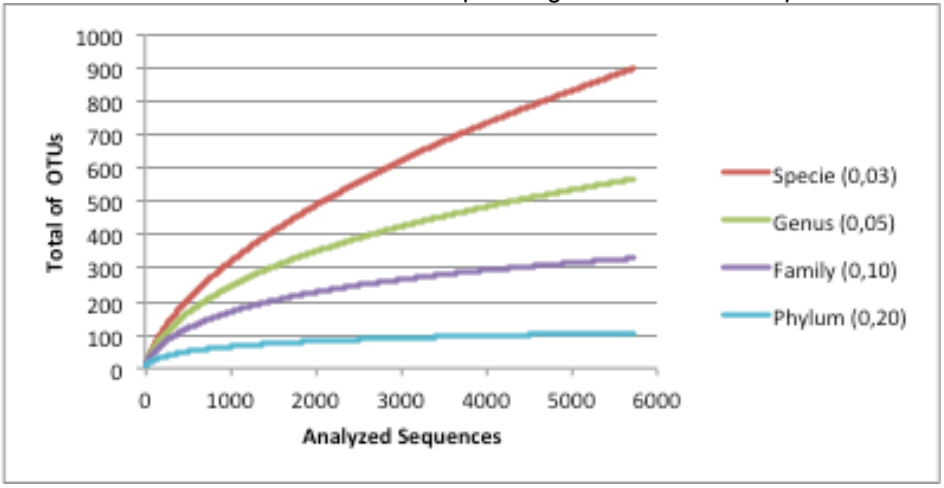
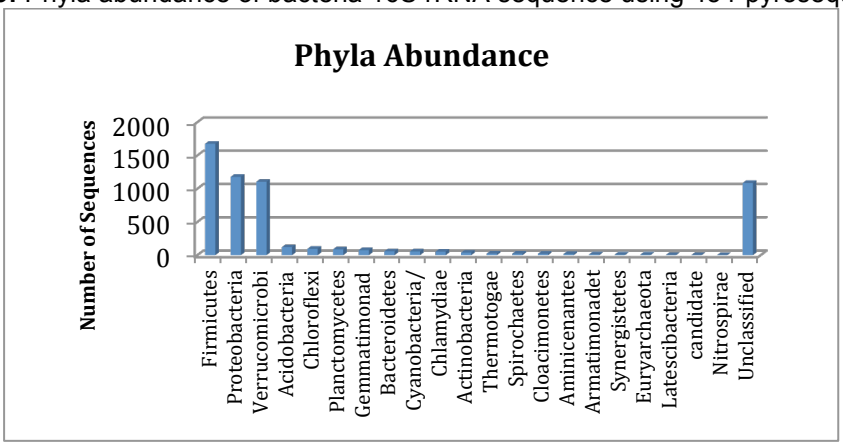


Table 5: Estimators used to estimate the maximum and the number of OTus.

Cutoff	N Seqs	OTUs	Ace	Chao1	Simpson	Shannon	Cover
0.03	5735	900	2701	1952	0.015	5.272	91%
0.05	5735	568	1108	945	0.021	4.789	96%
0.1	5735	330	428	470	0.038	4.161	98%
0.2	5735	107	132	132	0.133	2.693	100%

A total of 5735 sequences were obtained and then were compared to a database available in RPD Project website. It was possible identify around 900 species of organisms present in this database with 97% of similarity after estimating the number of OTUs. The most abudant phyla found was *Firmicutes* present in 29.2% of sequences, Proteobacteria 20.55% and Verrucomucrobia 19.26% of sequence in the sample (Graphic 3). Approximately 20% of the sequences remained unclassified.

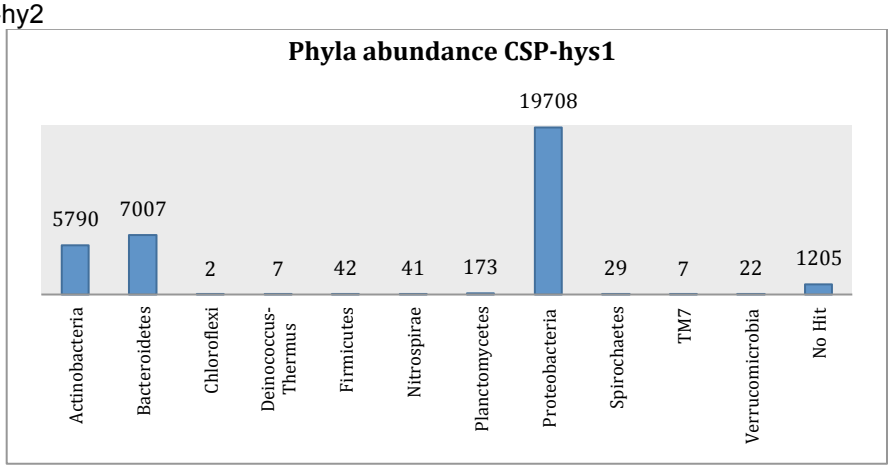
Graphic 3: Phyla abundance of bacteria 16S rRNA sequence using 454-pyrosequencing.

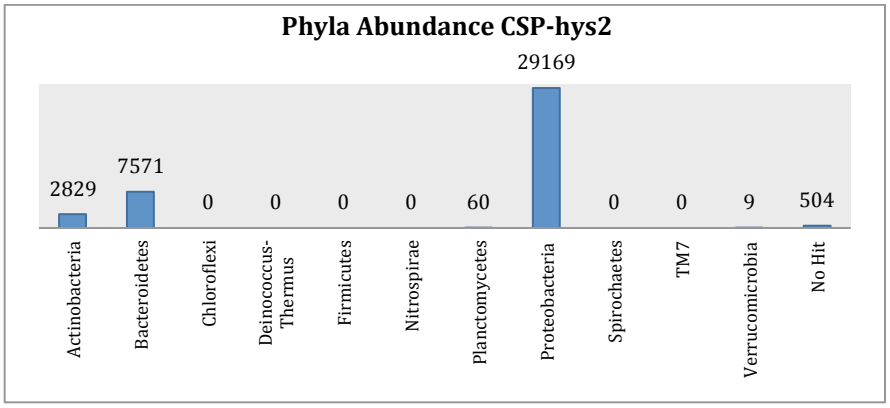


Rhizosphere from hydroponics: The sequencing was made using Ilumina miseq and then the data was analysed by Research and testing laboratory using a USEARCH classifier and in sequence a program for the diversity analysis, belonged to the refered laboratory, and with the OTUs numbers a full information about each taxonomic level (kingdom, phylum, class, order, family, genus and species) was aquired. Different sequencing data was collected for bacteria community and for the fungi population.

A total of 34.033 sequences for bacteria were obtained for the sample CSP-hys1 and 40.142 sequences for the second sample CSP-hys2. The results revealed the bacteria belonged to two different phyla: *Actinobacteria* with 36% of matches in the sequences being the most abundant genera of this phylum *Norcardiodes* with 27% of similarities for the sample CSP-hys1 and 21% for sample CSP-hys2 being in this sample the most abundant genera *Aeromicbrobium* with 62% of sequences matched. *Bacteriodetes* was the vastest phylum for both samples detaining 64% and 80% respectively. A graphic of the taxonomic abundance was plot (Graphic 4).[58]

Graphic 4: Bacteria taxonomic classification of the sequences for: A) Sample CSP-hy1 and b) sample CSP-hy2



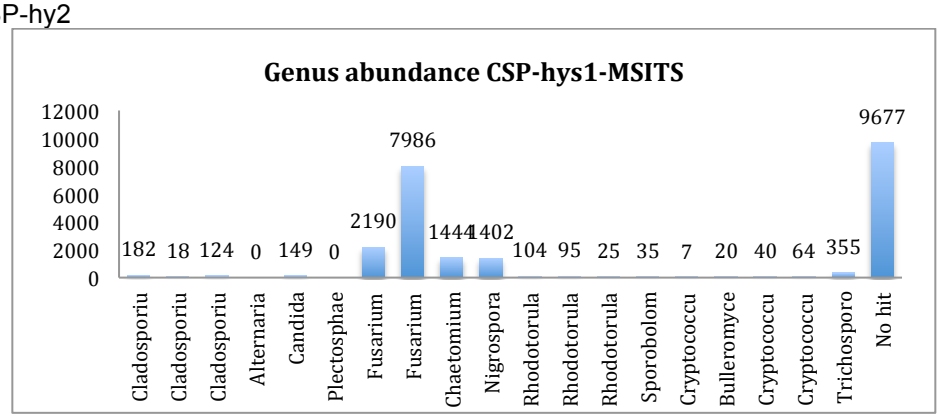


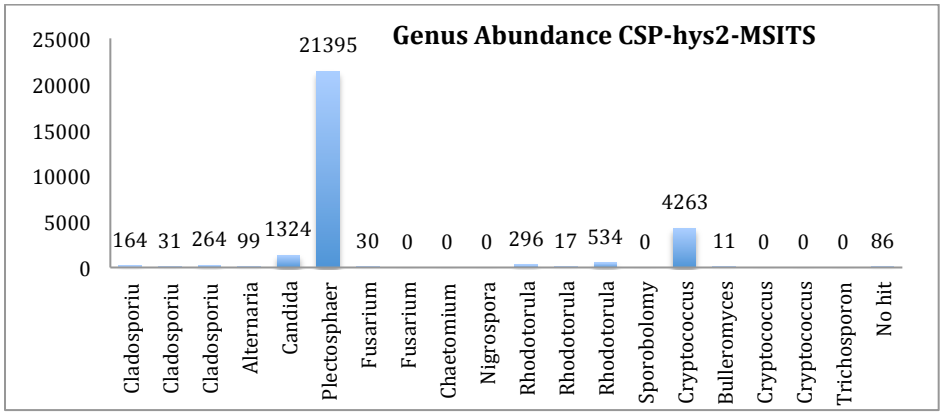
The phylogenetic analysis of the taxonomic groups that makes part of the fungi community was evaluated by 23.917 sequences obtained for sample CSP-hys1 and 28.514 for CSP-hys2 (table 6). The valid sequences were similar to Phylum *Basidiomycota* (3% CSp-hys1; 18%CSP-hys2) and *Ascomycota* (56% CSP-hys1; 82% CSP-hys2). The number of unclassified sequences were 40% CSP-hys1 and 0.3% CSP-hys2. The most abundant genus found in this study were CSP-hys-1: *Fusarium* detaining 33% of sequences followed by *Nigrospora* with 6% and *Chaetomium* also with 6% of the similarities; CSp-hys2 *Plectosphaerellaceae* is the most relevant genus responsible for 75% of the sequences, *Cryptococcus* 15% and for last the genus *Candida* detaining 5% of matches. Genus abundace is related to Graphic 5.

Table 6: Full taxonomic information for fungi ITS rRNA sequencing using Ilumina miseq

Kingdon	Phylum	Class	Order	Family	Genus	Species	CSP-hys1-MSITS	CSP-hys2-MSITS
Fungi	Ascomycota	Dothideomycetes	Capnodiales	Davidiellaceae	Cladosporium	cladosporioides	182	164
Fungi	Ascomycota	Dothideomycetes	Capnodiales	Davidiellaceae	Cladosporium	lignicola	18	31
Fungi	Ascomycota	Dothideomycetes	Capnodiales	Davidiellaceae	Cladosporium	sp	124	264
Fungi	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Alternaria	alternata	0	99
Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetales	Candida	albicans	149	1324
Fungi	Ascomycota	Sordariomycetes	Glomerellales	Plectosphaerellaceae	Plectosphaerella	cucumerina	0	21395
Fungi	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium	oxysporum	2190	30
Fungi	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium	sp	7986	0
Fungi	Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae	Chaetomium	sp	1444	0
Fungi	Ascomycota	Sordariomycetes	Trichosphaeriales	Trichosphaeriales	Nigrospora	sphaerica	1402	0
Fungi	Basidiomycota	Cystobasidiomycetes	Erythrobasidiales	Erythrobasidiaceae	Rhodotorula	minuta	104	296
Fungi	Basidiomycota	Cystobasidiomycetes	Erythrobasidiales	Erythrobasidiaceae	Rhodotorula	Erythrobasidiaceae	95	17
Fungi	Basidiomycota	Microbotryomycetes	Sporidiobolales	Sporidiobolales	Rhodotorula	mucilaginosa	25	534
Fungi	Basidiomycota	Microbotryomycetes	Sporidiobolales	Sporidiobolales	Sporobolomyces	sp	35	0
Fungi	Basidiomycota	Tremellomycetes	Filobasidiales	Filobasidiales	Cryptococcus	magnus	7	4263
Fungi	Basidiomycota	Tremellomycetes	Tremellales	Tremellaceae	Bulleromyces	albus	20	11
Fungi	Basidiomycota	Tremellomycetes	Tremellales	Tremellales	Cryptococcus	peneaus	40	0
Fungi	Basidiomycota	Tremellomycetes	Tremellales	Tremellales	Cryptococcus	rajasthanensis	64	0
Fungi	Basidiomycota	Tremellomycetes	Tremellales	Tremellales	Trichosporon	asteroides	355	0
No hit	No hit	No hit	No hit	No hit	No hit	No hit	9677	86

Graphic 5: Fungi taxonomic classification of the sequences for: A) Sample CSP-hy1 and b) sample CSP-hy2





3.3.3 Classical taxonomic identification of filamentous fungi

Detailed descriptions of known species of fungi are easily found on literature and were used in this work to identify the isolated species.[29] The genus and/or species identified are gathered in table 7. There was a predominance of the genus *Fusarium*, almost 80%, in the selected samples; the most identified specie in this genus was *Fusarium solani* followed by organisms belonged to the genus *Acremonium* (10%).

Table 7: Identification of isolated strain of filamentous fungi by classical taxonomy.

Isolated Fungi	Hydroponic cultivation	Rhizotron (sand)	Morphological identity
CSP-R19		X	*Acremonium sp*
CSP-26	X		*Fusarium sp*
CSP-30	X		*Acremonium sp*
CSP-R38		X	*Fusarium cf. solani*
CSP-4bII	X	X	*Fusarium cf. solani*
CSP-2	X		*Fusarium cf. solani*
CSP-12a	X		*Fusarium cf. solani*
CSP-09	X		*Fusarium cf. oxysporum*
CSP-17	X		*Fusarium cf. solani*
CSP-37a	X		*Fusarium cf. solani*
CSP-11e	X		*Fusarium cf. solani*
CSP-41	X		*Fusarium cf. solani*
CSP-21	X		*Fusarium cf. solani*
CSP-58/59	X	X	*Exophiala sp*
CSP-12Ia	X		*Paecilomyces sp*
CSP-1N	X		*Fusarium sp*
CSP-11a	X		*Fusarium cf. solani*
CSP-11c	X		*Fusarium sp*
CSP-5b	X		*Fusarium cf. solani*
CSP-28	X		*Fusarium cf. solani*

4 Discussion

The rhizosphere is a region rich in nutrients from plant's roots and for this reason it becomes a wealthy source for the development of microorganisms which are associated with plants into mutually beneficial relationships, for example the nitrogen-fixing bacteria that nodulate roots of certain plants such as legumes, or the various mycorrhizal fungi that form beneficial associations with the majority of plant species. Other bacteria and fungi have involved into parasites of plant roots, resulting in huge losses to agricultural production.[59-61] In this study the rhizosphere comunity of an important medicinal plant was evaluated using classical and molecular techniques of identification. The knowledge of the population in this environment is essencial to identify the impact of microorganisms on plant development in general and how these relations can disturb the area directly near the roots, the rhizosphere and consequently the areas nearby in the soil. In this work the focus on microbial community was in bacterial and fungi populations. Those organisms are the highest population in rhizosphere and take an important place in the plant development being responsible for a great number of interactions that happen at the rhizospheric area.[62-64] Bacteria are known to promote plant grow, adjust soil properties as pH, help plant with nitrogen fixation, contribute in the nutrients mobilization, interact with fungi and/or other organisms in the rhizosphere, can also have a negative effect as promoting plant disease or having a neutral relationship with the plant.[64] Fungi also play an importante role on plant nutrition, health and development. Some species of fungi can have negative effects on plants and promote diseases that can devastate crops, however other species can have positive effects stimulating the plant growth, e.g: strigolactone, or they can be antagonist with plant pathogen, decomposing plant residuals, contributing for the composition of bacterial community, stimulating plant to change host plant physiology and patters of roots exudation. In the present study, it was expected to verify the abundance of groups in the microbial community. Some characteristics of these groups can indicate the type of relation can be predicted between the plant and those microorganisms and the role they play during the plant development.[9]

It is importante to say that the rhizosphere community of *S. spectabilis* was not studied, by the time of this work, and this knowlegde is crucial to understand what

kind of interactions this plant possess with its microbial community and how and if these relations interfere on the plant metabolic production.

One of the difficulties encountered was to delimit the area of biological activity, since it depends on environmental conditions such as temperature, humidity and pH of the soil which vary according to geography and changes concerning the depth the roots are. Setting this region in adult trees was a conflicting factor and for this reason raising seedlings of *S. spectabilis* was chosen. In order to understand the development and behavior of rhizosphere region on the roots two different types of cultivation had been used: sand (physical support) and hydroponic (soiless) and through both systems being able to meet the rhizosphere of this important plant specie which has among its secondary metabolites strong biological activity of great social and commercial interests.

The rate of germination of the seeds was not as impressive as the values found in literature[26-27], which led us to believe it was due to the fact that they had been collected two years before this experiment and lost the viability through time. Although it can be said that the cutaneous dormancy was once broken, the adopted procedure was appropriate to speed up the germination process. Under these circumstances the immersion in acid solution was the best one. This method should have provided cracks in the tegument that promoted the entry of water and gas exchange between the enviroment and the seed which favored the development of the embryo.

Throughout the growing process of the seedlings in both kinds of cultivation, and due to the fact that plants sprouted out the seeds and started to grow producing roots, no stems, leaves nor insects were seen atacking the plants. This result was expected as it was found out in literature that this plant is toxic in parts due to the alkaloids composition. All speciems grew well and it was lost examplars only during an external issue at the green house (high temperature) a fact that did not compromise the following experiments. Both systems allowed observing the growing of the roots and the differences they were subjected when changing the environment conditions. In hydroponics the aqueous medium allowed the roots to grow with no friction nor barriers to overcome, which provided a rapid growth of the plant while in the rhizotron a slow growing of plants was observed. In hydroponics, concerning the roots, they hanged in the dynamic flow of the nutritive solution producing big roots that was only interrupted by the botton of the vessel they were in, however, they

started to weave in the neighboring plants and began to spread, forming multiple branches. The morphology of the root is not much different from the one found in the system only containing sand, but also with many more branches, and a thin and brittle root. The difference on growth rate between the systems can be explained by the oxygenation of the root zone. All quantities of water, mineral salts were controlled in hydroponics such as in the rhizotron except for the dissolved oxygen. These were possible to be controlled only on the hydroponics system and it may be one of the factors that favored the fast development of the roots. Lack of oxygen inhibits the formation and growth of roots, and reduces rooting percentage, mass and length. Moreover, it is in the presence of oxygen that the plant's nutritive elements, in the form of molecules, are transported.

The rhizosphere microbiota showed a diverse community of fungi and bacteria. It was possible to see how the microbial population changed possibly according to environmental factors such as temperature, humidity, the concentration of nutrients, exudates released by the plant, the structure of the roots and plant age. Sand cultivation (rhizotron) showed a more visible predominant growth of bacteria than filamentous fungi, the hydroponic, on the other hand, showed at least at first harvest a predominancy of filamentous fungi. The results obtained in here suggest that the difference in the arragement of root structures and the environment conditions by the time of harvest contribute for a predominance of one organism or another. Bacteria have a large physiologic capabilities, for example they can be aerobic or anaerobic and some species can survive with or without oxygen. They can live in association with the plants or be on the rhizosphere without directly interacting with them, only because of the exudates, specially sugars and aminoacids releasead by roots.[22] Fungi are all decomposers of dead and/or living organic material and can move more actively through soil, by growth of their thread-like hyphae.

The plates containing nutrient agar showed a plenty of fungi directly associated with each other during the plating and the separation was difficult, i.e.: it was not possible to isolate a white fungus that grows over a blue fungus - this fact can be an indication that the development of two directly connected microorganisms, one depends upon the other for survival, whether parasitic or beneficial. Something similar was observed in the czapek-dox agar plates with reference to bacteria and fungi. Both were hard to separate from each other and it was necessary to make the cultivation in liquid medium, the same used (nutrient and czapek) without the

presence of agar, and then plating the suspension to achieve easy separation of the pure strains.

All the isolation process for obtaining pure strains was performed after 60 days because of that directly association among some organisms. This association was mainly among fungi and minority bacteria and fungus. This delay in time was necessary to ensure that no other microorganism could grow on the plates from the dilutions and there was some difficulty in separating some organisms that were higly associated with other individuals.

The phylogenetic of taxonomic groups that compose the bacterial population of the rhizosphere of *S. spectabilis* were first evaluated individually using the isolated strains. Those pure individuals came from all vessels where the seddlings had been cultivated in nutrient solution and in sand. Most of the isolates of bacteria identified in this work were obtained from hydroponic cultivation and a few from rhizotron (sand). By comparing the sequences of cultures deposited in the database has been identified that distinct groups of bacteria were frequente such as *Pseudomonas*, *Achromobacter* and *Agrobacterium*. Bacteria from genus *Pseudomonas*, *Stenotrophomonas*, *Bacillus* and *Arthrobacter* are frequently found in soil; some organisms belonging to genus *Stenotrophomonas*; *Burkholderia* sp are easily found on rhizosphere and some are found as endophytic bacteria *Bacillus subtilis*; *Pseudoxanthomonas indica* and *Agrobacterium tumefaciens*. In general those bacteria interact with the plant in beneficial way. For example, Silva et al.[59] observed that *Stenotrophomonas* sp promoted the growth of *Brachiaria brizantha* for favoring nitrogen fixation. *Burkhoderia* species, nitrogen fixers also promoted the growth of corn and rice[65-66] and diazotrophic bacterium *Bacillus subtilis* can be applied to control pests of growing plants such as tomato.[66] *Pseudomonas*, for example, can cause the growth of maize due to the suppression of pathogenic microorganisms. Species of *Rhizobium*, *Enterobacter*, *Pseudomonas*, *Burkholderia*, *Achromobacter* and *Shigella* favor the growth of plants by solubilizing the phosphate.[67] *Arthrobacter* has been found in some plants and rhizosphere has the ability to produce indole acetic acid that favors the growth of wheat[68] as well as other biologically active metabolites. On the other hand, *Agrobacterium tumefaciens* is a Gram-negative bacterium that causes crown gall disease in plants.[69] Economically, this organism provokes serious problems in crops of walnuts, grape vines, stone fruits, nut trees and sugar beets. The infection by *A. tumefaciens* occurs due to the capacity of this

organism to transfer Ti plasmid DNA into plant cells where it is integrated into the host chromosome, this happens through the photoassimilates accumulated on rhizosphere. This property of *A. tumefaciens* in transferring Ti plasmid DNA has been used as a model of horizontal gene transfer in plants and in fungal transformations becoming a usefull tool in biotecnology.[70-71]

The 454-pyrosequencing was used in this work to investigate the roots' biofilm sample. The data were analysed using the free RDP database project so that the OTUs numbers and microbial diversity were determined. Through the rarefaction curve, it was observed the saturation is closely to be reached at philum level, while at species it would be necessary several sequences to reach it. Even though, the ecological cover results indicate that a big fraction of the bacterial community present on the roots biofilm were represented on the analysis. Additionally, a refined phylogentic analysis based on the OTUs was compared to database and indicated that three major phyla are dominant: *Firmicutes*, *Proteobacteria* and *Verrucomicrobia*.

Firmicutes contain around 2.475 species and has three classes: *Bacilli*, *Clostridia* and *Erysipelotrichi.*[72] All organisms of this phylum are Gram-positive.[72] From *Clostridia*, the genus *Anaerosporobacter* one of the most abundant in this work is a unknown genus with a few representative such as *Anaerosporobacter mobilis* gen. nov. sp. nov. isolated from a forest soil sample in Korea.[73]

The phylum *Proteobacteria* is represented by five classes α-; β-; γ-; δ-; and ε-*proteobacteria.*[74] They are Gram-negative and consist of a very abundant phylum in soil bacterial communities. α-proteobacteria, the most abundant class in this work, has as members nitrogen fixing bacteria such as *Rhizobium* and *Azospirilium*[67], one the most abundant genus with 4% of sequences;

Verrucomicrobia phylum is commonly found in soil and water enviroments. This work approaches three abundant genus: *Protheobacter*, Subdivision 3_genera_ incertae_sedis (the name means that the classification of this genus belonging to the subdivion3 class of the phylum *Verrucomicrobia* is uncertain) and *Opitutus* making up almost half of genus abundance.[75]

Members *Protheobacter* genus are Gram-negative, particularly aerobic bacteria, have tubulin-like genes and can grow with low concentrations of nutrients. They present prosthecae that increases the area of the cells that can help the heterotrophic bacteria enhance its respiration and nutrient uptake and thus enabling

it to fix in solid substrates.[75] They contain a few species related: *Protheobacter fusiformis, Prosthecobacter dejongeii* (Hedlund et al., 1997), *P. fusiformis* (Staley et al., 1976), *P. debontii* (Hedlund et al., 1997), *P. vanneervenii* (Hedlund et al., 1997), *P. Fluviatilis*[76] and *Prosthecobacter algae sp. Nov.*[75-77] The *Opitutus* genus is represented by one single specie *Opitutus terrae* and was related as being isolated from soil where rice plants are cultivated. According to other studies, reports of bacteria from *Verrucomicrobia* indicates that they are diverse depending on the type of soil and the predominant class is *Spartobacteria* which was reported as endosymbiont with nematodes.[78] The ecological factor involving this phylum is not totaly known, since some species are difficult to be cultivated, then compromises the knowledge about this phylum.

Microbial community of rhyzosphere from hydroponic cultivation was evaluated through molecular identification technique Ilumina smilieq and it showed a rich and vast species of microorganims. Both bacterial and fungi were studied based on the richest OTUs by comparing the sequences with data base, allowing to observe the general distribution of the sequences through those different groups bacteria and fungi. Differents regions of conserved genoma were used in the sequencing because they are distint for each group: 16S rRNA for bacteria and ITS for fungi so the primers used were the ones that are better recognized to be used on the sequencing. Two samples CSP-hys1 and CSp-hys2 were used, both from hydroponic but not the same vessels. For this reason some properties such as pH and roots structure were distint, so these characteristics can interfere on the microbial community as it was seen on the results. Similar phyla were found for bacteria and fungi in both samples; however, some species are present only in one of the samples and the percentage of genus or even phylum was different too. The number of non-classified samples also differs in the samples and with the type of rRNA region was sequenced. The database hold at RPD or USEARCH classifiers, the last one used in this experiment, is limmited by the sequences that are avaliable, which means, for example, that some new organisms cannot be described on the database, or the sequences are not adequate due to the chimeras formation even if a treatment to withdraw those had been done.

Bacteria were sequenced in this method using the V3-V6 regions of 16S rRNA differently from the previous experimente using 454-pyrosequencing focus on V4 region. A distint database for classification was also used. The effective sequences

selected were assigned to different taxa level: phylum, family, order, genus and species and the number of unclassified sequences were 4% and 1% of valid sequences. For both samples the major phylum was *Protebacteria* followed by *Bacteriodetes* and *Actinobacteria* of sequences respectively. Those results agree with the literature where these phyla including the *Verrucomicrobia* are the most abundant for soil samples. In this study the *Verrucomicrobia* is less than 1% of sequences for both.

Both samples, CSP-hys1 and CSP-hys2, have different percentages of genus population and species. *Proteobacteria* in CSP-hys1 is represented by α-*proteobacteria* family withing *Agrobacterium tumefaciens* representing 15% of species in *Agrobacterium* genus, and followed by *Betaproteobacteria* family with 86% of this group being represented by the specie *Simplicispira metamorpha* from *Burkholderiales* class. CSP-hys2 was delineated by sequences belonging to *Brevundimonas* sp from α-*proteobacteria* Family and *Pseudomonas* sp from γ-*proteobacteria* Family.

Agrobacterium are Gram-negative bacteria, generally found in soil, associated with plants through roots, tubers and underground stems. The bacteria can cause tumors and other diseases on plants as previously described to *Agrobacterium tumefaciens*[70], and although many species of this group are non-pathogenic, many were found in benefical associations with plants. *Comamonadaceae* is a group of Gram-negative bacteria characterized by a wide metabolic diversity including aerobic organotrophs, anaerobic denitrifiers and Fe^{3+} reducing, hydrogen oxidizers, photoautotrophic and photoheterotrophic.[79] They can be found in water, soil, Antartic and hot springs habitats, however, some are also pathogens and have been describe isolated from earthworms, rumen fluid, and human clinical samples.[80] *Brevundimonas*[81] is a group of Gram-negative bacteria, comprised in 16 species. This group was formed by species that once belonged to *Pseudomonas* and *Caulobacter* genera. *Pseudomonas* is a widespread group containing around 202 species related to this genus. The bacteria are non-sporulating, aerobic, Gram-negative, rods and are found inhabiting a large range of niches that include water, soil and associations with plants and animals, also can be found in biofilms and planktonic forms. Some studies show the diversity on biochemical pathway of this group such as nitrogen fixation, alginate production and respiratory mechanisms.[82] Due to its enormous metabolic versatility they are important on medicine and

biotechnology.[81] Most of the pathogenic members are related to plants and a few to animals.[81]

Actinobacteria has as majority genus *Microbacterium* in CSP-hys1.[83] This genus comprises around 71 species and it is composed by fermentative Gram-positive bacteria, characterized by yellow or orange rods. Some species can be nitrate redutase, fermenters or oxidase. In this work *Microbacterium phyllosphareae* was the major bacterium in the sequences matches and this organism is generally isolated from phyllophere of grass and wastewater and recently was reported being used as biosensor to determine BOD in wastewater.[83] *Aeromicrobium* was a majority genus in CSP-hys2 and the members of this group are a Gram-positive, motile, rod-shaped bacteria. The members are comprised in seven species isolated from soil and seawater, by the time of this writing, species were as follows: *A. erythreum*, *A. alkaliterrae*, *A. marinum*, *A. fastidiosum*, *A. panaciterrae*, *A. tamlense* and *A. ponti* sp nov.[84]

Bacteriodetes are lagely represented with the genus *Flavobacterium* in both samples. Species of this genus were found in a bunch of extreme and diverse environment such as glaciers, polar ice and sediments, warm springs, and hard water creek. They are characterized by rod shape, motile and no-motile Gram-negative bacteria consisting in 130 species decribed. Some species are fish pathogen and cause diseases as cold water disease and rainbow trout fry syndrome, others are related as opportunistic pathogen as *Flavobacterium johnsoniae.* Members of this group can produce enzymes, some cold adapted that can be used in biotechnology.[85]

The valid sequences for fungi were similar to two phyla that contain most known species of fungi: *Ascomycota* and *Basidiomycota*.

Phylum *Ascomycota* is a largest group in fungi division with around 32.000 species described. It is an important group for economy as yeasts used in food industries, molecular biology, pharmaceutical and plant and human pathogens. They can be found in terrestrial and aquatic environment such as saprophytes, parasit, symbiots or endophytes; in air generally are found conidia of *Fusarium*, *Cladosporium*, *Alternaria*, *Nigrospora* and *Acremonium*. *Ascomycota* presents two kinds of reproduction: sexual (ascus- a tube shaped vessel which contain sexual spores produced by meiosis) and asexual (conidia or mitospores-contain one nucleus and are products of mitosis cell division). After a refined analysis of the

sequences, three predominat genera in CSP-hys1 *Fusarium*, *Nigrospora* and *Chaetomium* were found. Species of genera *Nigrospora*[87- 89] and *Fusarium*[90-93] are knowm as seriuos plant pathogens specially fruits, seeds and leaves. Species of *Chaetomium* are densely setose, ovoid to pyriform ostiolate ascomata, clavate asci and pigmented, one-celled ascospores and are importante in plant decomposition and are isolated from dung, plant debris and soil.[94-96] Curiously those genera have not been found in the CSP-hys2 sample which can indicate some alteration in the cultivars as pH, roots structure or maybe the patter of exudation by the roots. CSP-hys2 has as majority phyla *Plectosphaerella*[97] and the yeast *Candida*[98], both know as pathogens, the first one plant pathogen attacking specially fruits and roots; and the second a human pathogen.

The *Basidiomycota*[86] phylum contains around 30.000 species described. The most known species are the ones that produce mushrooms which are the sexual reproductive structure and also beeing part of this group are yeasts and asexual species. They play an importante role on carbon cycle, some species are dead organic matters including wood and leaf litter decompositors. Economically they have been used as food in many countries, mushrooms, toxins can be used as hallucinogens it can be aldo deadly, paper production and bioremediation due certain enzymes. Some symbiont species are plant, animal and human pathogens but also can be useful fou its host as ectomycorrhizae (association with plants roots) and associations with insects. Basidiomycota members can be found in terrestrial or aquatic environments, are characterized by the basidia production. The basidium is a cell, which the nuclear fusion and meiosis occur; the basidia can be formed in the multicellular fruiting bodies or in single cells or yeast. One characteristic of this group is the production of ballistospores by the basidia, it can be sexual or assexual and are dispersed through the air; the aquatic species don't have those structures. Most sequences in CSP-hys2 were similar to *Cryptococcus* from *Filobasidiales* Family, this group is a teleomorph form of *Cryptococcus*[99] that belong to *Tremellaceae* Family. Both are found in the examplars studied in the present work but the filamentous fungus *Cryptococcus magnus* was the most abundant specie in CSP-hys2 with a few number of sequences in CSP-hys1. This fungus is a pathogen in plants, human and animals specially in the yeast form but a recente study showed that this specie can be used in control of anthracnose by *Colletrotricum gloeosporioides* in papaya fruit.[99]

The environment in which plants are is crucial for the development and maintenance of the microorganism's population. Any alterations in the conditions such as temperature, pH, nutrients concentration, roots structure, will contribute to regulate the microorganisms' community. Furthermore, the majority populations identified in this study is in agreement with the microbial populations described in the literature for rhizosphere, soil and hydroponics. Also when comparing the results of phylogenetic analysis it was possible to note a repeatability of the species of fungi and bacteria during all harvests and type of cultivation, indicating that the microbial community adjusts with the plant.

CHAPTER 2:

Cultivation of fungi and bacteria, co-culturing, obtainment of crude extracts and multivariate analysis

1 INTRODUCTION

1.1 Cross talking between microorganisms from rhizosphere

Plants have an intimate relation with microorganisms living inside or outside plant tissues. Microbes can be pathogens, have a mutualistic relationship or even have neutral interactions with plants.[101-103] These interactions occur especially in the environments that plants provide for the microorganisms; one of these zones is the rhizosphere. This interface region between plant and soil is not a region of definable size or shape but consist of a gradient of chemicals, biological and physical properties that change radially and longitudinally along the root.[101; 104]

Rhizosphere is the most dynamic region of the bulk soil because of the exudates released by roots that are responsible for maintaining a diverse species of microbes that are attracted by available nutrients.[101;104-105] These microorganisms compete which other and some are successful whilst others not. Interactions can be intra and/or interspecies and they are based on the exchange of signaling molecules between two partners.[101;106] As result of this interaction, partners acquire new traits and properties and eventually change their surrounding environment, therefore influencing a bunch of other neighbors and the host. Many of these interactions are regulated by the exudates released by roots, affecting the rhizosphere organisms that will later interact with the plant and its neighbors.[101; 103-105]

Some of main interactions that happen and are important are the ones between fungi/bacteria.[101;107] Those organisms are the majority of the population in the rhizosphere and are responsible for the important communications between plant/bacteria; plant/fungi; plant/fungi/bacteria and fungi/bacteria; these processes take place with distinct classes of organisms, demonstrating that the partnership is diverse and holds a great number of different organisms. The nature of these relationships is still not certain; there is some speculation that one of microbe benefits the other by facilitating the nutrients acquisition, phosphate transportation and nitrogen fixation, in a symbiotic side.[101-102;107] For example, Partida-Martinez et al[102;108] demonstrate that the toxins rhizoxini and rhizonin are produced by the endophytic bacterium belonging to *Burkholderia* family (the parasite) and not to the fungus *Rhizopus microspores* (the host), so the fungal pathogenicity is activate by the symbiont. Still on symbiosis, roots have great interaction with mycorrhizal fungi

even though all mechanisms of this talk are not totally clear.[101] It is known that some flavonoids and strigolactones released by plants roots attract the arbuscolar mycorrhizae (AM) fungi but not other species of fungi.[10;64;101] Also, studies have demonstrated that AM fungal exudates affect soil bacterial composition and bacteria associated with AM can improve colonization, root branching and antifungal properties. *Burkholderia* not only can be a parasite of AM fungi but have also been found as plants endophytes and some relates connect the growth promoting of plants being favorable because of the endophytic bacteria.[101-102;108] On the contrary, some endophytic bacteria have a negative effect on plants causing diseases.[64] Bacterial/fungal pathogens interactions are widely studied due to molecular techniques which can easily answer questions about distribution and occurrence of specific modes of actions and also due to the capability of these interactions on providing information concerning basic host/pathogen molecular communication.[10;101-103] Bacteria produce antifungal compounds and can be used to control fungi pathogens.[103] Furthermore, species of bacteria from *Pseudomonas* are broadly cited in many papers for a variety of reasons[101;107]: they are easy to genetically manipulate in lab, are fast growing, are common organisms in the rhizosphere and produce antifungal substances; e.g. *Pseudomonas aureofaciens* have been used as control of *Sclerotina homeocarpa* (dollar spot) on bent grass.[110]

The search for new bioactive compounds has turned to small molecules derived from the cellular metabolism produced by microorganisms, which, however, there is still a poor understanding of their functions and, in most cases, little knowledge on their true biological roles.[111-112] The communication methods on the rhizosphere can be helpful on human health, agriculture and the environment.[101-102;113] Microorganisms are not alone and they have to interact with their neighbors and this exchange through signaling molecules is an important way to know how this dynamic works.[101] For this reason, understanding how mechanisms of communication work and the discovery of the small molecules involved in these processes offers potential for expansion in the repertoire not only of antibiotics as well as other important medicines for humanity.

The rhizosphere, for being an area of strong biological and chemical activities, it has become a rich source for understanding these biosynthetic pathways, which are stimulated by the interaction among the various organisms present in rhizospheric region and their interactions with the host plant.[115] Understanding the dynamics of

biological systems combined with an advanced chemical separation and characterization techniques promote the construction of large chemically diverse libraries of bioactive compounds for therapeutic applications.[114-116]

1.2 Stimulating cross talking between microorganisms

Maximizing the productivity of a single microbe through turning on silent or cryptic biosynthetic genes by varying accessible cultivation parameters such as media composition, pH, temperature, oxygen supply, quality and quantity of light, bioreactor platform or addition of precursors or enzymes inhibitors, are some of the parameters that can be used to stimulate a stress in the microorganism and force it to alter its primer characteristics when alone in a synthetic media.[115;117] Another way to reach this perspective is using a different microorganisms and adding this new specie to co-culture experiments.[117]

Co-culturing is a cultivation of different microorganisms in the same spot, i.e. same agar plates. This type of experiment is useful to understand the interactions that happen when a determined microbe is under a presence of another individual, so it can be either bacteria-bacterial; bacterial/fungal and fungal/fungal. It is a tool that allows inducing inter-species talking.[117]

Different situation can come about during the co-culturing such as competition for space and nutrients or symbiosis, alteration on metabolic production stimulating a new compound or increasing another or even provide gene expression or biotransformation. Some of these transformations can be noted looking on the agar plates.[117-118] In addition, Bertrand et al[118] demonstrate that only four types of interaction were registered during two months of cultivation in agar plates using 600 different fungi species from clinical, soil, plant derivate species and species from *Fusarium* genus: distance of inhibition (can be caused by production of antimicrobials); formation of a black precipitate (possible new compounds); contact of inhibition (no apparently new compounds released) and overgrowth (complete invasion of one colony into another).

Furthermore, the stimulation of interactions among different organisms, which may be made by co-cultures or mixed cultures, mimics natural conditions found in the rhizosphere, not only providing new metabolites and therefore increasing some

substances of biological activity with pharmacological interest, but also helping in understanding the interactions that occur in the rhizosphere.[117-118]

1.3 New approaches on metabolic identification

Metabolites are small molecules that are specific to a type of cells or organism. They interact with macromolecules (proteins and nucleic acids), are involved in fundamental metabolic processes to maintain an organism alive such as communication (protection- e.g.pigments, competitive interactions – antibiotics or signaling molecules, defense mechanism - some flavonoids and alkaloids toxins) between neighboring plants and other organisms or improving nutrient availability - e.g. in the form of chelating agents such as siderophores.[119-120]

The advanced on "omics"[121] sciences such as genomic, transcriptomic, proteomic and metabolomics has helped to reveal that microorganisms can produce more metabolites that was originally expected. They have the objectives to characterize and quantify biomolecules in the same group. The integration of information obtained by all those platforms above is the main goal of System Biology.[120-121;123-124]

System Biology approaches uses all information of secondary metabolites and their place in the whole metabolic context (chemical composition, metabolism and functions) not only the studying parties, but especially the interrelationships among them, resulting in the functioning of organisms. The essential key of System biology is metabolomics. Metabolomics is defined as quantitative and characterizing analysis of all small metabolites in a given sample under normal conditions and under some kind of stress.[125-126] It has been used in various fields and in a number of purposes to facilitate the understanding of taxonomic and chemosystematics of secondary metabolites and also in the quality control of pharmaceuticals and biosynthesis and genetic regulation.[127-128] Metabolomics uses the spectrometric platforms such as Nuclear Magnetic Resonance, Mass Spectrometry or hyphenate techniques such as LC-MS, GC-MS and LC-SPE-NMR.[129-132] Otherwise, metabolomics also combines the application of spectroscopic techniques with multivariate statistical analysis in studies of the molecular composition and dereplication methodologies to facilitate the detection and structural characterization of chemotypes.[132]

In co-culturing experiments the goal is to detect changes in the metabolic production of the organisms involved (identify compounds either produced de novo or are up/down regulated upon interspecies interaction).[133-134] This can be reached by a simply comparing of a chromatographic profile obtained by HPLC-UV, for example.[118] In addition, MS and NMR derivate and/or hyphenate platforms such MALDI-imaging, LC/MS, one and bi dimensional NMR have been widely used to detect de novo and identify new compounds in mixed cultures by associating with mathematical analysis of the data collected such as multivariate analysis that provides a rapid view of raw data that in case of metabolomics experiments are expressive.[118; 129; 131-132]

1.3.1 Mass Spectrometry in metabolomics

The application of mass spectrometry (MS) and its coupling with separation techniques have classical approach advantages to purification, isolation and elucidation due to its versatility, speed and use of smaller amounts of sample in the analysis, so as to provide a quick prioritization of extracts and substances, while avoiding the re-isolation of substances already known. [131;132;135] MS is highly sensitive and can provide molecular information in fragments patterns for a wide range of metabolites. However, it requires confirmation from standard compounds that are often not available, especially for unknown substances.[136] It is an excellent tool for methodologies of dereplication, which include the coupling techniques of separation and detection, as well as bioassays and the comparative analysis of the data sets obtained through virtual libraries containing chemotaxonomic data, pharmacological and molecular weights among others.

The virtual libraries or databases are essential tools in applying dereplication strategies because they facilitate the detection of metabolites known and described. Among the available public databases, the following can be included:

METLIN[137]- free access metabolites database to perform searches using the information generated from MS. It was developed in order to facilitate the identification of metabolites from different sources: MS analyzes of known sequences of reference metabolites, derivatives and profiles LC/MS in human metabolites and some model organisms, physical and structural data known endogenous metabolites, drugs and metabolites drugs, FT-MS data of the reference samples;

MASSBANK[138] - free database established to assist research in the life sciences. It is composed of mass spectra of primary metabolites, flavonoids, gibberellins, saponins, carotenoids, phospholipids and oligosaccharides. This database contains ESI/MS and EI/MS, these data are not standardized and, therefore, the information was used per compound under different experimental conditions;

DNP – is also free a database containing more than 226,000 natural products. It includes descriptive data on the chemical, physical and biological properties of compounds; systematic and common names of compounds; references; structure diagrams.

1.3.2 NMR for detection of secondary metabolites in metabolomics

NMR requires a simple preparation of sample, is a universal detector for all compounds since they have NMR-active nuclei, the intensity of proton signals is proportional absolutely to the molar concentration of the metabolite, so if an internal standard is used, it is easy to calculate the concentration of metabolites in the sample. NMR possesses high reproducibility due to the physical properties of compounds.[130;139] Following the metabolomics methods, the data itself is not enough to complete a metabolomics analysis. The raw data needs to be analyzed by static methods in order to extract all the information that is possible. For this, it is necessary accuracy and correctness of data, and the NMR is advantageous due to its high reliability; with a few exceptions, chemical shift, constant and integral coupling of each signal is the same once the same conditions of acquisition is applied (field strength, pH, temperature, solvent).[140-143] The disadvantage of NMR is the low sensitivity.

1.4 Chemometric techniques

Chemometrics is the application of mathematical and statistical methods for drawing information from various processes and to improve understanding of chemical information and correlate the quality parameters and physical properties data of analytical instruments.[144] It is used for multivariate data and analysis protocols obtained from different equipments, also calibration, process modeling, pattern recognition and classification, signal correction and compression, and statistical process control.[144-146] It is helpful on the analysis of a vast data collection

generated by the latest automated instruments in research labs. Furthermore, patterns in the data are modeled and can then be applied routinely to predict future data with even quality parameters.[144]

The results of the chemometric approach are gaining efficiency in the chemistry of natural products, since the quality control of analytical processes and methodologies has enabled obtaining a richer metabolic profile, improved separation and facilitated the analysis of spectroscopic data.[145] In chemometrics it is possible to use unsupervised and supervised methods. In the unsupervised methods the data is primarily created or implemented an algorithm for high confidence, which performs the statically calculations that will group individuals in classes. Subsequently each class is manually reviewed to correct some wrong classification by this algorithm.[148] In the supervised methods it is necessary to first define the classes to be evaluated with patters identified as predefined classes. Then it is necessary a creation of a model to input the data and subsequently this model is inserted in the recognition algorithm for classification that will perform mathematical calculations to identify which individuals are in the same data set. The most similar are the samples the closer to each other they will be in a multidimensional space.[145-148]

An applicability of chemometrics in the analysis of natural products is in getting the "fingerprints".[145] This concept was introduced and accepted by the WHO (World Health Organization) as a strategy to identify and qualitatively evaluate a medicinal herb, especially when there is lack of authentic standards for the identification of the active components. It consists of spectrograms, chromatograms and other spectroscopic data and provides chemical data unique for each sample, thus allowing the comparison.[145]

Exploratory multivariate analysis mainly employed is the principal component analysis. PCA is a non-parametric one-dimensionality reduction technique that allows you to design the most information in the fewest number of uncorrelated dimensions.[149] The PCA can be used to identify correlations between the structures of variables and to find changes in these correlations. The variables have a variance and the variables can be associated with each other and then will have a covariance between them. Another use in chemometrics is the PLS tool.[150] PLS is a method to construct predictive models used to model the relations among data sets of variables. This method modifies the relations of the data set into a small number of latent variables by incorporating regression, dimension reduction techniques and modeling

tools. The vector of this latent variable maximizes the covariance found in the data sets. It is similar to PCA and can be used as a discriminating and reducing dimension tool. PLS-DA is used to refer to a discriminant analysis of PLS. In addition PLS-DA admits efficiently working with data sets where there are highly correlated variables and present considerable random noise.[150]

2 METHODOLOGY AND MATERIALS

2.1 Crude extracts and analysis from pure fungi cultures

2.1.1 Obtainment of the crude extracts

The fungi strains (80 species) were cultivated in czapek-dox agar solid medium, for 10 days, at 28 °C and then transfered (plugs 1cmx 1cm) to 500 mL erlenmeyer flasks containing 200 mL of sterile (121°C, 15 min.) liquid media czapek-dox and nutrient broth, kept at 28 °C for 21 days, static mode.

At the end of the incubation period the mycelium suspensions were subjected to filtration separating the mycelia from the filtered. The filtrates containing secreted metabolites by fungi were subjected to liquid-liquid partition with EtOAc using three 200 mL portions of EtOAc extraction. After evaporation of the solvent the crude extracts were obtained.

2.1.2 Analysis of the crude extracts HPLC

For eliminating non-polar interfering, the samples were subjected to solid phase extraction (SPE) cartridge filled with chemically derivatized silica with octadecyl silane groups (C-18). Initially, the adsorbent was activated with pure MeOH (6 mL), and subsequently equilibrated with the chosen eluent, MeOH/H_2O (8:2; 6 mL). The samples were prepared by diluting 6.0 mg dry weight in 1 mL MeOH/H_2O (8:2) ultra pure. The resulted solutions were stored in vials for HPLC. The cartridge was cleaned with MeOH and then DCM.

The mobile phase used was a mixture of solvents: Ultra Pure Water (pump A) with 0.1% acetic acid and MeOH or CAN (pump B) in exploratory gradient:

Monolithic column C18 phenomenex: 5% to 100% B in 25 min, 25- 30 min. 100%; 30-33 min. returning the initial condition of 5%; 33-38 min. remaining at 5% for

the balance of the column. The flux used in each assay was 2.5 mL/min, injection volume of 30 µL. B=MeOH.

Knetex column C18 phenomenex: 1% to 5% B in 10 min, 5% to 9% in 10-15.5 min, 9% to 35% from 15.5min to 32.5 min., 32.5- 40.0 min. 100%; 40.0-42.5 in 100%, 42.5-45.5 min. returning the initial condition of 1%; 45.5-53.5 min. remaining at 1% for the balance of the column. The flux used in each assay was 1.0 mL/min, injection volume of 30 µL. B=ACN.

The chromatograms were recorded programming the diode array detector (DAD) to scan 190-800 nm.

2.1.3 Analysis of the crude extracts HPLC-DAD/HRMS

The chromatographic conditions were the same as mentioned above for monolithic column and the MS: the eluent from the column was divided by a splitter in a 4:1 ratio, with the greatest flow directed to the DAD and the rest of the mass spectrometer. The chromatograms in the UV region generated by DAD were recorded at 280 nm (wavelength that exhibited the best absorption for chromatographic bands evident). The chromatograms generated by MS were recorded in the region between *m/z* 50 and *m/z* 1000 and analyzes were performed in positive ionization mode.

We analyzed all possibilities for the formation of precursor ions that could be formed by forming cationic adducts (positive ionization) with H^+, Na^+ or K^+, among others.

The detection of *in silico* analysis of the data obtained by HPLC-DAD/HRMS was performed using the Bruker software Data Analysis version 3.2 coupled with the natural product dictionary databases (DNP), massbank[138] and metlin.[137]

Co-cultured (pure strains mixed) - The co-cultures experiments were made in different combinations using randomly selected microorganisms - bacteria CSP-1 and CSP-51, belonging to the genus *Burkholderia*, fungi: CSP-11c, CSP-11e, CSP-09 belonging to the genus *Fusarium* and CSP-12Ia the genus *Paecilomyces*. All co-culture experiments were performed in triplicate.

2.2 Crude extracts and analysis from co-cultures

2.2.1 Obtainment of the crude extracts

- Bacterium-fungus (CSP-1/ CSP-11c)

In the bacterium-fungus (table 8) cultivation was used czapek-dox agar medium, for both microorganisms to grow well in that environment. The experiments were performed in triplicate.

Agar plugs 5 mm were transferred to Petri dish containing culture medium and 3 cm away from the center of the plate, CSP-1 bacteria plates (pre-inoculated agar Czapek-dox, 30 °C, 48h) the fungus CSP-11c (pre-inoculated agar Czapek-dox, 30°C, 6 days) was inoculated to start the co-cultivation experiment (Figure 22). The collection of the interaction zone occurred after 72h and 120h elapsed cultivation beginning. For extraction, 2 x 5 mL EtOAc followed by 2 x 5 mL of MeOH were used. The solvent was dried in rotary evaporator apparatus and then weighed.

- Fungus-fungus (CSP-11e/CSP-11c/CSP-09/CSP-12Ia)

For co-cultures of different fungi (table 8), eight distinct experiments were designed in petri dish containing agar medium Czapek-dox. Fungi were co-cultured in pairs: 6 combinations - CSP-11c/CSP-11e; CSP-11c/CSP-09; CSP-11c/CSP-12Ia; CSP-11e/CSP-09; CSP-11e/CSP-12Ia; CSP-09/CSP-12Ia and the complete set of 4 species in two different arrangements in the petri dish.

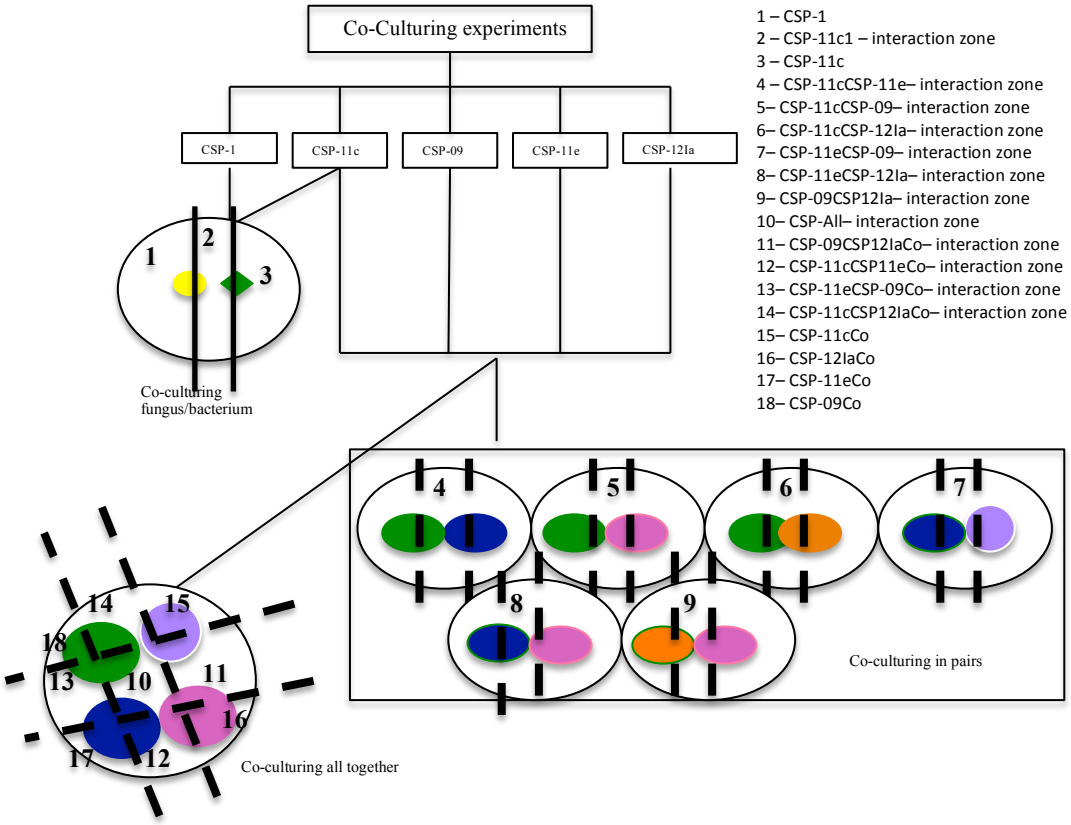
Table 8: Identification codes for the fungi and bacterium used in the co-culturing experiments.

Code	Identification	Origin (isolated from)
CSP-1	*Burkholderia* sp	Hydroponics and sand
CSP-11c	*Fusarium* solani	Hydroponics
CSP-11e	*Fusarium* sp	Hydroponics
CSP-09	*Fusarium* oxysporum	Hydroponics
CSP-12Ia	*Paecilomyces* sp	Hydroponics

In the experiments using pairs of fungi the collection was done after 12 days of cultivation. On the other hand, the experiments with the four fungi together the collection was performed after 7 days of cultivation. All extracts were extracted with 2

x 5 mL MeOH, followed by evaporation of the solvent under Rota evaporator. The dried samples were weighed.

Figure 22: Scheme of the experimental design applied to the co-culturing experiments.



2.2.2 ^{1}H NMR

The dried extracts were dissolved in 800 μL a KH_2PO_4 buffer (90 mm, pH = 6) in D_2O containing 0.05% (w/v) of 2,2,3,3-d_4 trimethylsilyl propionic acid sodium salt (TMSP) and transferred to 5mm NMR tubes. The mixture was shaken for 2-3 minutes in vortex at room temperature. The one-dimensional ^{1}H spectrum was acquired in Varian equipment operating in the frequency 400 MHz. D_2O was used as an internal lock. Each ^{1}H NMR spectrum consisted of 520 scans with 3.17s time of acquisition, 0.16Hz / point, pulse 30˚ and relaxation time = 15s. The FID was Fourier-transformed with line broadening = 0.3Hz. The resulting spectra were manually manipulated to phase correction, baseline and calibrated with TMSP to 0.00 ppm using MestReNova (v. 8.1.2, Mestrelab SL research, Santiago de Compostela, Spain). The above methodology was adapted from Pimenta et al, 2013[143].

2.2.3 Multivariate statistical analysis

Principal components analysis (PCA) and Principal linear squares discriminant analysis (PLS-DA) were performed using SOLO PLS Toolbox (v. 7.9.3, Eingenvector Research Incorporated, Wenatchee, WA, USA). The data were pre-proceeded using mean center and pareto (Sqrt Std) scaling functions in PLS toolbox.

3 RESULTS

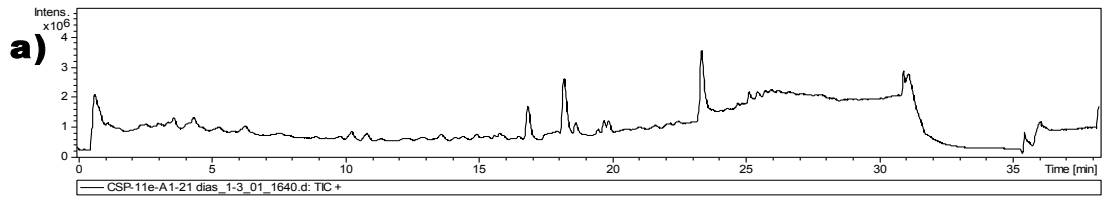
3.1 Fungi pure strains

The extracts were analyzed by HPLC-DAD and by HPLC-DAD/MS in positive mode, under the same conditions described for exploratory gradient in 0.1% acetic acid mobile phase (MeOH/H_2O, page 88). From all the 80 extracts evaluated, nine extracts that were submitted to analysis via HPLC-DAD/MS were selected, making it possible to detect a series of compounds as it can be seen in the chromatograms of total ions (TIC) of these extracts, conducted in positive mode (Figure 23).

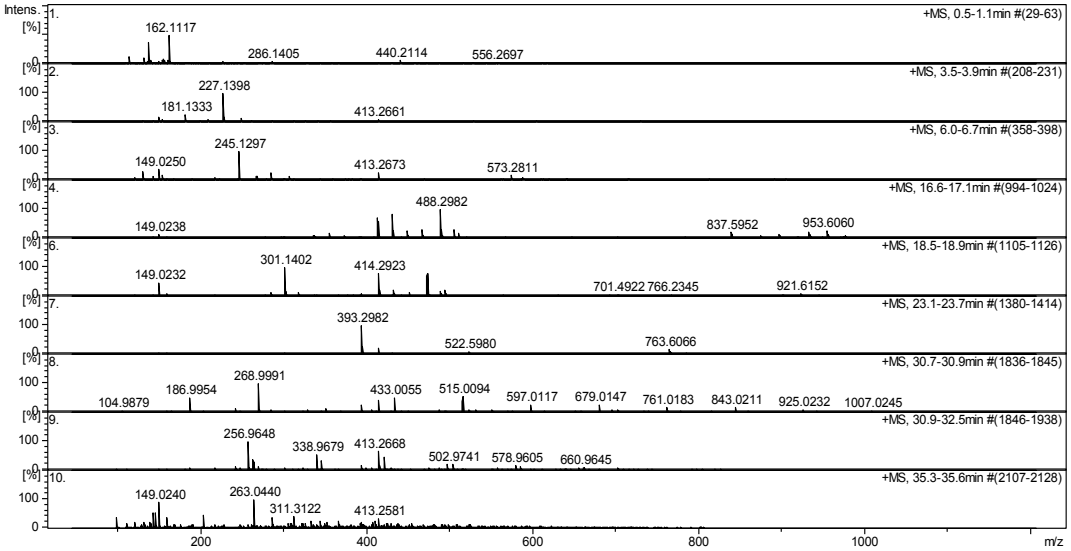
- Extract CSP-11e

In Figure 23a and 23b relates the obtained positive TIC and some major ions are detected: *m/z* 162- 0.5 min.; *m/z* 227 to 3.5 min.; *m/z* 245- 6.0 min.; *m/z* 301 to 18.0 min.; *m/z* 317 to 19.5 min.; *m/z* 413 to 16.6 min.; *m/z* 393 to 23.1 min.; *m/z* 299 -30.7 min.; *m/z* 256 -30.9 min., and *m/z* 268 to 30.7 min.

Figure 23: a) TIC in positive mode LC/MS of EtOAc extract of CSP-11e after 21 days of culture; b) Major positive ions present in TIC.

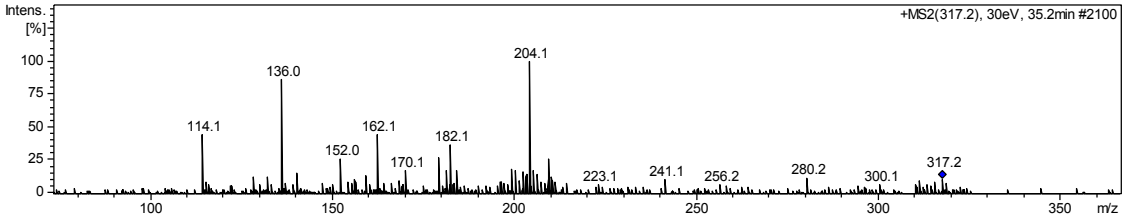


b)



The results were compared with the DNP database using as search guide the genus and species, *Fusarium* and *Fusarium solani*, which owns the fungus from which the extract was generated. From those results, ESI (+)-MS/MS experiment were performed, in which the observed fragmentations are the ions*, m/z* 317*, m/z* 301 and *m/*z 136 in in Figure 24.

Figure 24: Cromatogram MS2 obtained from MS/MS experiment for the majority ions of the EtOAc extract of CSP-11e after 21 days of cultivation.

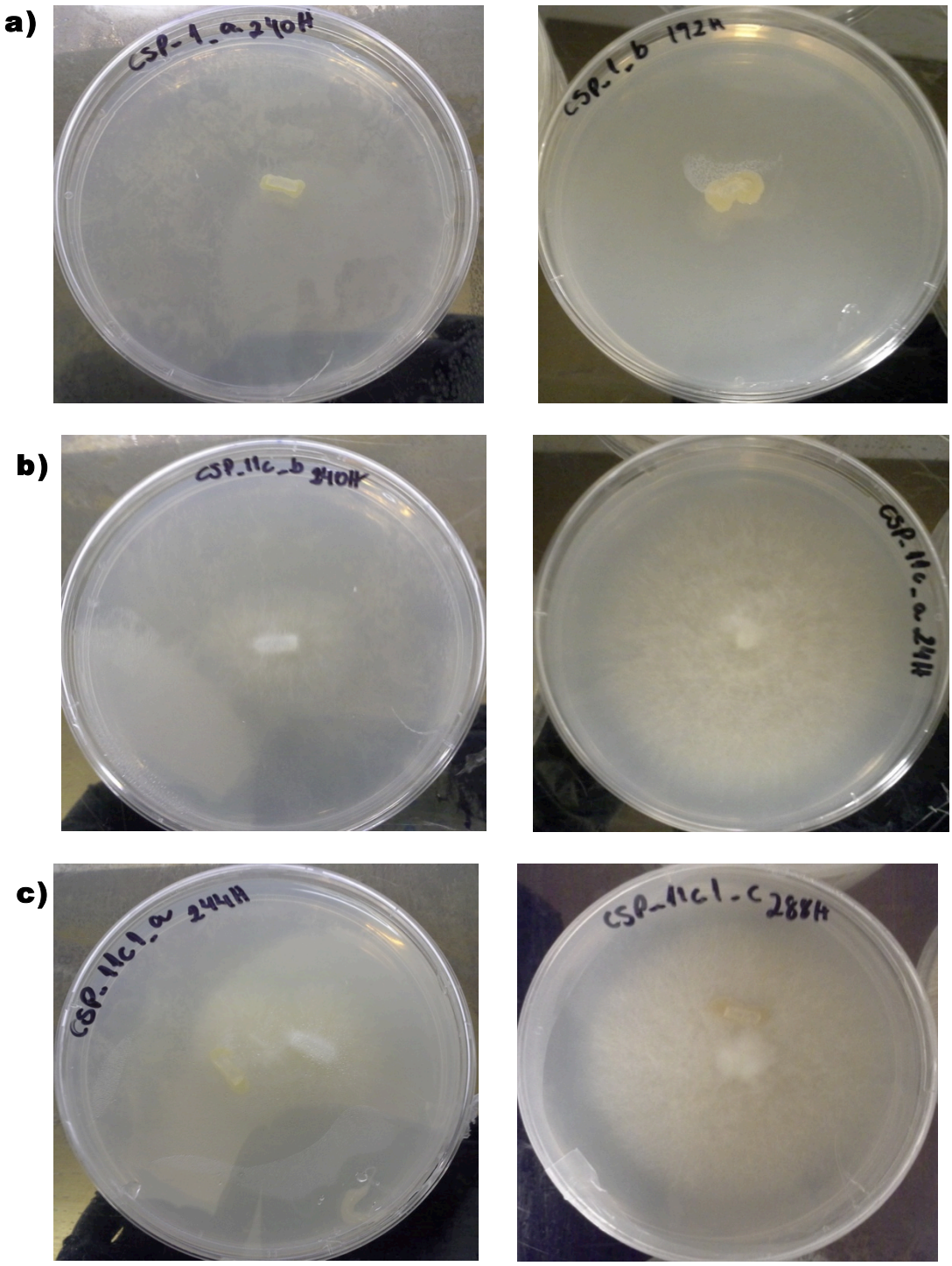


3.2 Co-Culture

- Observations on morphological changes in co-culture experiments

In the co-culture of bacterium/fungus, CSP-1/CSP-11c is observed that a CSP-1 bacterium had its normal growth inhibited when compared with the isolated culture (Figures 25a and 25b). During the course of the experiment there were no apparent changes in the color of the two microorganisms. After 48 hours the bodies reached a contact zone and after 72 hours the CSP-11c fungus crossed the zone of inhibition and grew over the bacteria, covering it completely and continuing its growth spreading in the petri dish (Figure 25c). The interaction zones were collected at the time of contact, 48h, and 120h.

Figure 25: Growth in czapek-dox agar of a) pure strain CSP-1 in 48h and 120h; b) pure strain CSP-11c in 48h and 120h and c) co-cultured CSP-11c1 in 48 hours and in 120 hours.

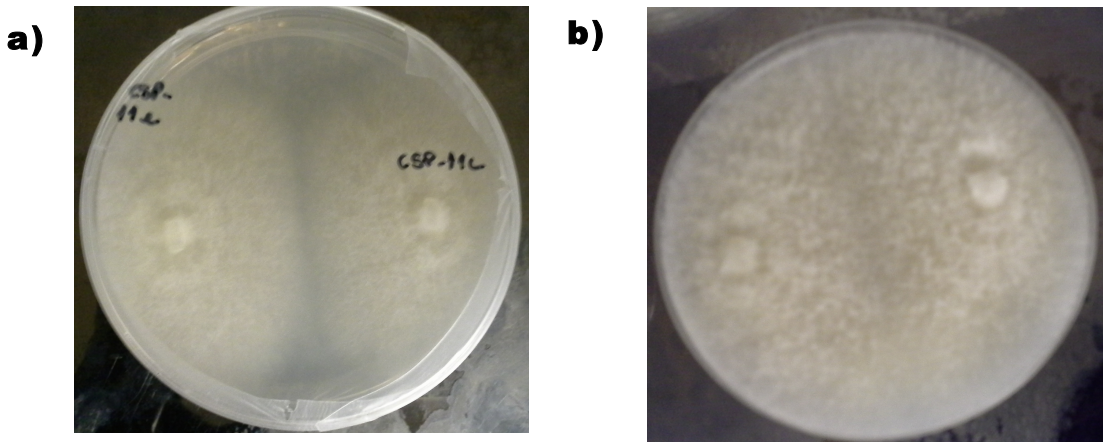


While growing experiment among different fungal species observed several different events happening in the experiment in pairs and the same effects by repeating the experiment with 4 fungi placed on the same plate. The interactions seen during the course of cultivation had been a total of 12 days when the collecting

of the zone of interaction was performed, as listed in figures 26-33. The first contact between the mycelia in experiments using fungi pairs occurred after 10 days of growth, where a "barrier" was formed. So far we did not observe changes in the color of the fungus only a minor growth when compared to the individual organism (figure 32). It was then awaited an additional 48 hours (day 12) for the most fungi interact with each other, and this time some changes were intensified as follows:

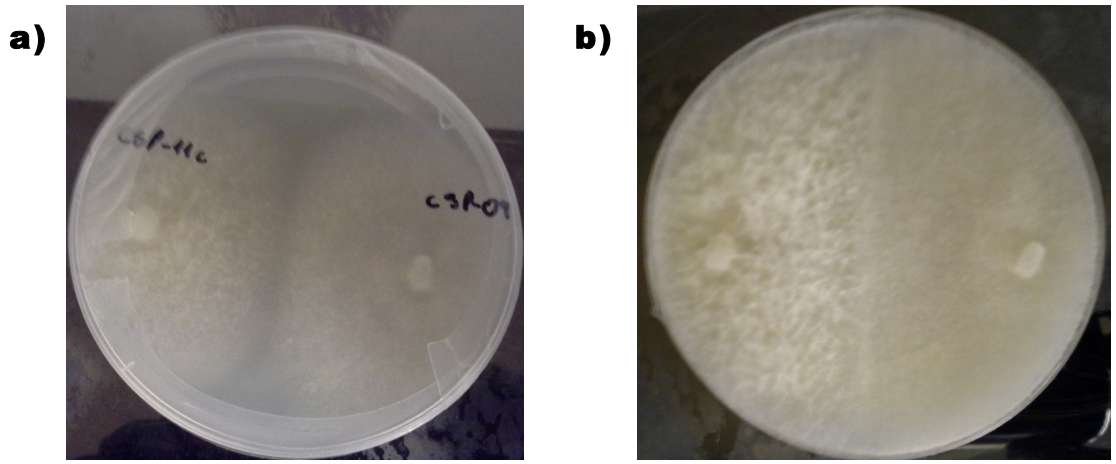
• CSP-11c/CSP-11e: The fungal growth occurred at the same speed, keeping pace to the contact interfaces was then maintained when a small contact distance with no apparent growth of either organisms was observed. Both remained with the same staining characteristics during growth (Figure 26).

Figure 26: Cocultivation CSP-11c /CSP-11e: 4 days after beginning of the culture and 12 days of culture.



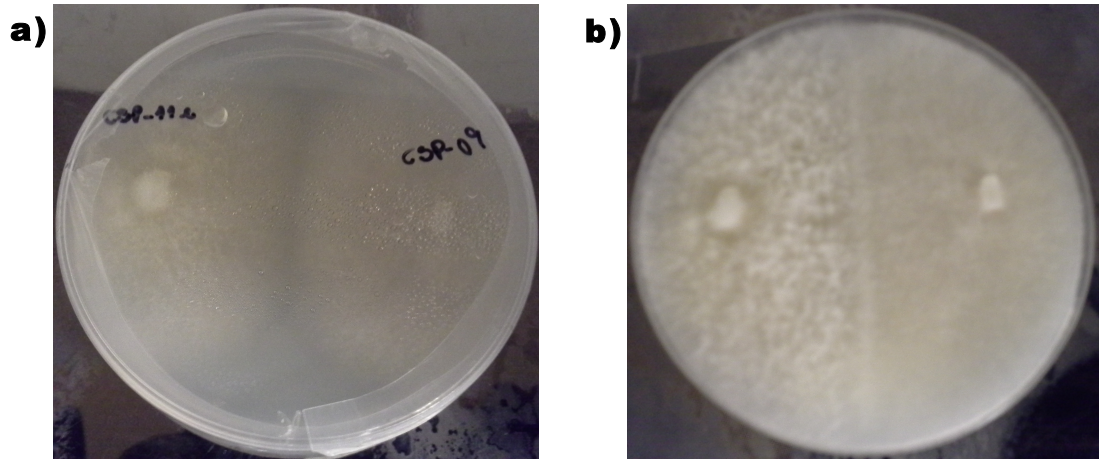
• CSP-11c/CSP-09: a white line was formed on partition separating the two bodies in a "contact-line", that can be seen in Figure 27b, in turn, CSP-11c showed a small change in its normal color, from white to yellowish, especially at the edges and around the zone of inhibition (Figure 27).

Figure 27: Cocultivation CSP-11c/CSP-09 em: 4 days after beginning of the culture and 12 days of cultivation.



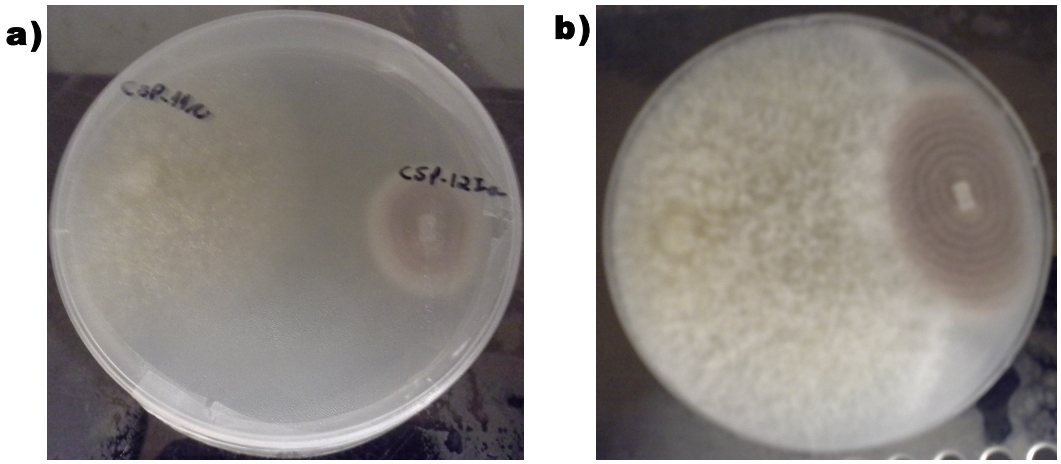
• CSP-11e/CSP-09: a white visible dividing line was formed on day 11th and intensified on day 12th separating the two bodies in a "mutual inhibition zone" that can be seen in Figure 28. Change of white CSP-11e coloring to a yellowish tone, day 12, with a formation of a "mutual barrier" as a contact line. The growth on the plate was proportional to the two fungi (Figure 28).

Figure 28: Cocultivation CSP-11e/CSP-09: 4 days after beginning of the culture and 12 days of culture.



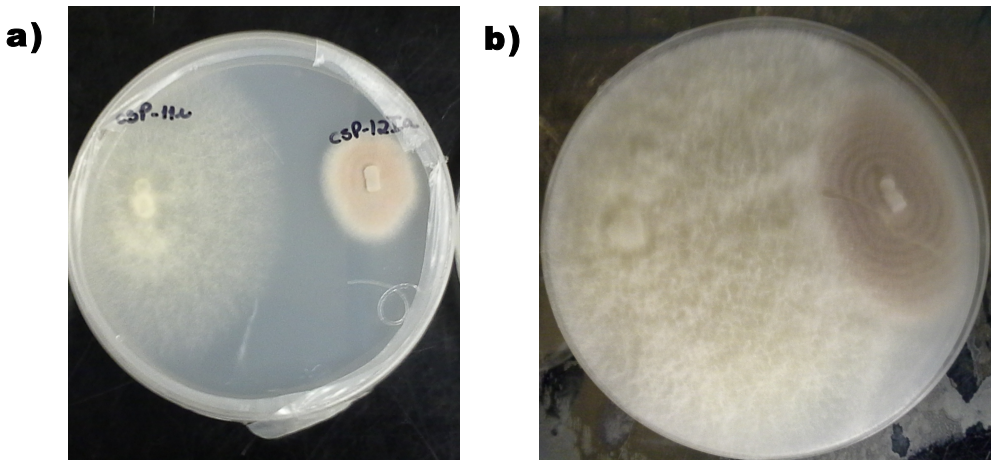
• CSP-11c/Csp-12Ia: The CSP-12Ia fungus grew less and seems to have its growth inhibited by CSP-11c, CSP-11c did not overgrow CSP-12Ia but the first grown all around the second (Figure 29) and two fungi lies and "touch". The color of CSP-11c becomes yellow in the center of the colony and remains white at the edges.

Figure 29: Cocultivation CSP-11c/CSP-012Ia 4 days after beginning of the culture and 12 days of culture.



• CSP-11e/12Ia: aerial mycelium formation along the entire length of the contact zone by CSP-11e. CSP-12Ia seems to grow along the contact zone, without going beyond the barrier formed between them. There was no CSP-11e overlap on CSP-12Ia (Figure 30).

Figure 30: Cocultivation CSP-11e/CSP-12Ia: 4 days after beginning of the culture and 12 days of culture.



• CSP-09/CSP-12Ia: CSP-09 clearly increased well over the period of 12 days and appears to inhibit the growth of CSP-12Ia. This in turn had its thicker edge and a sharp white color when compared with the fungus alone. The molds are maintained at an unchanged distance from the inhibition 10th even with CSP-09 growing around CSP-12Ia (Figure 31).

Figure 31: Cocultivation CSP-11e/CSP-12Ia: 4 days after beginning of the culture and 12 days of culture.

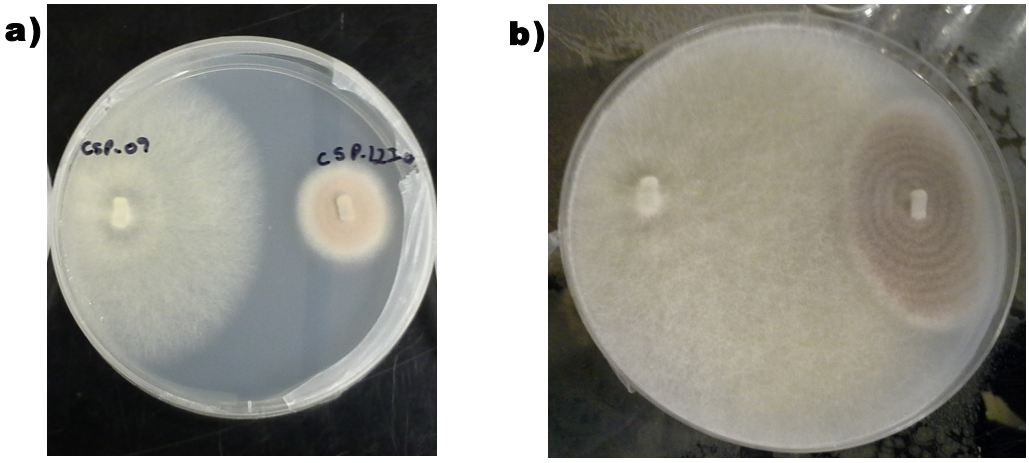
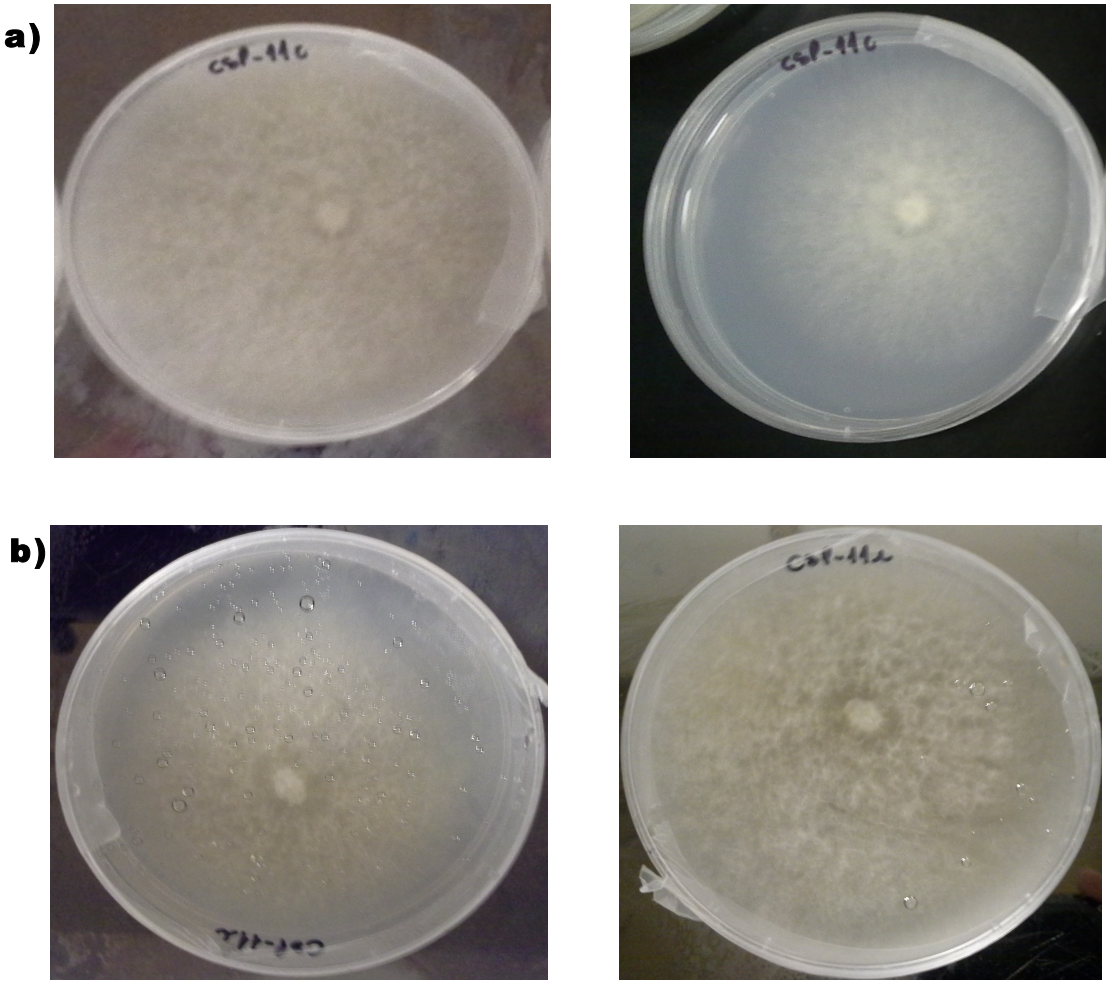


Figure 32: Controls – isolated strain of a) CSP-11c, b) CSP-11e, c) CSP-09 and d) CSP-12Ia, respectivaly, in: 4 days after the beginig of cultivation and after 12 days of cultivation.



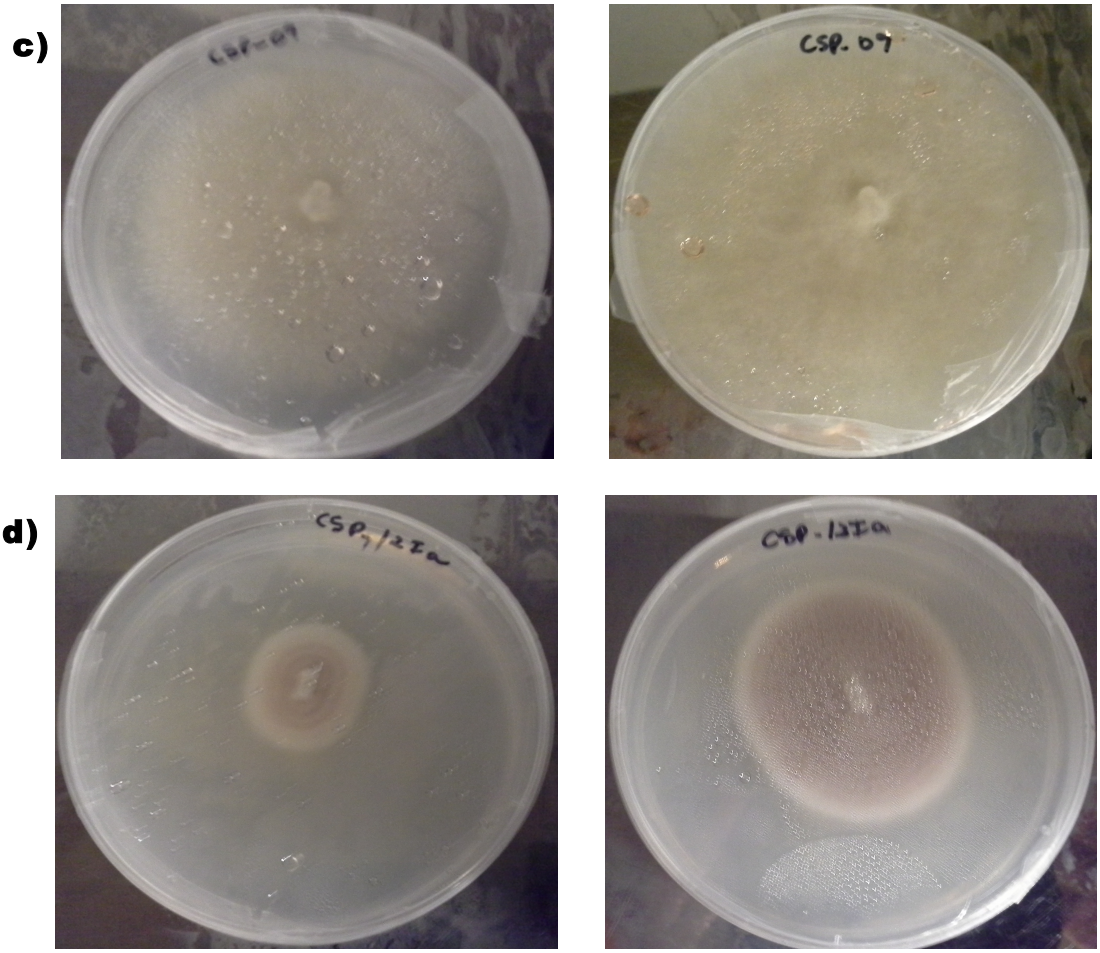
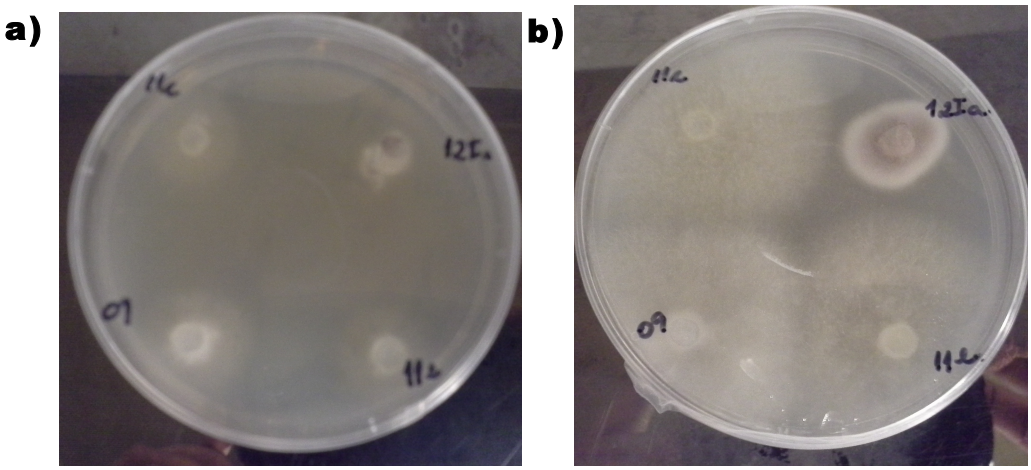
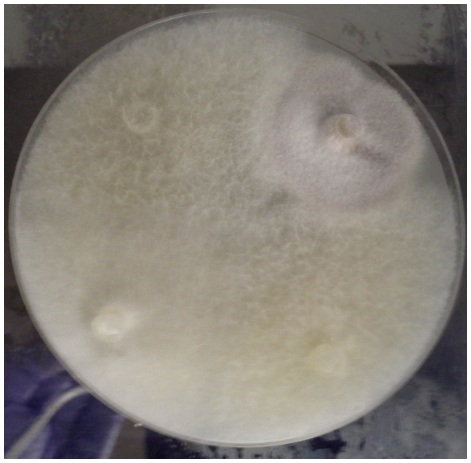


Figure 33: Co-culture experiment with the four fungi: CSP-11c, CSP-11e, CSP-09 and CSP-12Ia, respectivaly, in: a) at the beginning of experiment, b) 4 days after the beginig of cultivation and c) after 12 days of cultivation.



c)

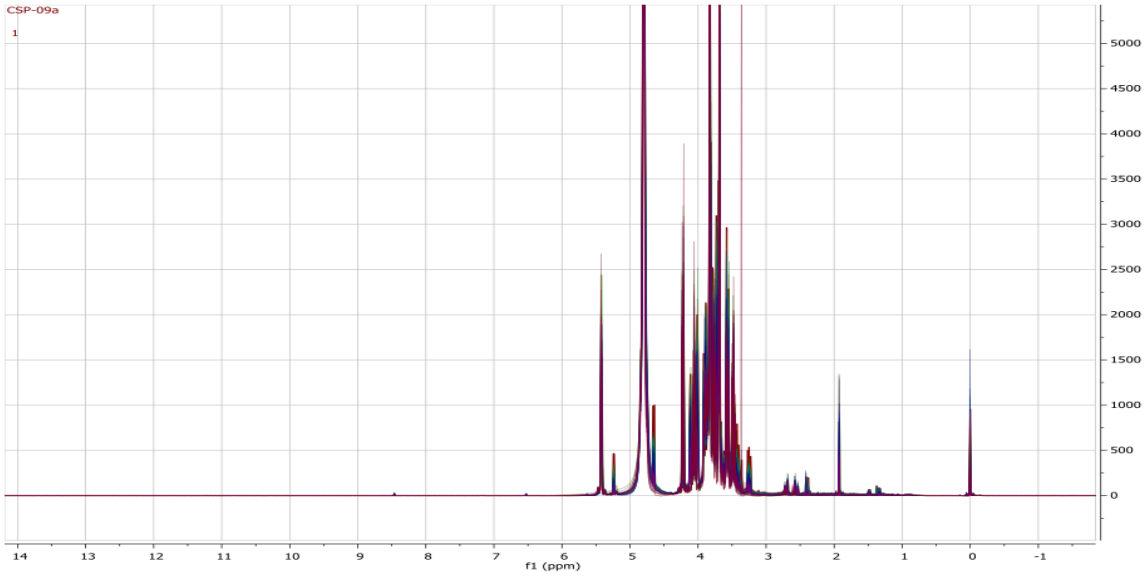


3.3 ^{1}H NMR Multivariate analysis of co-cultures

All co-cultures, monocultures and medium without microorganisms extracts were analyzed by ^{1}H NMR using D_2O buffered 90mm KH_2PO_4. Other solvents were tested as: $CDCl_3$; MeOD, different proportions of MeOD and D_2O buffered 90mm KH_2PO_4 however the extracts dissolved better only in D_2O.

In total there were obtained 141 ^{1}H NMR dimensional spectra of monocultures, co-cultures and culture medium. The superimposed NMR spectra are related on figure 34.

Figure 34: Superimposed ^{1}H NMR spectra of the four fungi co-culturing interaction zone and controls.



The extracts obtained spectra show differences, some other subtler sharp, as in the co-culture bacteria-fungi CSP-1/CSP-11c, especially in the spectral region phenolic compounds. Also noted were typical signs of sugar chemical shift mainly in culture medium but also in the other extracts of mono- and co-culture.

The plots of scores for the three principal components are shown in figures 35-38. CSP-11c separated from the others groups along with PC1 in negative side. CSP-1 is along the PC3 on negative side but is close to the interaction zone that is separated along PC3 positive. CSP-11c overlaps with zone of interaction along PC3. The samples are very distant from each other, indicating variability in the data of replicates. In the PLS-DA plot scores, the separation between the classes are more evident than in PCA. Along PV2 the interaction zone clearly separates from other classes (figure 36).

In the 120h co-cultivation experiment, the PCA plots scores (figure 37) separate from other two along PC2 negative side. Along PC1 negative side, CSP-1 is located and in the positive side is the CSP-11c. The score plots of PLS-DA show a good separation of the zone of interaction along V2, while CSP-1 and CSP-11c are separated throughout the LV1, negative and positive side, respectively (figure 38).

Figure 35: Plot of Scores in PC1xPC2 and PC1xPC3, respectively, of the three principal components analysis of zone of contact of CSP-1/CSP-11c in 72h of cultivation.

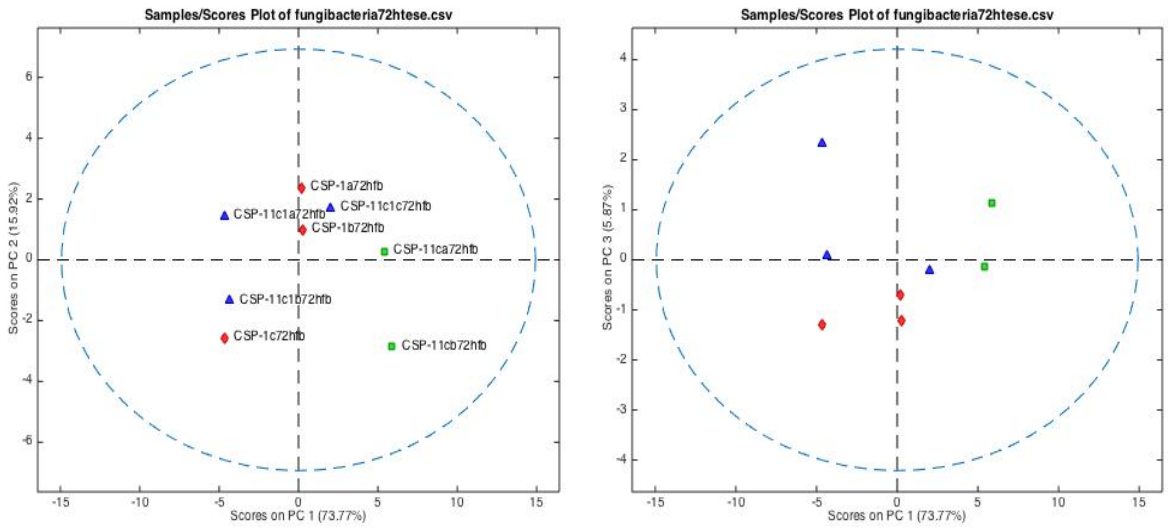


Figure 36: Plot of Scores in PLS-DA: VL1xVL2 and VL1xVL3, respectively, of the three principal components analysis of zone of contact of CSP/1/CSP-11c in 72h of cultivation.

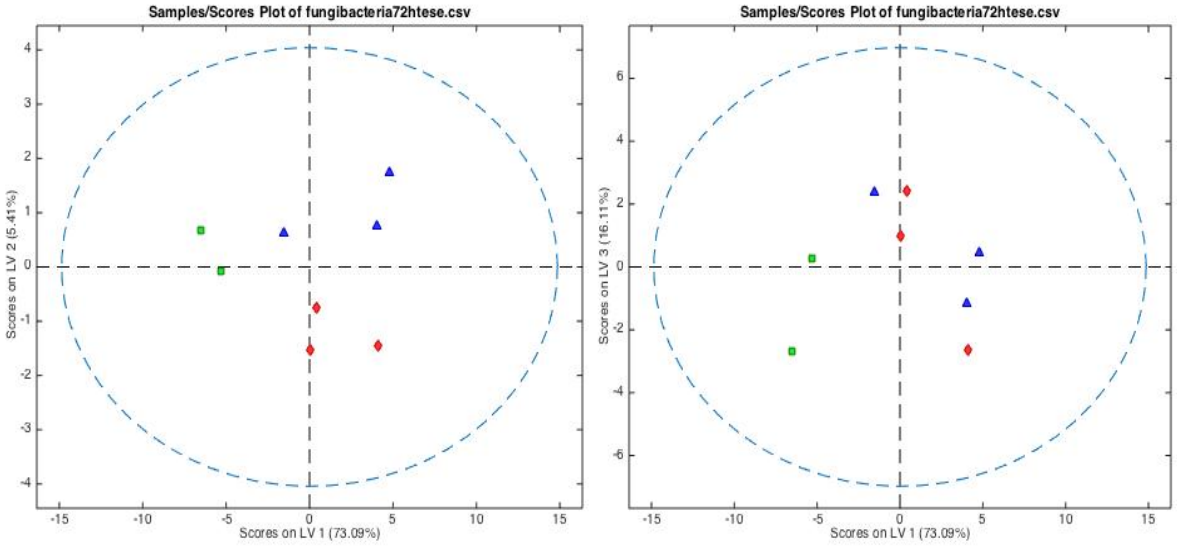


Figure 37: Plot of Scores in PC1xPC2 and PC1xPC3 respectively, of the three principal components analysis of zone of contact of CSP-1/CSP-11c in 120h of cultivation.

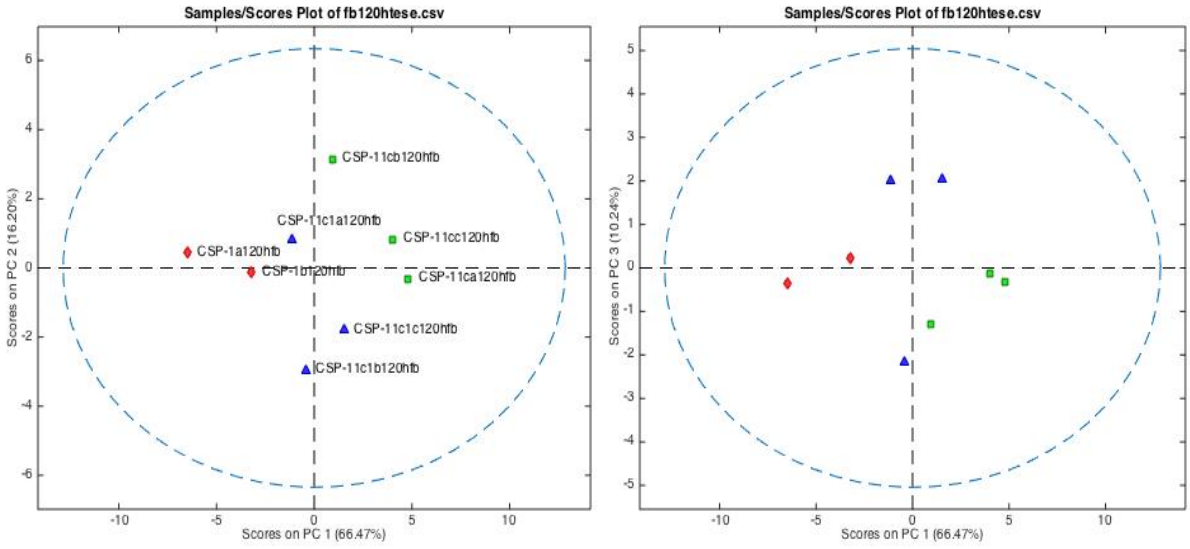
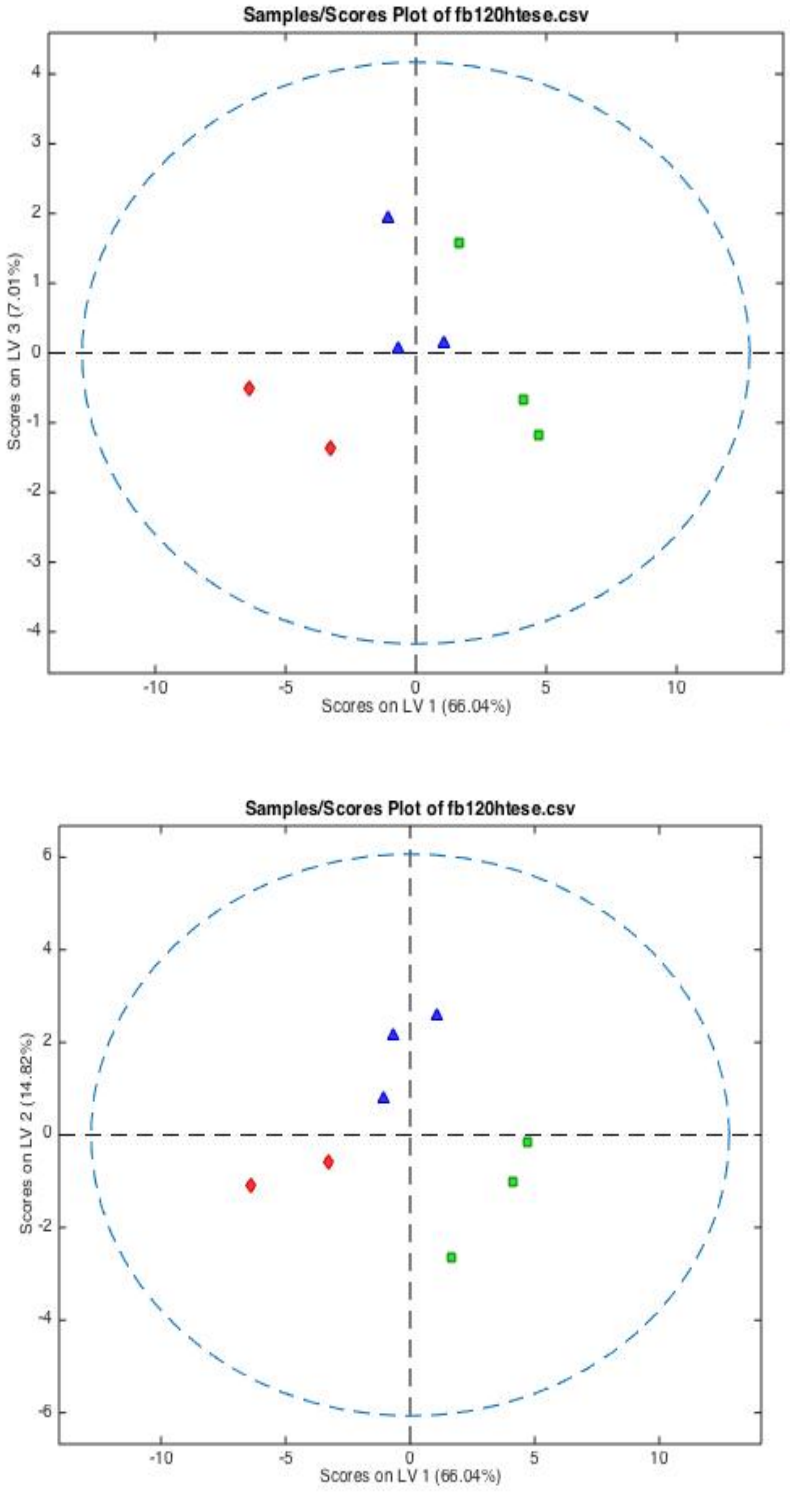


Figure 38: Plot of Scores in PLS-DA: VL1xVL2 and VL1xVL3, respectively, of the three zones in co-culturing experiments: zone of contact of CSP/1/CSP-11c in 120h of cultivation.



The figures 39-45 show the results obtained by PCA with the data collected in the interaction zone of the co-culture experiment of fungi in pairs and for the controls (fungi growing in another Agar plate alone). The score plots of the correlation

between the different PCs are related on figures 39-44. The PLS-DAs score plots did not provide different separations, and when compared with the PCAs the results of separation were the same. Each plot represents the interaction zone of the pair of fungi and the relation with the controls (fungi alone in Agar plate). In all experiments three regions can be noted with some partial overlapping plus some anomalous points.

The interaction zones are separated from the isolated fungi along PCs as follow:

CSP-09/CSP-11c – The interaction zone is separated from the isolated fungi along PC1 in the positive side of PC; PC2 positive represents CSP-11c while PC3 correlates CSP-09, are anomalous points in positive and negative sides of this PC indicating variations in the replicates.

CSP-09/CSP-12Ia- PC1 is related to the separation of the interaction zone and CSP-09 is fully separated along PC2 and CSP-12Ia along PC3;

CSP-11c/CSP11e - CSP-11e is separated from the other groups along PC2 positive while CSP-11c is in the negative side of this PC; the interaction zone separation can be explained by PC3 positive;

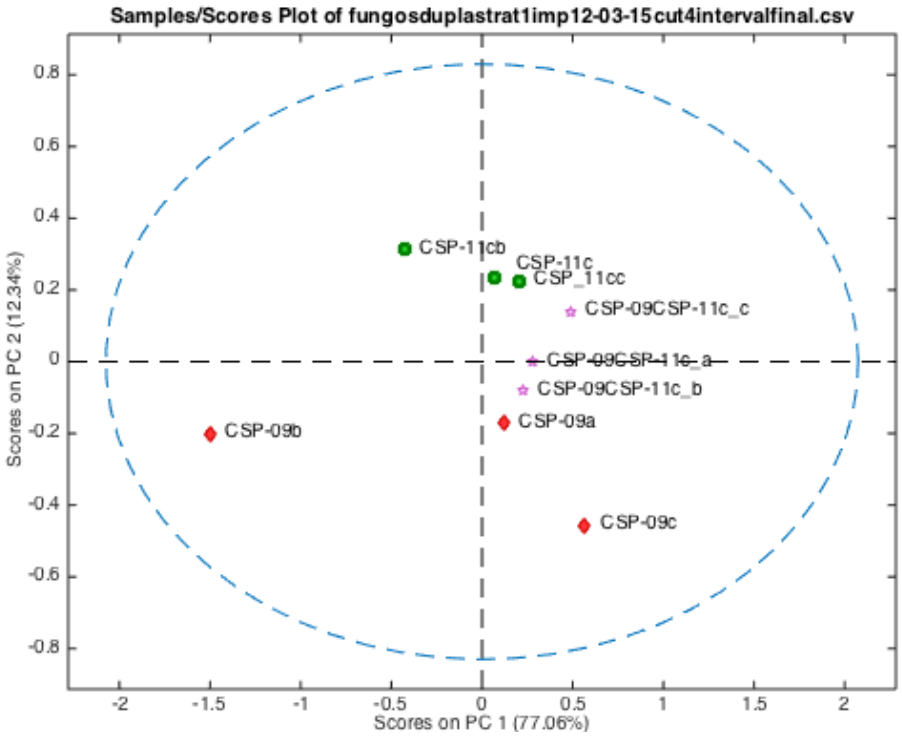
CSP-09/CSP-11c – CSP-09 is totally separated along PC2 positive while the interaction zone and CSP-11c are closely separated along PC1;

CSP-09/CSP-11e- Zone of interaction is separated along PC1, PC2 explains the separation of CSP-09 e PC3 is related to CSP-11e;

CSP-11c/CSP-12Ia- CSP-12Ia is separated along PC2 positive and CSP-11c has a slightly separation with overlapping interaction zone along PC3.

CSP-11e/CSP-12Ia - CSP-12Ia separation is represented by PC2 and the interaction zone is located along PC3 negative and in the positive side of this PC is CSP- 11e.

Figure 39: Plot of Scores in PC1xPC2 and PC1xPC3, respectively, of the three principal components analysis of CSP-09/CSP-11c co-culturing feat isolated controls (CSP-09 and CSP-11c) data.



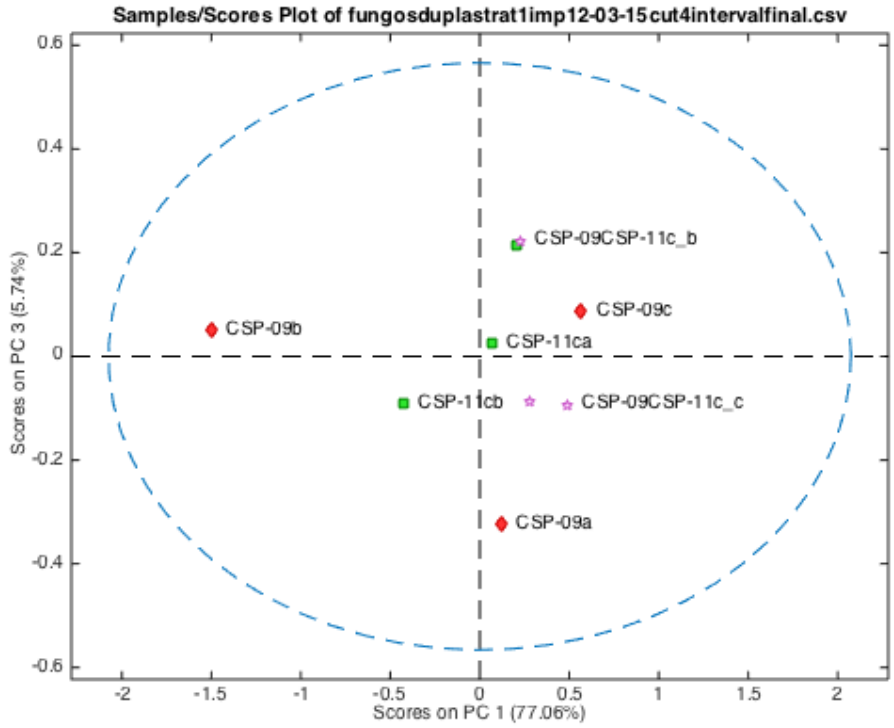


Figure 40: Plot of Scores in PC1xPC2 and PC1xPC3, respectively, of the three principal components analysis of CSP-11e/CSP-11c co-culturing feat isolated controls (CSP-11e and CSP-11c) data.

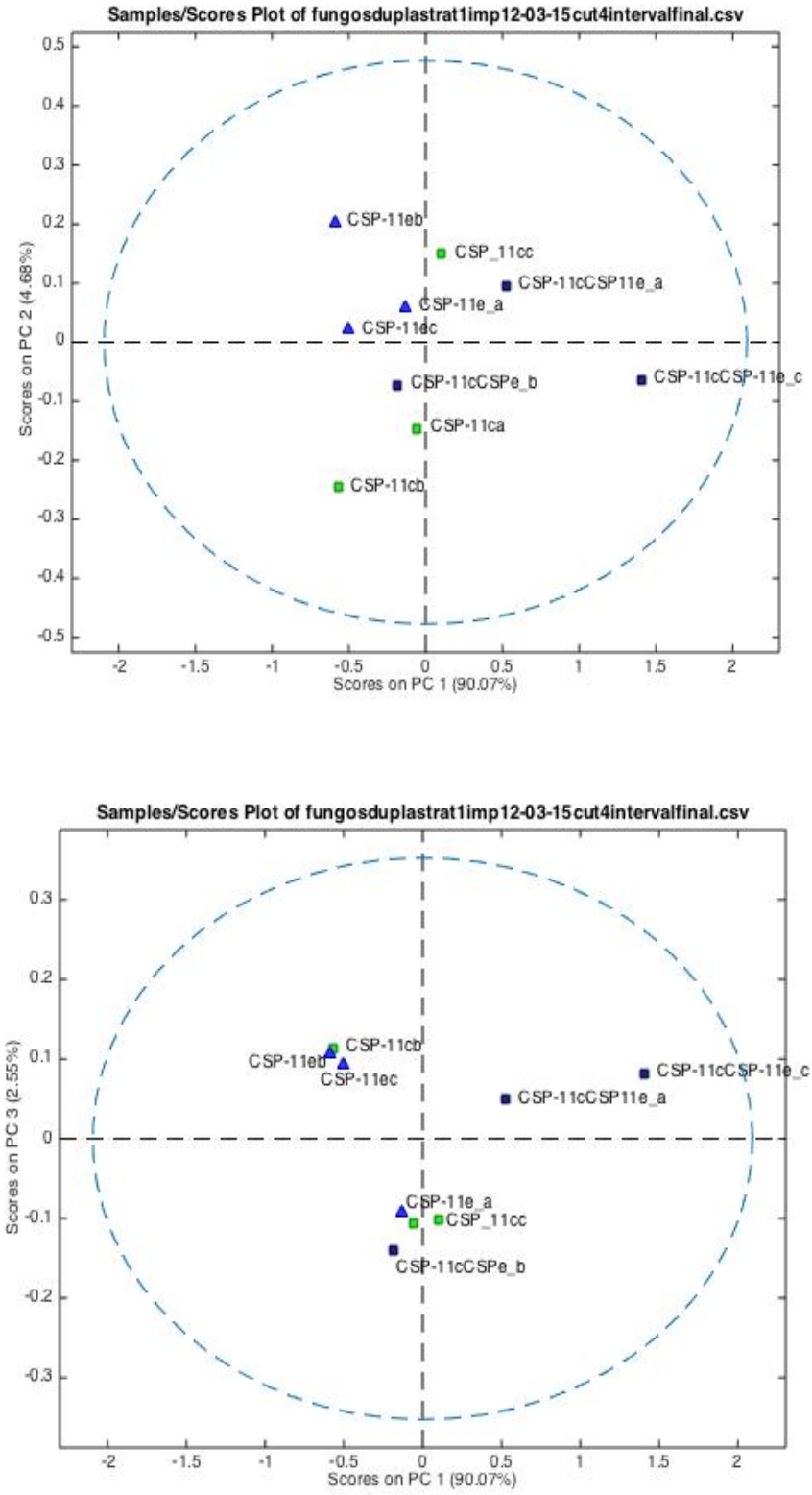
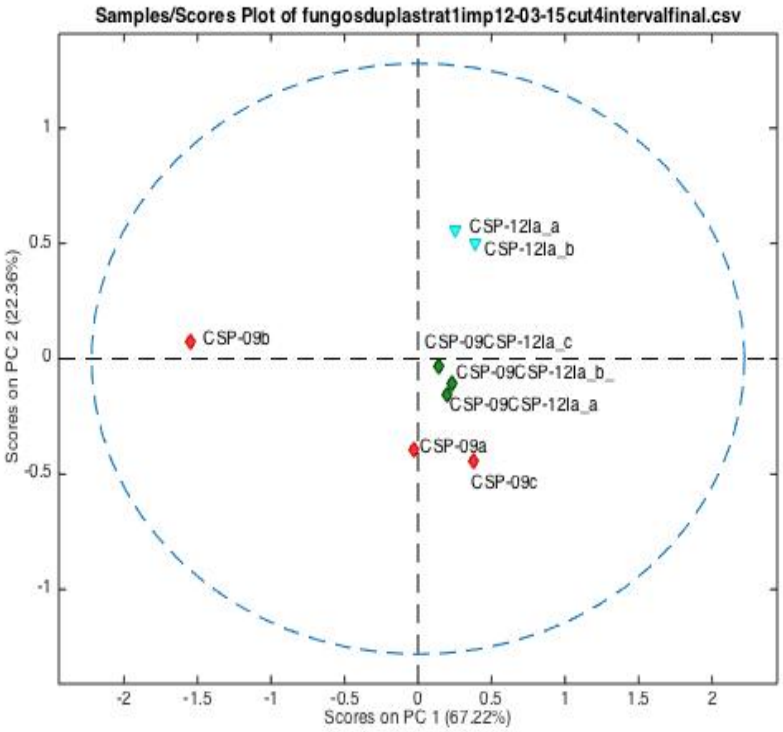


Figure 41: Plot of Scores in PC1xPC2 and PC1xPC3, respectively, of the three principal components analysis of CSP-09/CSP-12Ia co-culturing feat isolated controls (CSP-09 and CSP-12Ia) data.



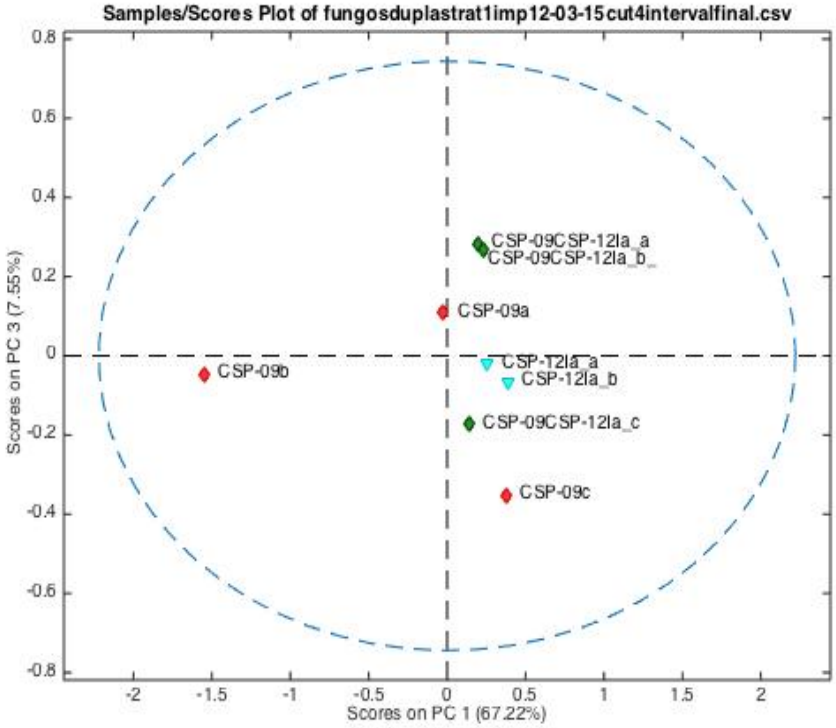


Figure 42: Plot of Scores in PC1xPC2 and PC1xPC3, respectively, of the three principal components analysis of CSP-09/CSP-11e co-culturing feat isolated controls (CSP-09 and CSP-11e) data.

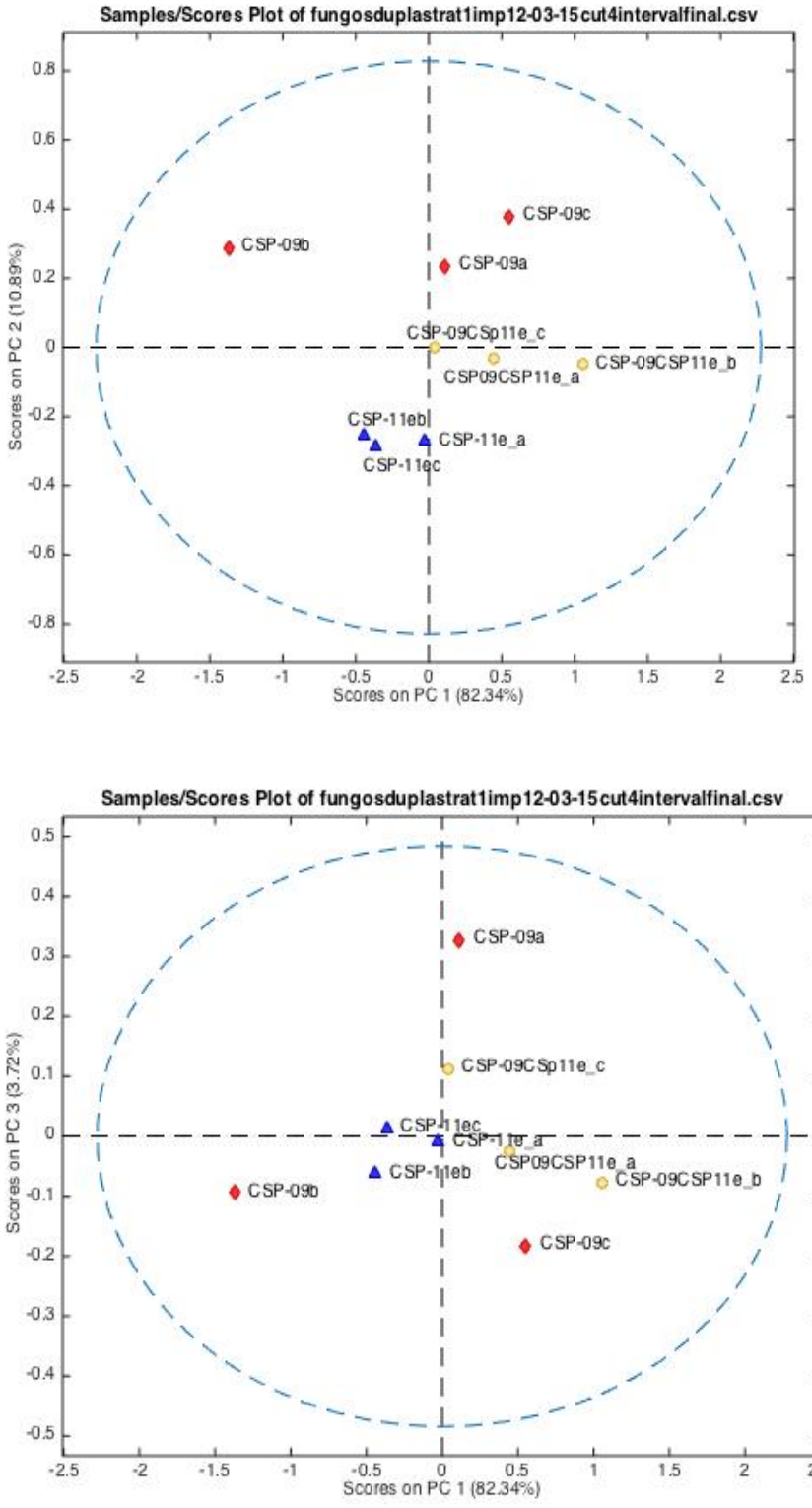
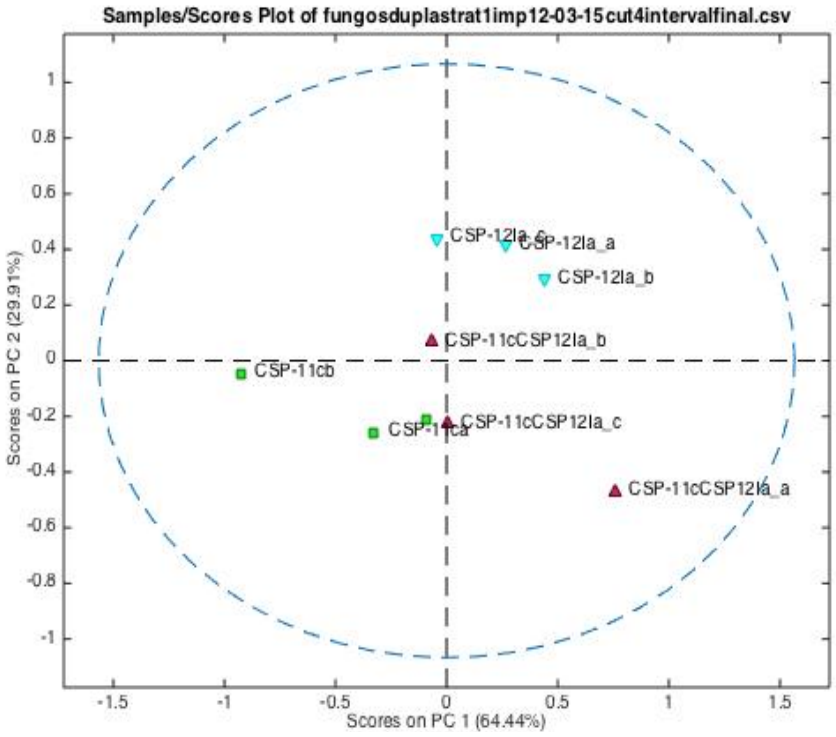


Figure 43: Plot of Scores in PC1xPC2 and PC1xPC3, respectively, of the three principal components analysis of CSP-12Ia/CSP-11c co-culturing feat isolated controls (CSP-12Ia and CSP-11c) data.



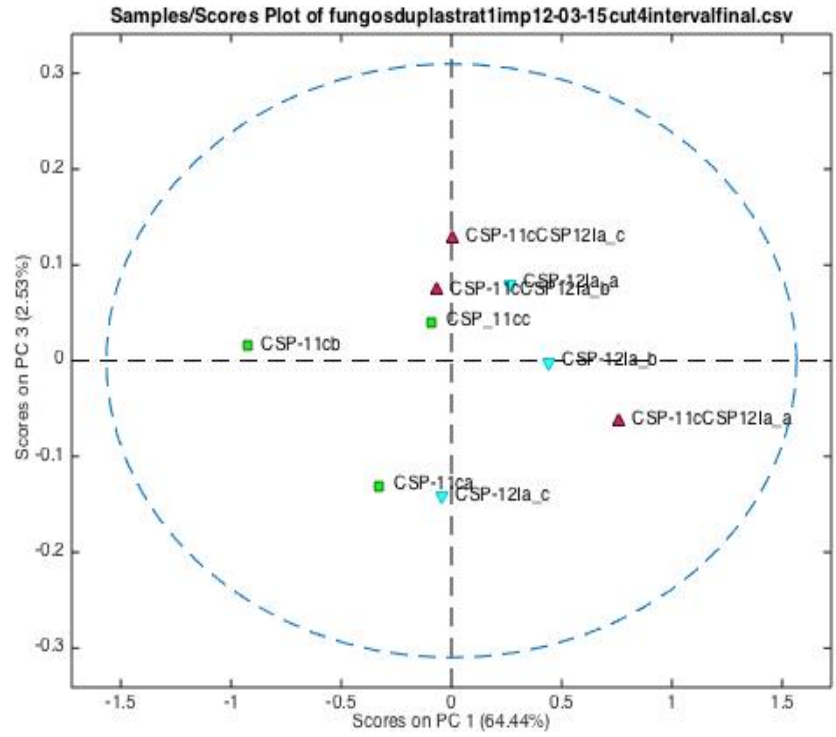


Figure 44: Plot of Scores in PC1xPC2 and PC1xPC3, respectively, of the three principal components analysis of CSP-12la/CSP-11e co-culturing feat isolated controls (CSP-12la and CSP-11e) data.

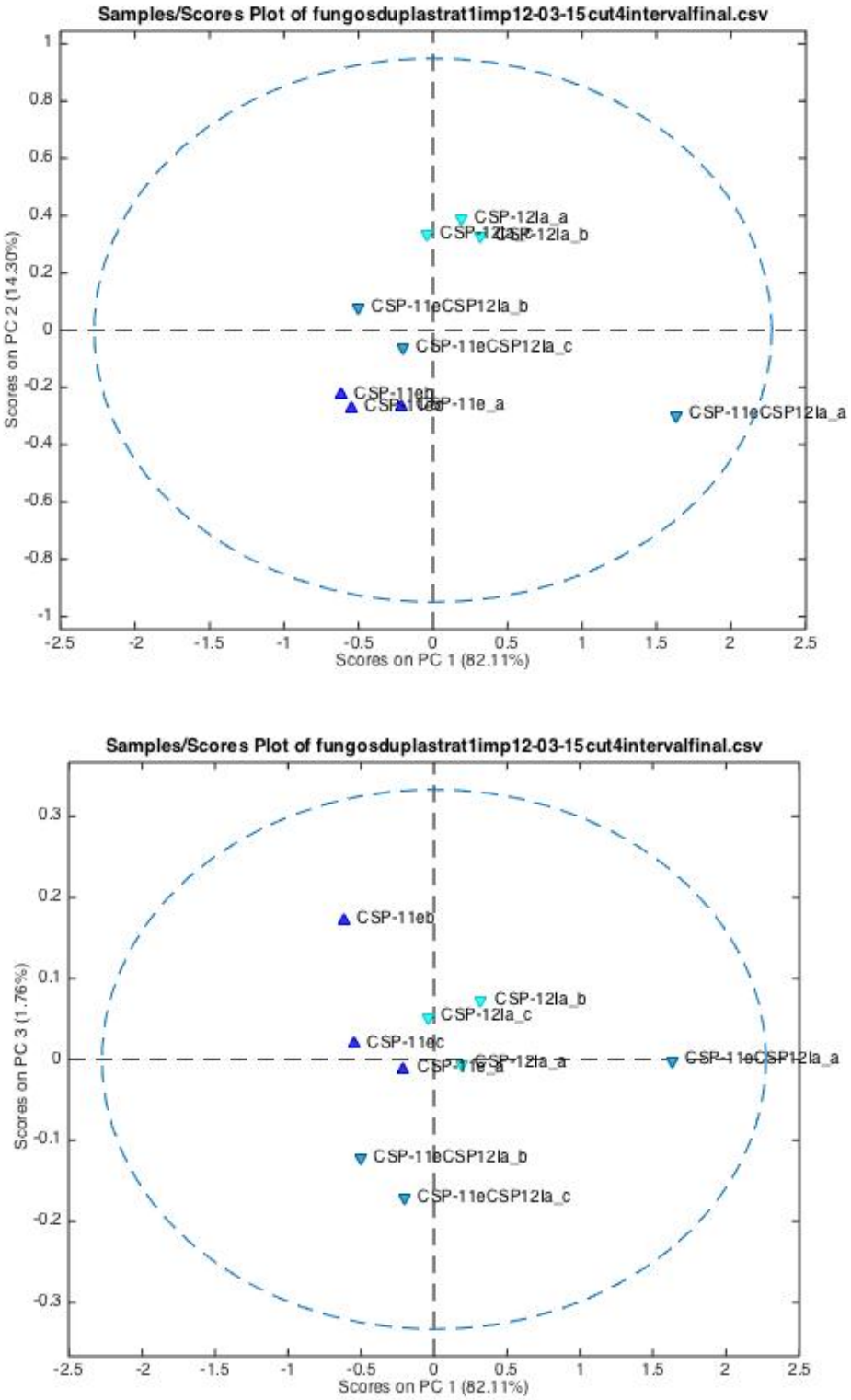


Figure 45: Plot of Scores in PLS-DA: VL1xVL2 and VL1xVL3, respectively, of the three zones in co-culturing experiments: zone of contact of pairs (CSP-11c/CSP-09; CSP-11c/CSP-12la; CSP-11e/CSP-12la, CSP-11c/CSP-11e; CSP-09/CSp-12la and CSP-09/CSP11e) and isolated controls (CSP-12la; CSP-11c; CSP-09 and CSP-11e).

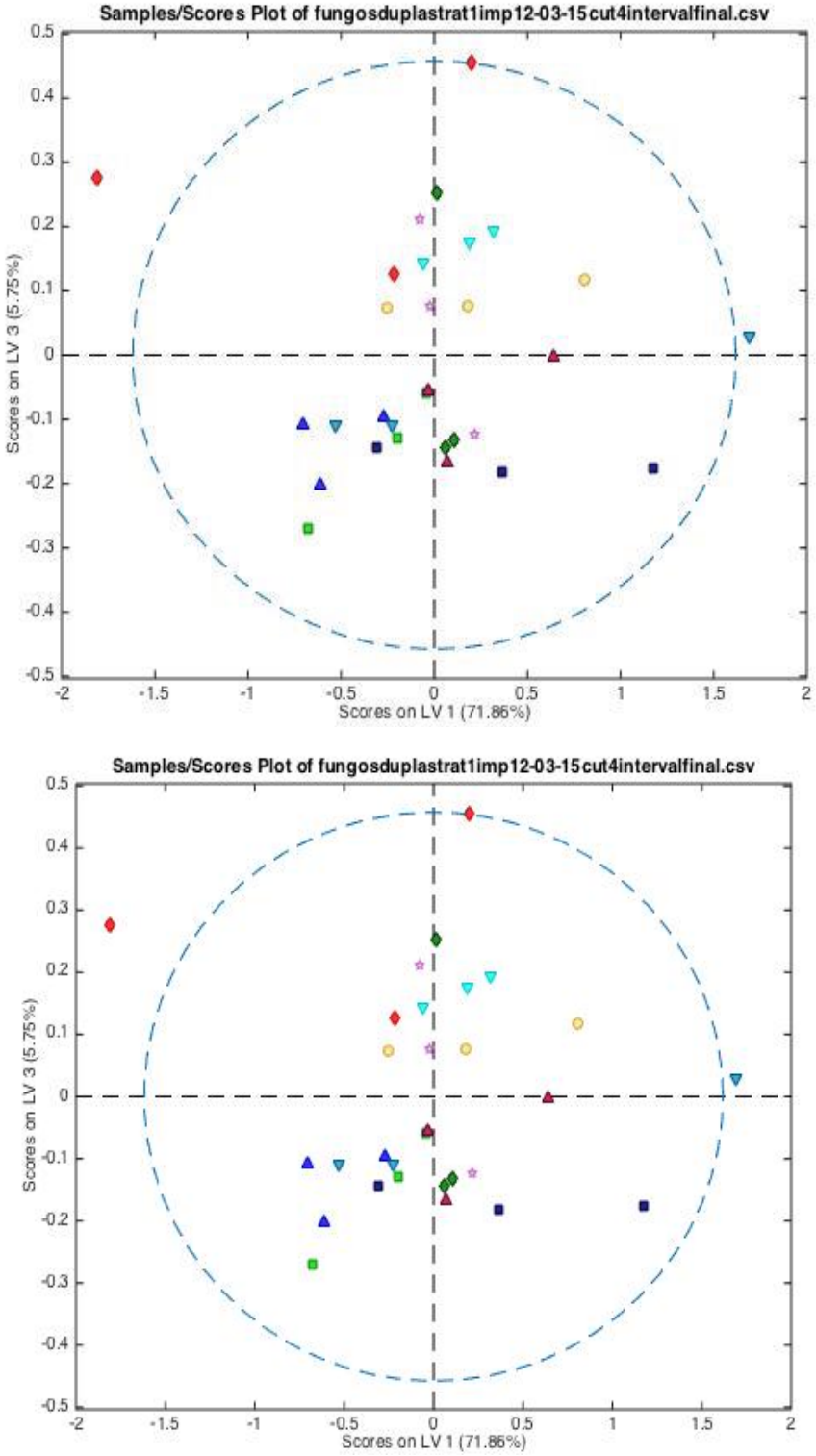


Figure 46 shows the resultant plots of scores in PCA obtained for the four fungi co-culturing experiment. The score plots show the relations among the four fungi growing together, the interactions zone in pairs, the interaction zone among the

four and the isolated strains. In figure 47 is the plot of PLS-DA of the corresponding PCAs values. PCA does not offer a good separation of the isolated strains from the strains remaining in the co-culture experiment. However, along PC2 in both sides of this PC it is possible to see a separation of the interaction zones (located between the pairs). The contact zone of the four fungi is located between the interaction zone of the pair and the isolated strains but it can be separated from the other groups along PC3 positive. In PLS-DA score plots it is easier a separation of the classes, the co-culture interaction zone of the pairs is explained along VL2 and the four interaction zone is located along LV3 negative. Along LV1 negative side it is the isolated cultures. There is an overlap between the isolated fungi and a slightly one in the co-cultured regions of pairs.

Figure 46: Plot of Scores in PC1xPC2 of the three principal components analysis of zone of contact of pairs (CSP-11c/CSP-09; CSP-11c/CSP-12Ia; CSP-11e/CSP-12Ia and CSP-09/CSP11e); zone of contact with four (CSP-11c/CSP-09/CSP-11e/CSP-12Ia); the remaining parts of colonies (CSP-12IaCo; CSP-11cCo; CSP-09Co and CSP-11eCo) isolated controls (CSP-12Ia; CSP-11c; CSP-09 and CSP-11e). Fungi 1- CSP-09; Fungi 2-CSP-11c; Fungi 3-CSP-11e and Fungi 4:CSP-12Ia.

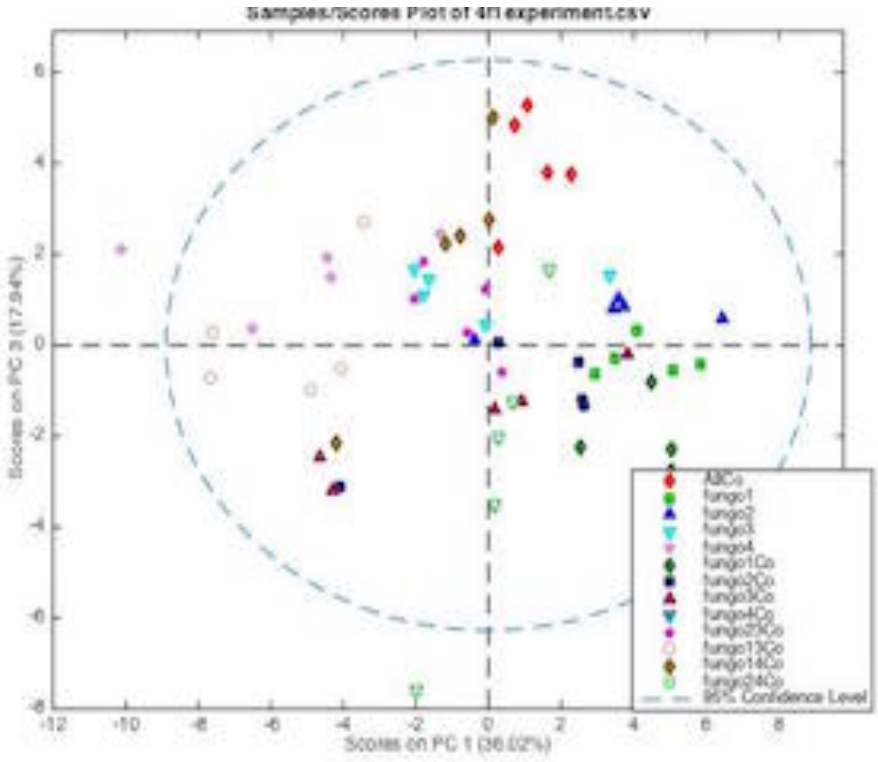
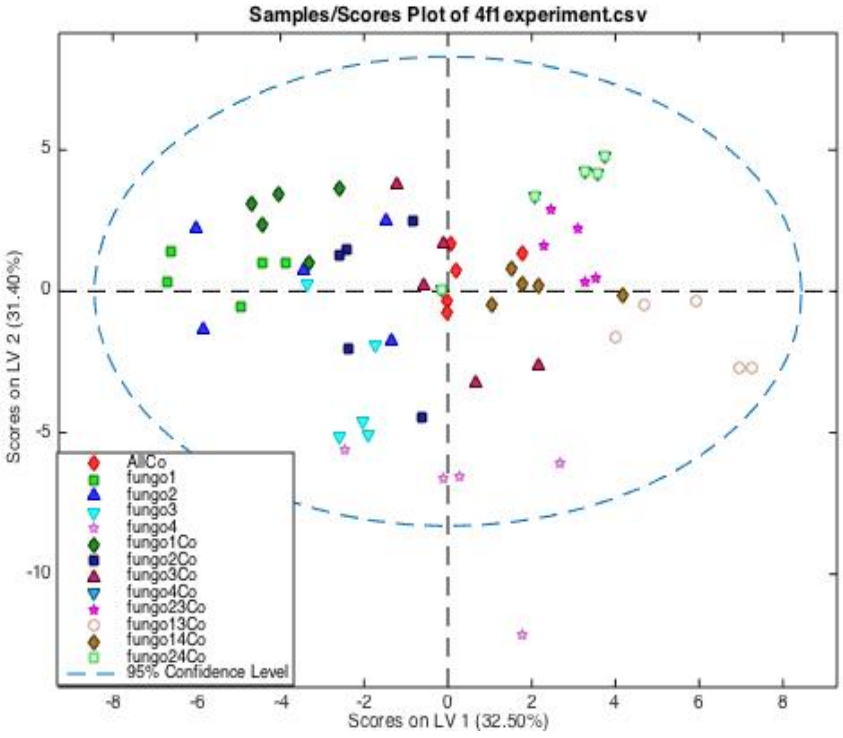
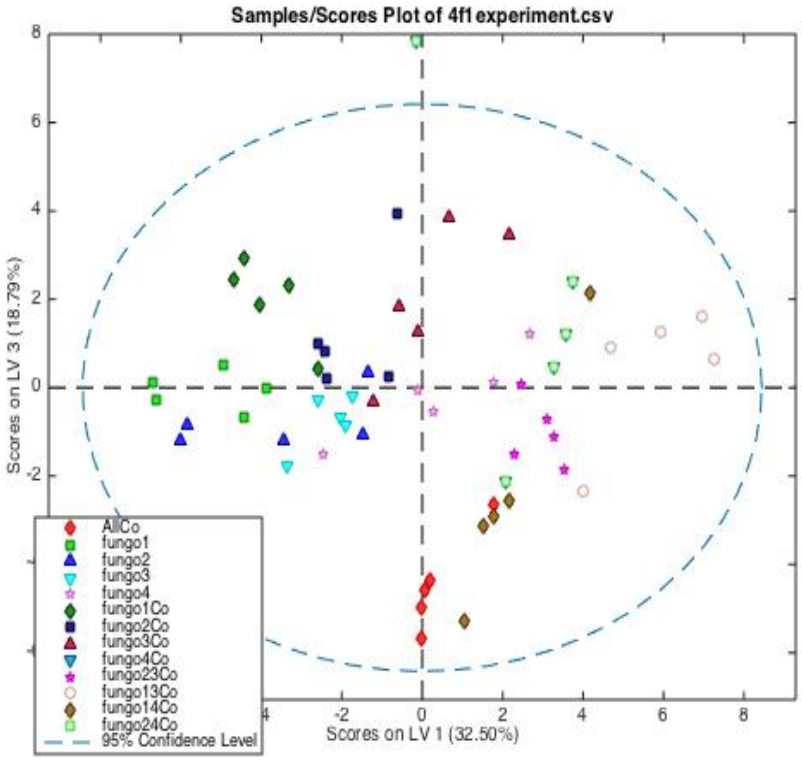


Figure 47: Plot of Scores in PLS-DA: VL1xVL2; VL1xVL3 respectively, of the three zones in co-culturing experiments: zone of contact of pairs (CSP-11c/CSP-09; CSP-11c/CSP-12Ia; CSP-11e/CSP-12Ia and CSP-09/CSP11e); zone of contact with four (CSP-11c/CSP-09/CSP-11e/CSP-12Ia); the remaining parts of colonies (CSP-12IaCo; CSP-11cCo; CSP-09Co and CSP-11eCo) isolated controls (CSP-12Ia; CSP-11c; CSP-09 and CSP-11e). Fungi 1- CSP-09; Fungi2-CSP-11c; Fungi3-CSP-11e and Fungi 4:CSP-12Ia.





The existence of these separate zones is a consequence of the differences among the values of the variables used in the PCA. These differences indicate that are changes in metabolites that were released by the fungi or the bacteria in the interaction zones.

3.4 HPLC-DAD/MS of the interaction zone of the four fungi in co-culture

The TIC positive and negative of the interaction zone of the four fungi in the coculture experiment are related on figure 48. From the TIC a lot of *m/z* can be noted. The following figures (figure 49-50) are the mass spectra for each retention time detected on the HPLC/DAD. The results were compared with the DNP database using as search guide the genus and species, *Fusarium* and *Fusarium solani*, which owns the fungus from which the extract was generated and the databases Metlin and Massbank. For those results ESI (+)-MS/MS experiment was performed, which observed fragmentations of enniatins (figures 47-48) as it can be seen in the spectra for the ions*, m/z* 683, *m/z* 669, *m/z* 556*, m/z* 551, *m/z* 343 and in *m/z* 313.

Figure 48: a) TIC in positive and negative mode of the interaction zone of the four fungi cultivation.

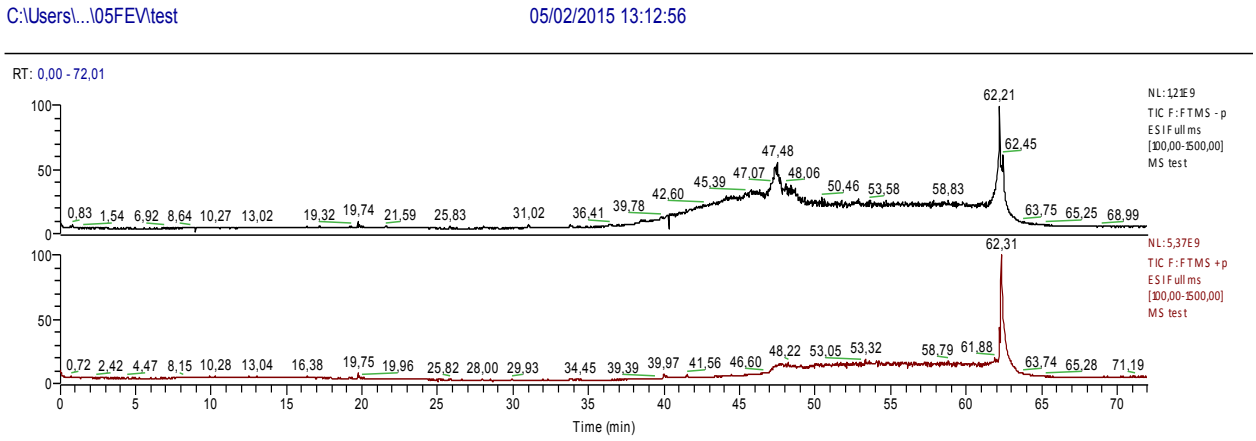


Figure 49: a) Amplified region of RT 54.03 min. in a) positive mode of the interaction zone of the four fungi cultivation.

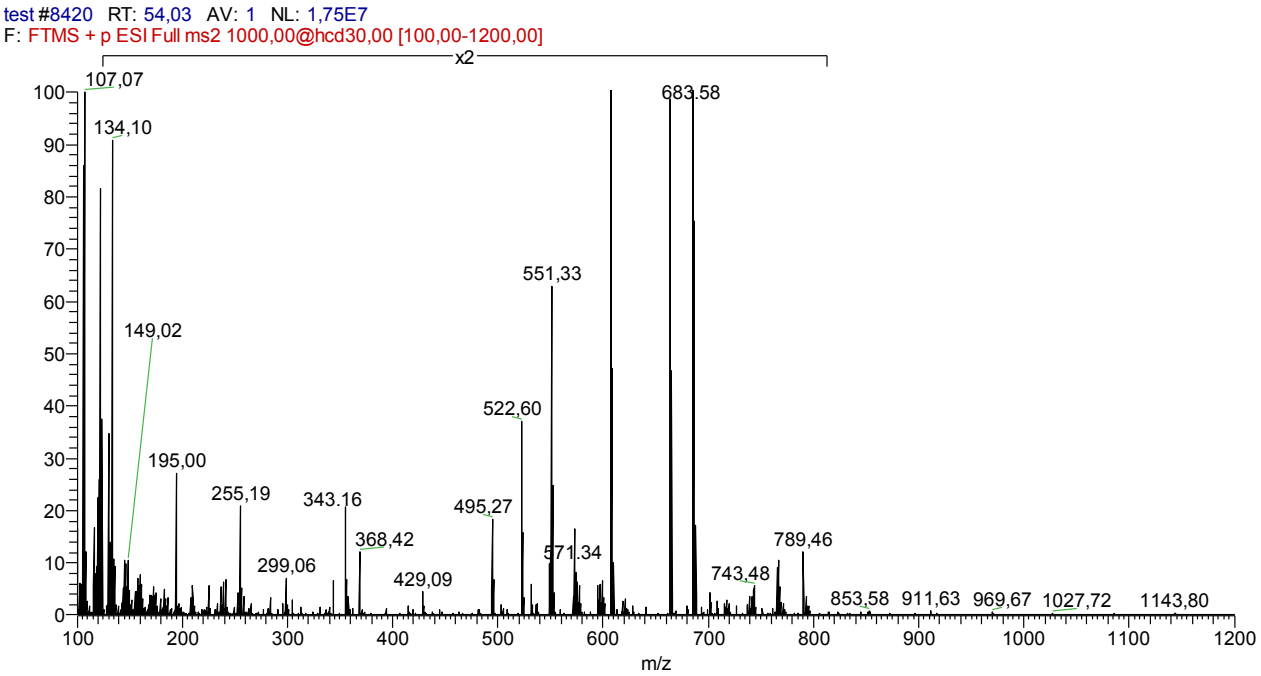
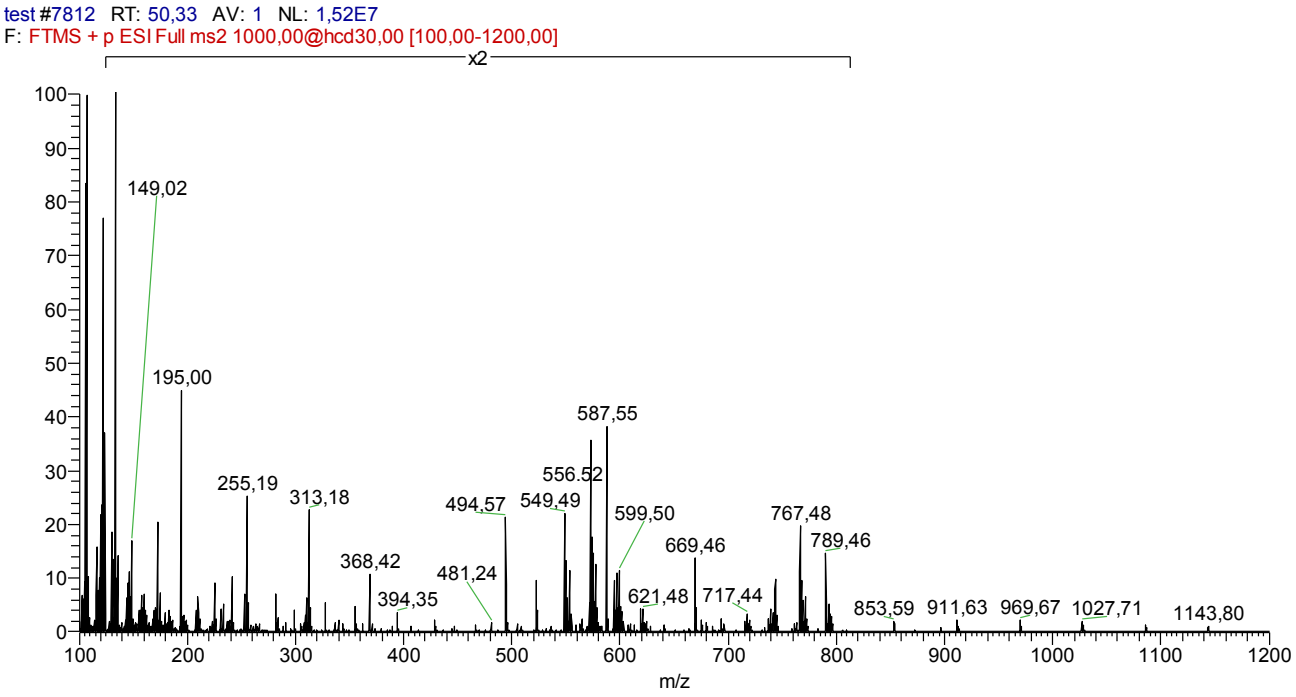


Figure 50: a) Amplified region of RT 50.33 min. in a) positive mode of the interaction zone of the four fungi cultivation.



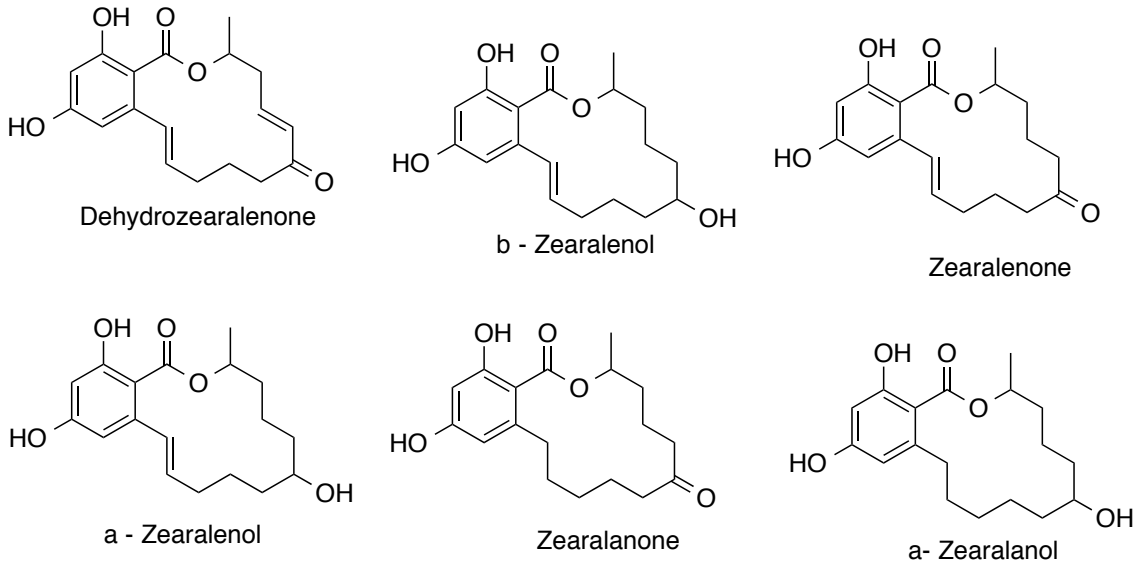
4 DISCUSSION

By chromatographic data it was possible to evaluate the metabolic profile and check the possible presence in these extracts the markers of interest: piperidine alkaloids - cassine and spectaline - produced by the plant *S. spectabilis*. With the information obtained we observed that the markers of interest are not present in any of the extracts tested to date. However, it has been observed that the possibility of alkaloids in the samples present significant bands in the regions close to the standards. By comparing the profile of the extracts with the chromatographic profile of the extract from the culture medium without the fungus it was observed that the bands corresponding to the metabolites produced by the fungus coincide with some less intense bands with culture medium lacking the fungus. Thus it becomes possible to determine with certainty which microorganism exclusively produces the metabolites.

The cultivation of microorganisms in liquid media was chosen randomly among the 185 microorganisms isolated.

In the evaluation of *Fusarium* extracts by HPLC/MS-MS a series of zearalenone[151] compounds were detected (figure 51).

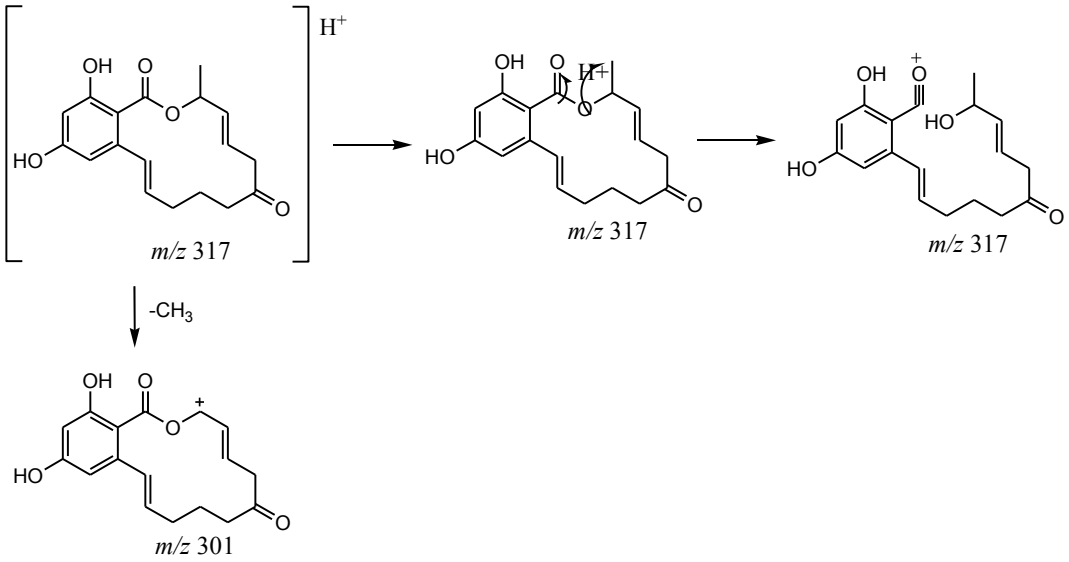
Figure 51: Zearalenones: derivate structures.



Zearalenone is a β-resorcyclic acid lactone mycotoxin produced by *Fusarium* species.[151] They are polyketide derivates and are very harmful to crops as corn, wheat, sorghum, and rice and are also toxic for humans and animals. The following

figure (figure 52) shows the proposal of the fragmentation observed for these compounds in the *Fusarium* extracts. An opening of the lactone ring occurs provoking the formation of *m/z* 317 ion. A loss of 15 Da produces the ion in *m/z* 301.

Figure 52: Fragmentation proposal of Zearalenone derivated structures.



From de previous analysis using with HPLC-DAD, four species of fungi and one of bacteria (*Burkholderia* sp) were selected for the co-culture cultivation. Those fungi strains belong to the *Fusarium* genus but different species and *Paecilomyces* genus. The goal of choosing only a few organisms was to submit them to co-culture experiments and it would not be possible using all microorganisms isolated in this work. The choice was random, but it was made with microorganisms that were in the same “mother agar plate”, with the aim of imitating environmental conditions, and in this case, with the interactions among other microorganisms in the rhizosphere and consequently observe if there would be alterations in metabolic production when compared with pure strains. *Fusarium* strains were selected because they are very common microorganisms found in soil environments and chemistry is also widely involved with this fungi. In addition it would be a good opportunity to see how organisms from the same genus behave when submitted to grow together. A different fungus from *Paecilomyces* genus was selected as a differential factor among the *Fusarium* strains. *Burkholderia* species are great antimicrobial producers and is also common organisms in rhizosphere.

The experimental design was made based on: first see how two strains behave when submitted to a co-culture, so six different experiments were evaluated using combinations of the four fungi selected and second mix all together and the results compared with the previous co-culture experiment.

The first step in the co-culture experiments was observing the visual changes that daily occurred. Changes in color of the colony may be due to production of some extracellular enzymes that can act in the hydrophobicity of the cell wall modification, these changes act as fungal defense mechanisms.[153] The distance of inhibition, where the fungus grows, does not compete directly with other colony and this fact can be associated with the production and release of compounds that inhibit the growth of both fungi. For example, some of compounds produced by *Fusarium* species as enniatins A, A1, B and B1[153] have been reported as antimicrobial activity against species of bacteria (*Bacillus*, *Mycobacterium*) and phytopathogens (*Botrytis cinerea*; *Beauveria bassiana* and *Trichoderma harzianum*. The formation of the white line between the two molds in the contact zone may be associated with production of new (s) metabolite (s) by one of the individuals since this type of display is not found when the organisms are growing isolated or some of the pigment elapsed action of some enzyme, such as melanin production which acts as a physical barrier against enzymes of other organisms.[118;152] Direct contact of the fungus after normal growth may be due to the formation of inhibitory compounds, but no apparent evidence for this was observed.

The same interactions described for paired co-cultures were observed during co-culture with 4 arranged in the same mold plate. The main changes here was the duration of the period of cultivation that it was 7 days, with the first contact on the 5th. In the contact area among the 4 fungi there was a clear "inhibition distance" between the four species. CSP-11c/CSP 11e have acquired a yellow color from the 5th day of growth; CSP-09 remained white all the time with no formation of pigment in the typical pink aerial mycelia for this fungus at 5 days of culture; CSP-12Ia had extended its borders with an intense white color, not so evident in the isolated colony and grew very little compared to the other. CSP-11c and CSP-11e remain at such a distance inhibition as evidenced in the previous experiment. CSP-09 and CSP-12Ia are also far apart. CSP-11e and CSP-12Ia no formation of aerial mycelium in the contact zone between the two by CSP-11e was observed. CSP-11c and CSP-12Ia meet but no one overlaps the other, thus maintaining a line of contact. Among CSP-

11e/CSP-09, CSP-09 and CSP-11c there is the formation of a white dividing line in the contact zone.

The second step to evaluate the co-culture experiments was acquiring ^{1}H NMR data and subsequently analyzing by multivariate analysis.

Methanol was used for the metabolites extraction because detectable metabolites are not always extracted completely in the polar phase, since a semi polar extraction system might contribute to the large-scale detection of various metabolites. Furthermore, it was more effective for extractions in solid agar medium than EtOAc. In addition, to use other systems of solvents as different proportions of CH_2Cl_2/EtOAC/MeOH - reported been effective for solid agar media extractions - would not contribute to an easy one-solvent-step extraction that is expected in metabolomics analysis as a high throughput platform reported as been effective for the detection of metabolites. However, even using a semi polar extractor, the samples were not soluble when dissolved in MeOD or other common NMR solvents, so it was necessary to use D_2O buffered with KH_2PO_4. This may occur due to the highly polar compounds in the extracts. Of course the line broadening provoked by water peak (4.8-5.0 ppm) is a serious barrier to detecting signals but it can be removed by suppressing the water signal or in the time of the data processing the interfering signal is easily removed from final spectra and it was used in this work without compromising of the detection of observation in changes during the co-culturing experiments.

Within the data acquired it was possible to say that the ^{1}H NMR can be used to detect and locate differences in the metabolic profile of the co-cultures compared monocultures and help to identify which compounds are being produced and which ones differ in the extracts. The disadvantage of NMR is its low detection limit, i.e. some compounds that are present in low amounts in the extracts were not detected.

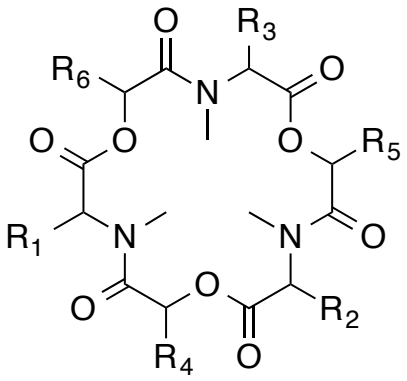
PCA identify the largest variation in data via the principal components and represents the data in a coordinate system defined by PC's. The results reported in this work showed that for all co-culture experiments there is a separation between the interaction zone and the single fungi. Only the CPS-12Ia fungus in all experiments is really close to the medium composition. One hypothesis for this result is that the fungus produces few compounds that could not be detected by NMR or it was inhibited by the substances released by other fungi strains. The most differences are detected by the interval of δ 4.2-4.4ppm.

Analyzing the graphics scores and weights resulting from this analysis it was possible to identify patterns of association and similarity among the samples as well as among variables. These standards form the basis for establishing spatial relationships between the co-cultures and the isolated strains.

In order to complement the results obtained with NMR, with the analysis of PCA and PLS-DA, a LC/MS experiment was carried out using the extract obtained from the co-culturing experiments containing the interaction zone of the four fungi.

The compounds detected in the pure strain cultivation can also be found in the co-culture experiment but also a series of enniatins can be detected: enniatin L, M1 and M2 (Figure 53). Those detected substances were found in the interaction zone of the four fungi in the four fungi co-culture experiment. These results indicate the metabolic production have changed during the co-culturing experiments in agreement with the observations noted on the agar plates (phenotypic) during the experiments. However, it is not possible detecting if fungi in the partnership are producing those compounds because enniatins are produced by a plethora of different species of *Fusarium*.

Figure 53: Chemical structure of enniatins.

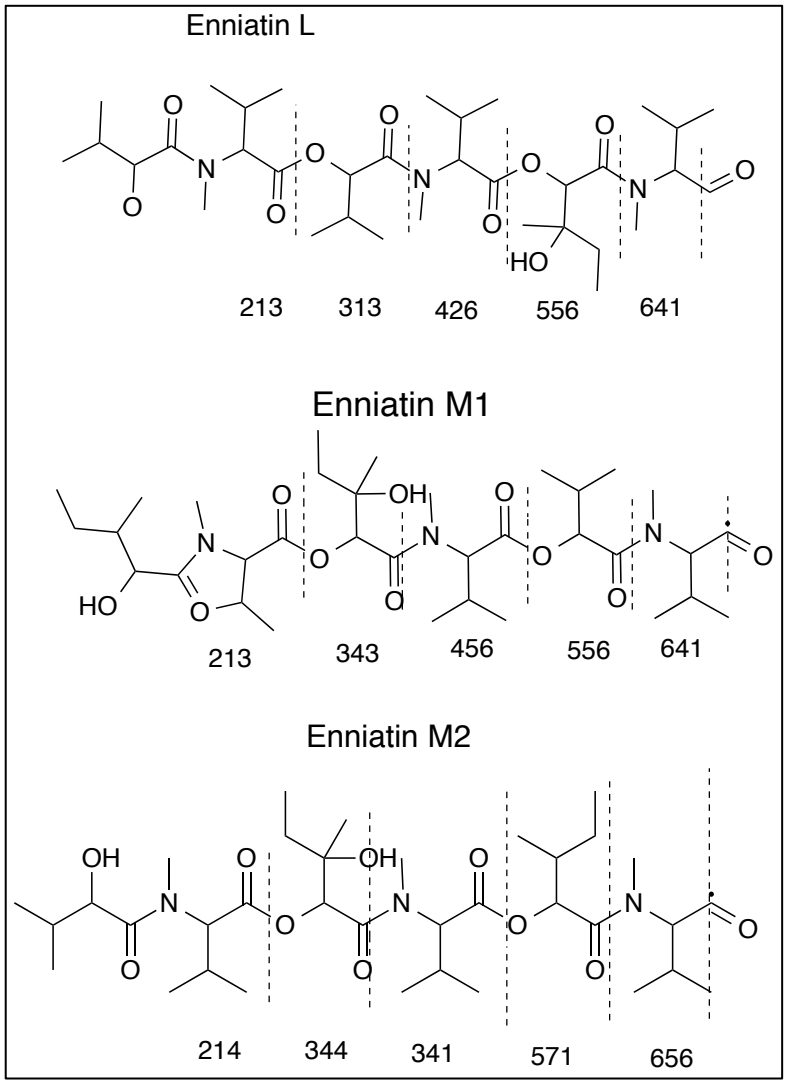


Enniatin L R_1=R_2=R_3=R_4=R_5:$CH(CH_3)_2$; R_6=$C(CH_3OHCH_2CH_3)$
Enniatn M_1 R_1=R_2=R_3=R_6:$CH(CH_3)_2$; R_4=$C(CH_3OHCH_2CH_3)$;R_5 =$C(CH_3CH_2CH_3)$
Enniatin M_2 R_1=R_2=R_3=R_5:$CH(CH_3)_2$; R_4=$C(CH_3OHCH_2CH_3)$;R_6 =$C(CH_3CH_2CH_3)$

Enniatins are cyclohexadepsipeptides composed of alternating residues of three N-methyl amino acids, commonly valine (Val), leucine (Leu), and isoleucine (Ile), and three hydroxy acids, typically hydroxyisovaleric acid (Hiv) and are commonly found in *Fusarium* species. There are 29 enniatins described in the literature from natural

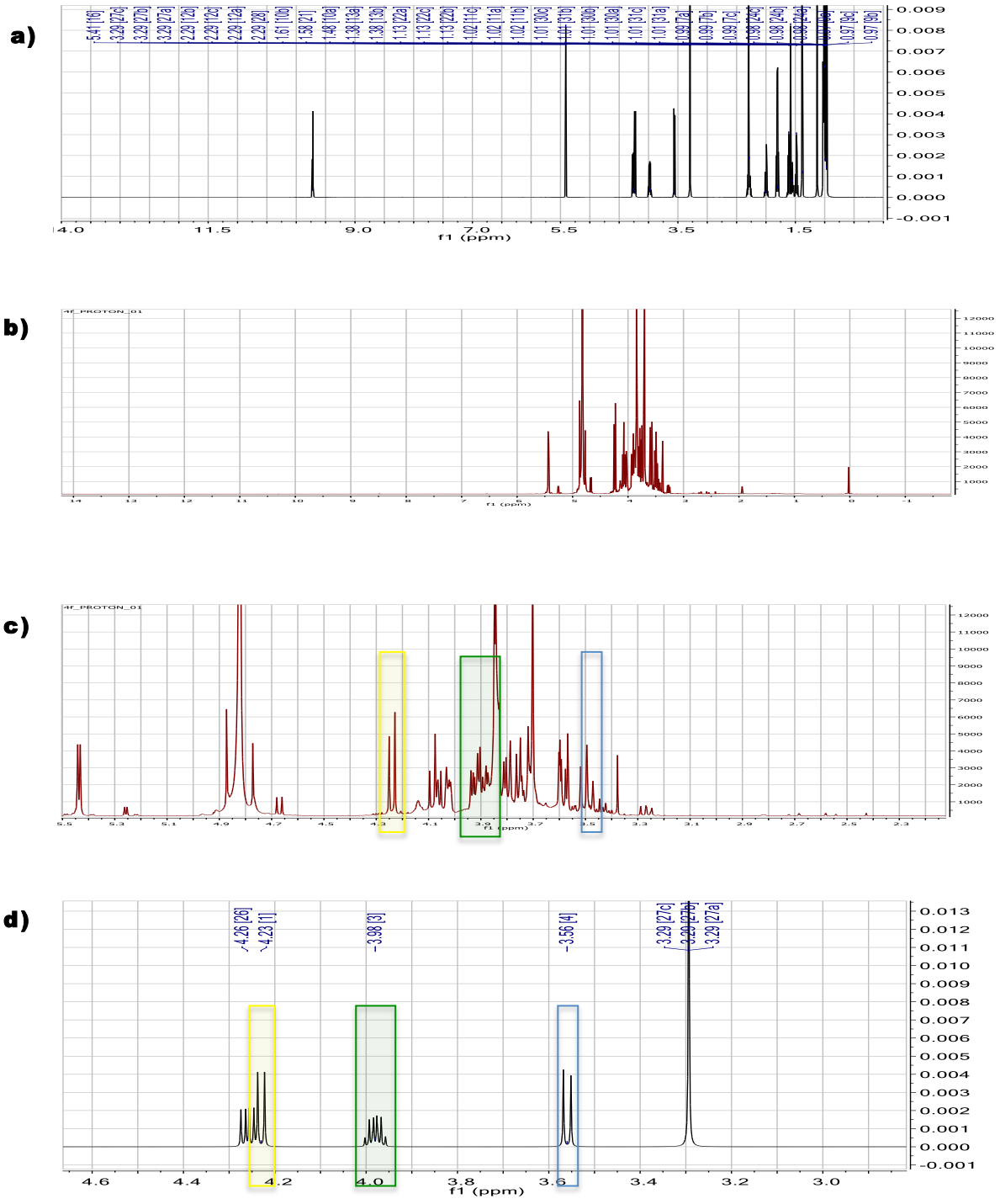
source and revealed biological activities as antimicrobial, enzyme inhibitor, cytotoxic and anticancer properties.[154-155] They can be easily detected by positive mode fragments *m/z* 670 $[M+H]^+$ to enniatin L and *m/z* 684 $[M+H]^+$ to enniatins M_1 and M_2. In general, an opening ring composes the fragments observed to the enniatins by cleavage C-O bond followed by loss of CO (28Da) and loss of amino acids in sequence as observed in figure 54.

Figure 54: Proposal of fragments of Enniatins L, M1 and M2.



Comparing the NMR obtained for the crude extract of the four-region interaction and a simulated NMR spectra it was possible observed a few signals referent to oxazolidinic ring of the Enniatin M1 in the extract, which can indicate the presence of this molecule in the interaction zone (figure 55).

Figure 55: Comparision of ^{1}H NMR a) simulated of partial Enniatins M1 with the b) ^{1}H NMR obtained for the interaction zone of the four fungi in co-culturing experiment. Amplified regions (δ 3.56 ppm; δ 3.98 ppm and δ 4.25 ppm) referent of the signal of proton in the oxazolidinic ring of Enniatin M1 c) simulated spectrum of partial Enniatins M1 and d) obtained spectrum for the interaction zone of the four fungi in co-culturing experiment.



CONCLUSIONS

In this work seedlings of *Senna spectabilis* were cultivated in sand and in hydroponics cultivation and the rhizospheric microorganisms community were studied.

The focus on the analysis of the microbial community was on fungi and bacteria. The first steps were isolating the microorganism from the rhizospheric region in both sand and hydroponics cultivations. The most dynamic and numerous microorganisms were found in the hydroponic cultivation. A total of 197 microorganisms were isolated: 136 fungi and 61 species of bacteria.

The knowledge of which organisms were part of the rhizospheric community a taxonomic identification was made. In bacteria the most diverse species included in eleven different phyla were found: *Actinobacteria*, *Chloroflexi*, *Deinococcus-Thermus*, *Nitrospirae*, *Planctomycetes*, *Bacteriodetes*, *TM7*, *Verrumicrobia*, *Firmicutes*, and *Proteobacteria*. The fungi community detected was also rich and shows 2 major phyla for this organisms Ascomycota and Basidiomycota species. The relevant genus identified in this group is *Fusarium*. It consists of a known group of fungi that can be harmful for plants and animals by producing toxins highlighting the derivates from diketopeptides: fumonisins, trichothecenes and zearelenones but also for the antimicrobial components such as enniatins, bouvericins and bikaverin. Besides using different techniques and instruments of identification it was possible to see for the bacterial community the same phyla.

A good knowledge about the composition of the rhizospheric community of *Senna spectabilis* was reached in this work, supplying the lack of existing studies of the rhizosphere and the microbial population associated with it. Of course a seasonal and deeply study of the maintenance or changes in this community it is required in order to evaluate how much environmental factors are responsible for the community.

The fungal extracts evaluated by HPLC-DAD showed, specially for the *Fusarium* species, not only a great variability of compounds but also that another detector needs to be used in order to detect compounds commonly produced by these species, such as the trichothecens and fumonisin that are not detected by UV. This

hypothesis was detected when the HPLC-DAD/MS was used. Several compounds that are not possible to be identified in the UV chromatogram are detected by mass.

It was detected by HPLC-DAD/MS a mixture of zearelenones in the *Fusarium* extracts from the liquid medium cultivation and fragments obtained in the MS/MS experiments allowed to indicate possible pathways for the fragmentation. For the other fungi a more elaborate data package is necessary in order to conclude the components of the extracts.

The data collected of the liquid cultivation provided an indication of which species of microorganisms could be used in the co-cultured experiments. The strains selected in here were in the same vessel, which means they were affected by same conditions when they were associated with the plants. So the idea was to detect and see how different species from the same genus would be affected by each other. A different species of fungi (*Paecilomyces sp*) and bacteria (*Burkholderia* sp) was also used in order to compare if the changes provoked by a totally strange in the same media would be the same or more significant than the one provoked by the similar organisms.

PCA's and PLS-DA's resultant from the ^{1}H NMR data showed that these are differences in co-culture experiments when compared with the isolated strains especially with the different genus species. Besides that in the agar plates during the experiments it was noted that *Fusarium* species seemed to inhibit the development of *Paecilomyces* and the *Burkholderia* is totally covered by the *Fusarium*. This can indicate that the mycotoxin or antimicrobial compounds have been produced and released on the medium by *Fusarium* species.

The dynamics of the rhizosphere can be discreetly understood by the experiments involving mixed cultures since it is a complex mix of different organisms living together in the same space itself. For this, it is neecessary a more insightful evaluation of the results obtained by making use of rational methodologies of detection and evaluation of complex mixtures.

Mass spectrometry and ^{1}H NMR is an essential tool in a rational methodology for detection of metabolites in microorganisms. Conducted with the statistical tools PCA and PLS-DA provide an efficient way to identify and locate differences in the metabolic profile of the co-cultures compared monocultures and help to identify which compounds are being produced and which differ in the extracts. PCA and PLS-DA provide a rapid tool in order to reduce dimensionally the data and allow an easy

identification of the differences and the classification of similar individuals. The implementation of chemometric techniques with a use of analytical techniques capable of providing accurate results in a simple and rapid manner proved to be a good method in the analysis of a lot of data.

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APPENDIX

APPENDIX A: Chromatograms obtained by HLPC-DAD using C18 column onyx phenomenex. 5% to 100% B in 25 min, 25- 30 min. 100%; 30-33 min. returning the initial condition of 5%; 33-38 min. remaining at 5% for the balance of the column. The flux used in each assay was 2.5 mL/min, injection volume of 30 µL. B=MeOH.

Figure A1: Chromatograms of EtOAc extract of CSP-1 after 21 days of cultivation– a) 280 nm and b) 366 nm.

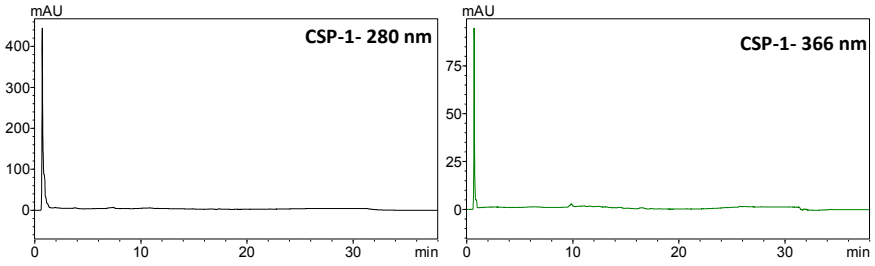


Figure A2: Chromatograms of EtOAc extract of CSP-02 after 21 days of cultivation – a) 280 nm and b) 366 nm.

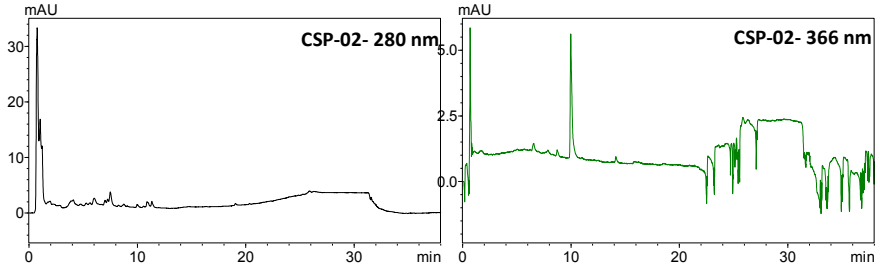


Figure A3: Chromatograms of EtOAc extract of CSP-03 after 21 days of cultivation – a) 280 nm and b) 366 nm.

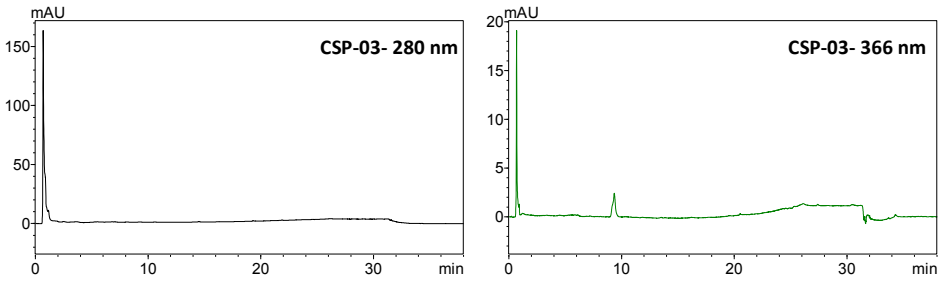
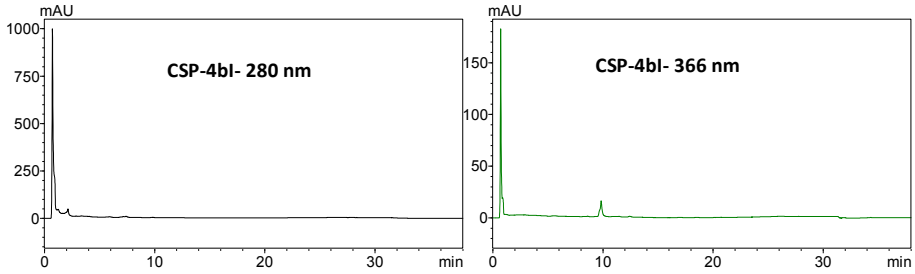


Figure A4: Chromatograms of EtOAc extract of CSP-4bI after 21 days of cultivation – a) 280 nm and b) 366 nm



. **Figure A5:** Chromatograms of EtOAc extract of CSP-4bII after 21 days of cultivation – a) 280 nm and b) 366 nm.

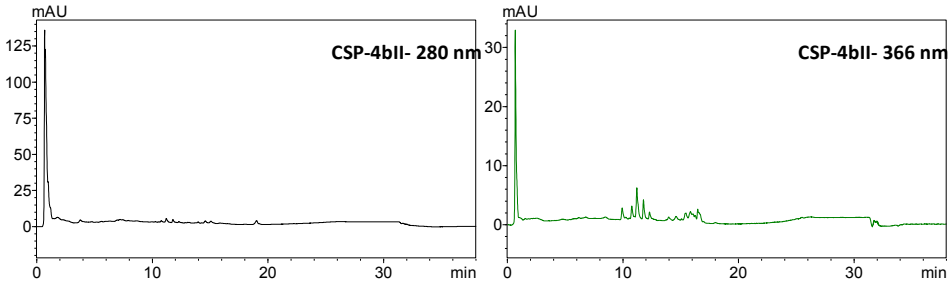


Figure A6: Chromatograms of EtOAc extract of CSP-5a/05 after 21 days of cultivation – a) 280 nm and b) 366 nm.

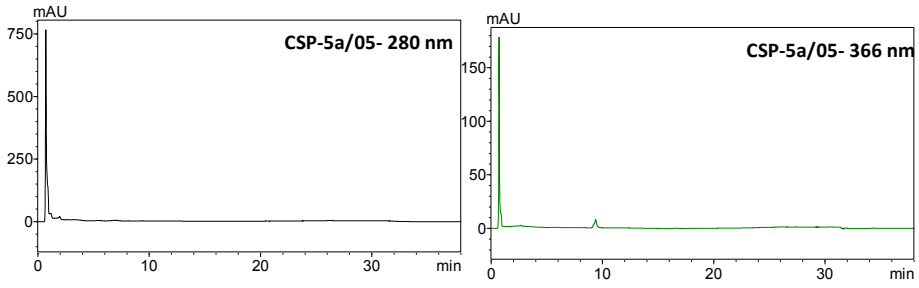


Figure A7: Chromatograms of EtOAc extract of CSP-5b after 21 days of cultivation – a) 280 nm and b) 366 nm

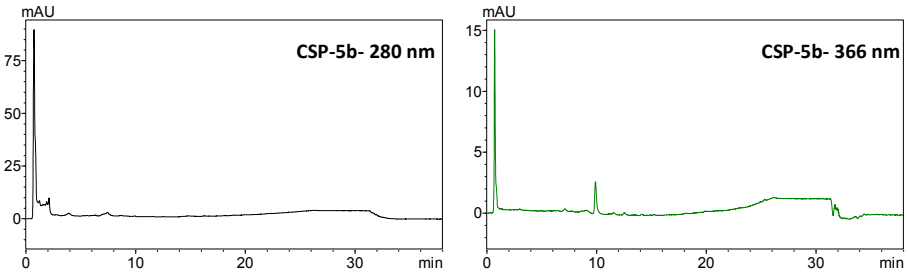


Figure A8: Chromatograms of EtOAc extract of CSP-6b after 21 days of cultivation – a) 280 nm and b) 366 nm.

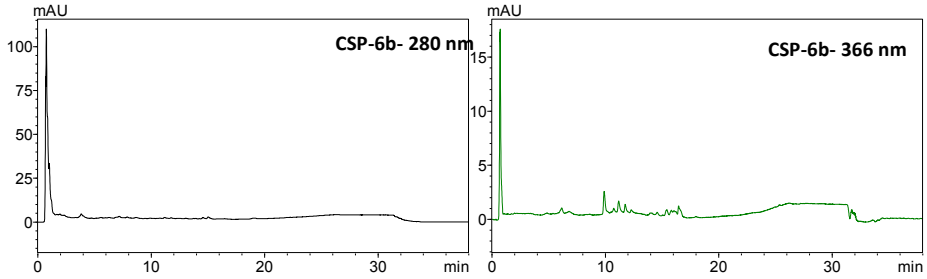


Figure A9: Chromatograms of EtOAc extract of CSP-7 after 21 days of cultivation – a) 280 nm and b) 366 nm.

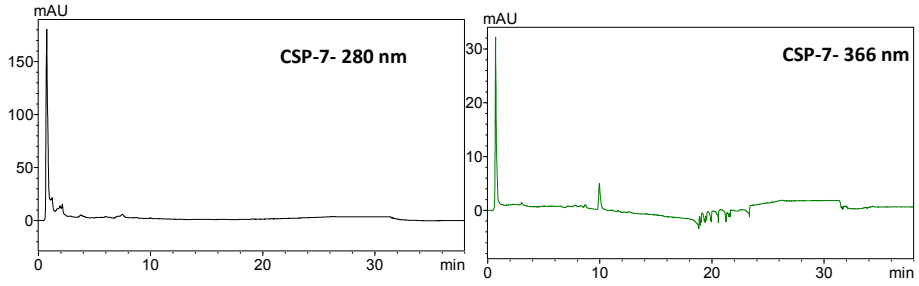


Figure A10: Chromatograms of EtOAc extract of CSP-09 after 21 days of cultivation – a) 280 nm and b) 366 nm.

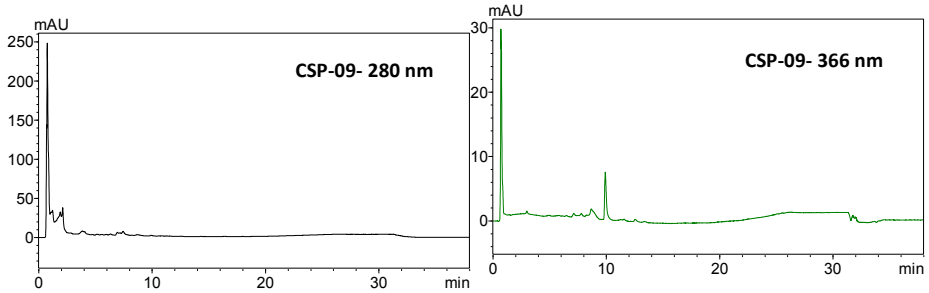


Figure A11: Chromatograms of EtOAc extract of CSP-9 after 21 days of cultivation – a) 280 nm and b) 366 nm.

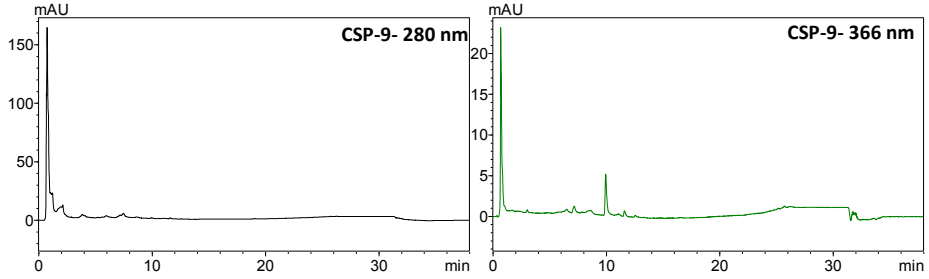


Figure A12: Chromatograms of EtOAc extract of CSP-10 after 21 days of cultivation – a) 280 nm and b) 366 nm.

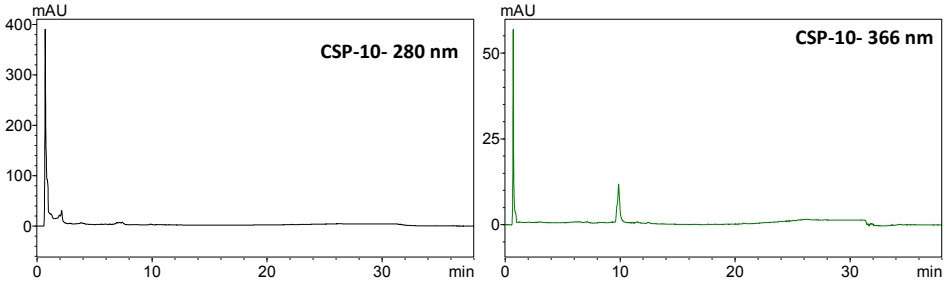


Figure A13: Chromatograms of EtOAc extract of CSP-11a after 21 days of cultivation – a) 280 nm and b) 366 nm

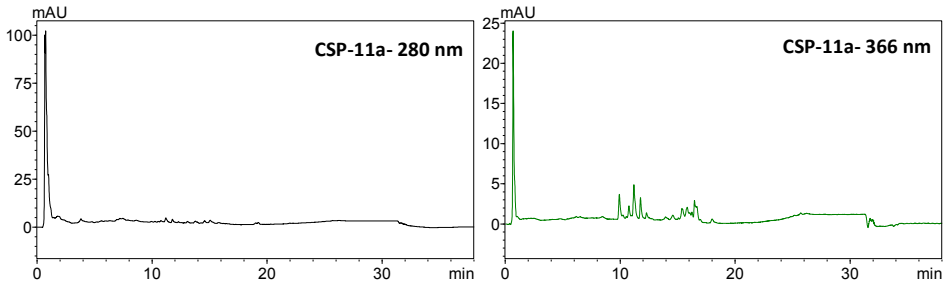


Figure A14: Chromatograms of EtOAc extract of CSP-11c after 21 days of cultivation – a) 280 nm and b) 366 nm.

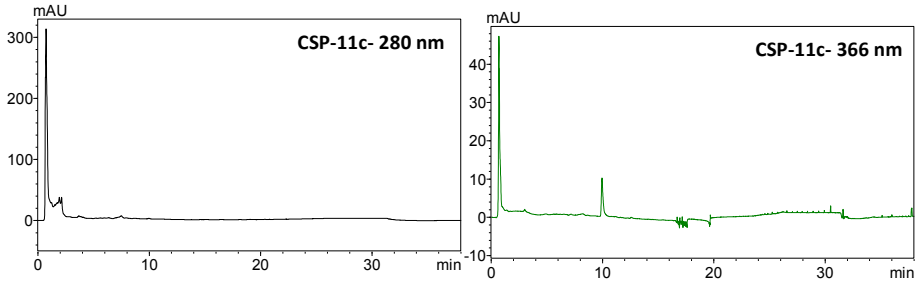


Figure A15: Chromatograms of EtOAc extract of CSP-11e after 21 days of cultivation – a) 280 nm and b) 366 nm.

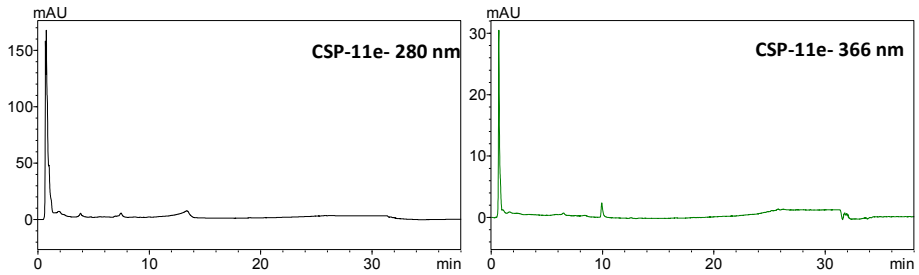


Figure A16: Chromatograms of EtOAc extract of CSP-12a after 21 days of cultivation – a) 280 nm and b) 366 nm.

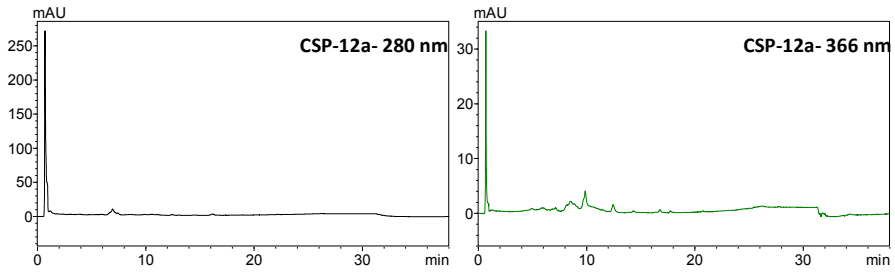


Figure A17: Chromatograms of EtOAc extract of CSP-02 after 21 days of cultivation – a) 280 nm and b) 366 nm.

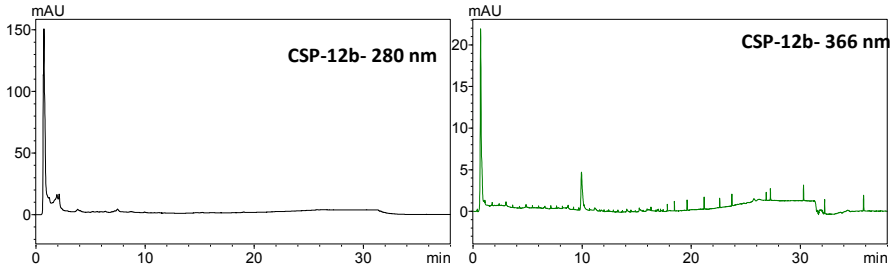


Figure A18: Chromatograms of EtOAc extract of CSP-13II after 21 days of cultivation – a) 280 nm and b) 366 nm.

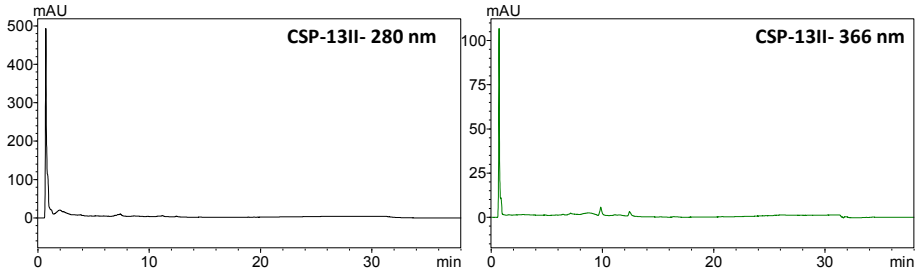


Figure A19: Chromatograms of EtOAc extract of CSP-13IIa after 21 days of cultivation – a) 280 nm and b) 366 nm

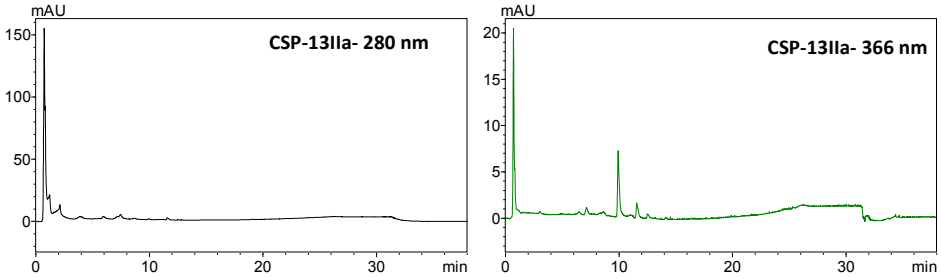


Figure A20: Chromatograms of EtOAc extract of CSP-17 after 21 days of cultivation – a) 280 nm and b) 366 nm.

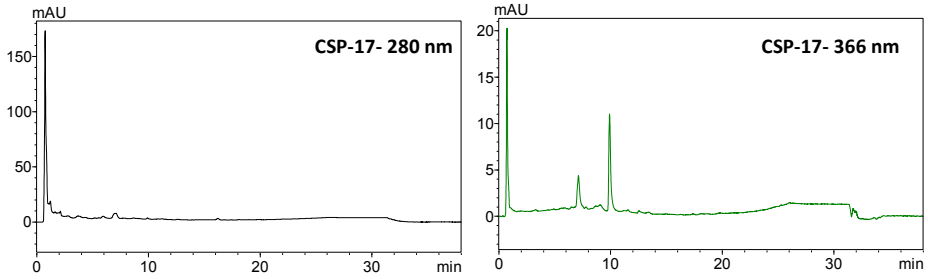


Figure A21: Chromatograms of EtOAc extract of CSP-19a after 21 days of cultivation – a) 280 nm and b) 366 nm.

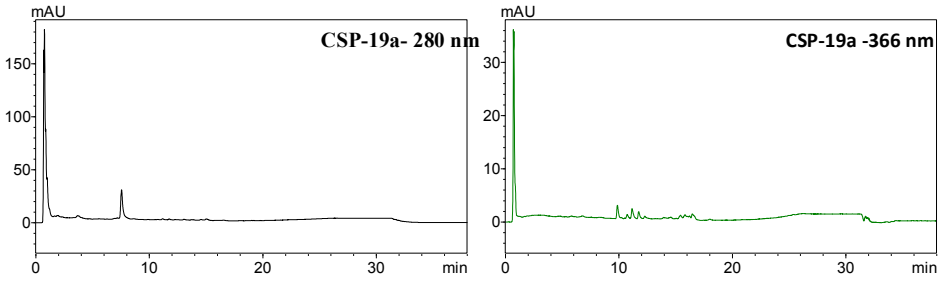


Figure A22: Chromatograms of EtOAc extract of CSP-20 after 21 days of cultivation – a) 280 nm and b) 366 nm.

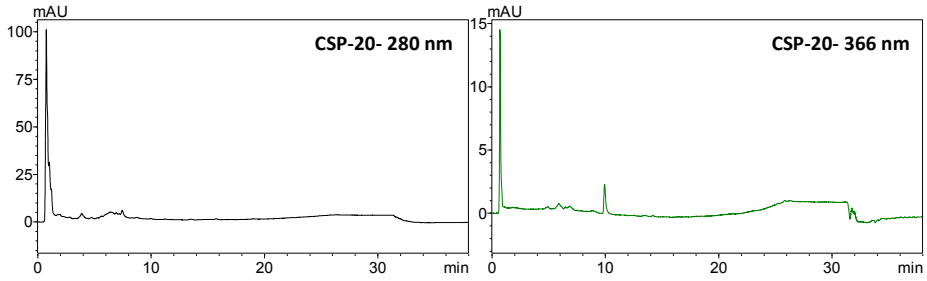


Figure A23: Chromatograms of EtOAc extract of CSP-24 after 21 days of cultivation – a) 280 nm and b) 366 nm.

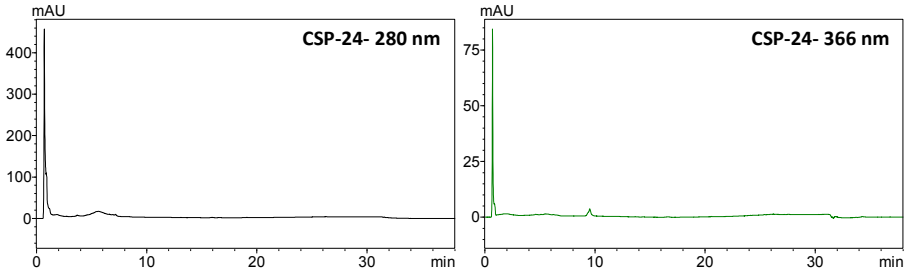


Figure A24: Chromatograms of EtOAc extract of CSP-25 after 21 days of cultivation – a) 280 nm and b) 366 nm.

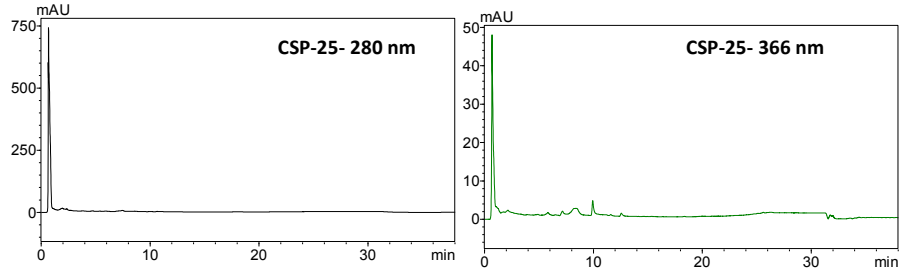


Figure A25: Chromatograms of EtOAc extract of CSP-25/26 after 21 days of cultivation – a) 28 0nm and b) 366 nm.

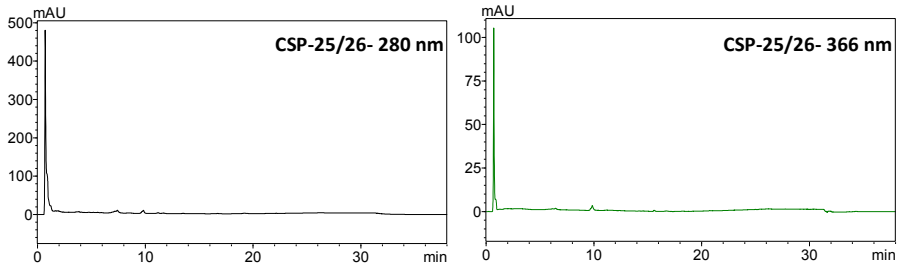


Figure A26: Chromatograms of EtOAc extract of CSP-26 after 21 days of cultivation – a) 280 nm and b) 366 nm.

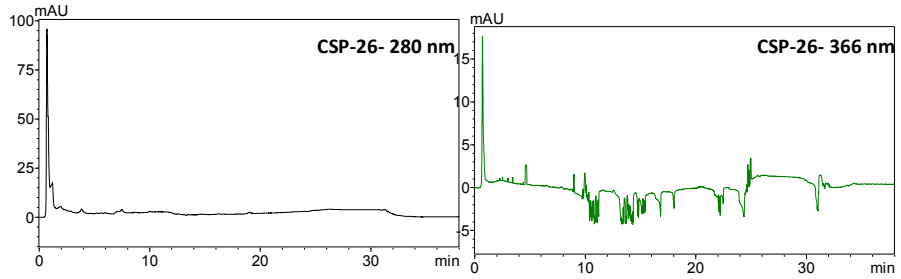


Figure A27: Chromatograms of EtOAc extract of CSP-28 after 21 days of cultivation – a) 280 nm and b) 366 nm.

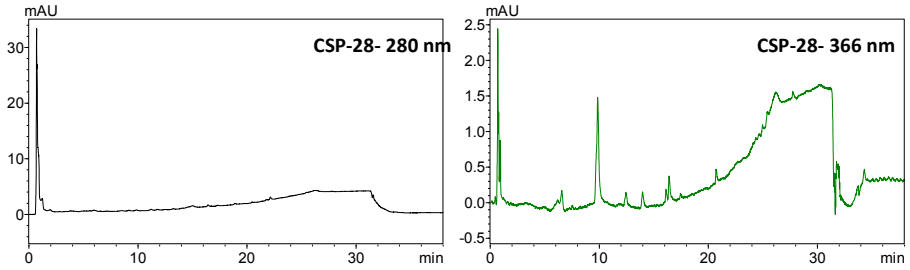


Figure A28: Chromatograms of EtOAc extract of CSP-29 after 21 days of cultivation – a) 280 nm and b) 366 nm.

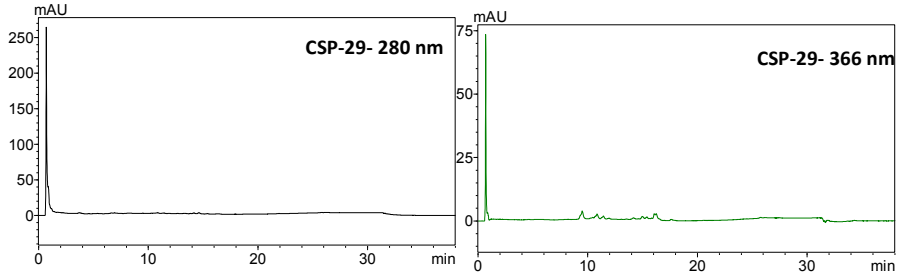


Figure A29: Chromatograms of EtOAc extract of CSP-30 after 21 days of cultivation – a) 280 nm and b) 366 nm.

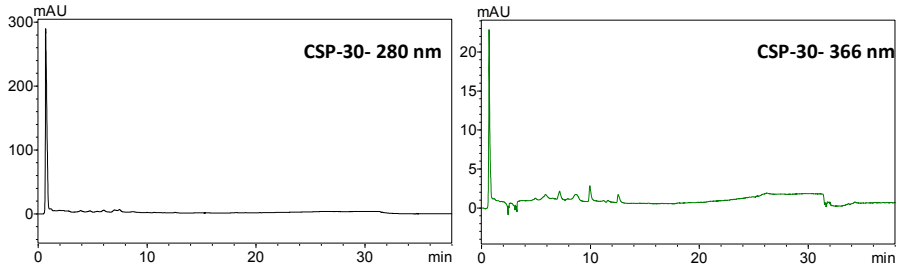


Figure A30: Chromatograms of EtOAc extract of CSP-32a after 21 days of cultivation – a) 280 nm and b) 366 nm.

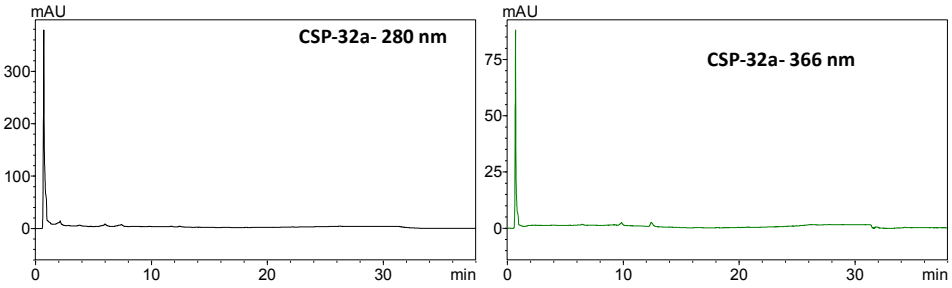


Figure A31: Chromatograms of EtOAc extract of CSP-33 after 21 days of cultivation – a) 280 nm and b) 366 nm.

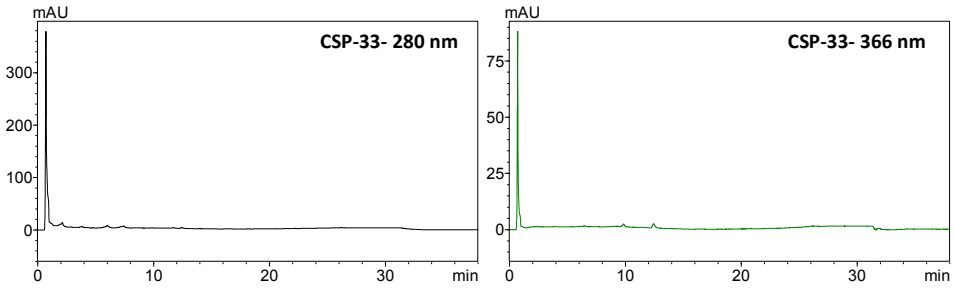


Figure A32: Chromatograms of EtOAc extract of CSP-34 after 21 days of cultivation – a) 280 nm and b) 366 nm.

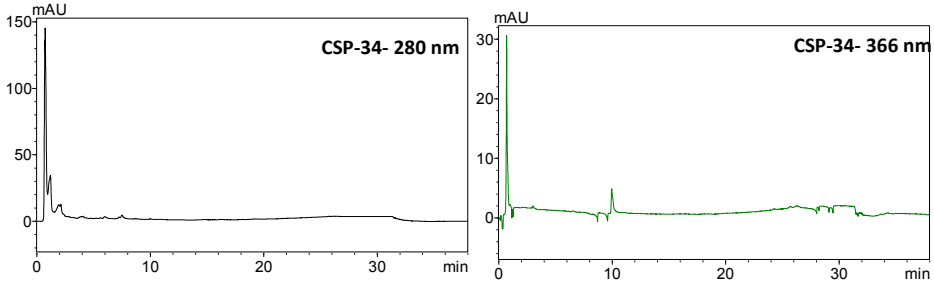


Figure A33: Chromatograms of EtOAc extract of CSP-34a after 21 days of cultivation – a) 280 nm and b) 366 nm.

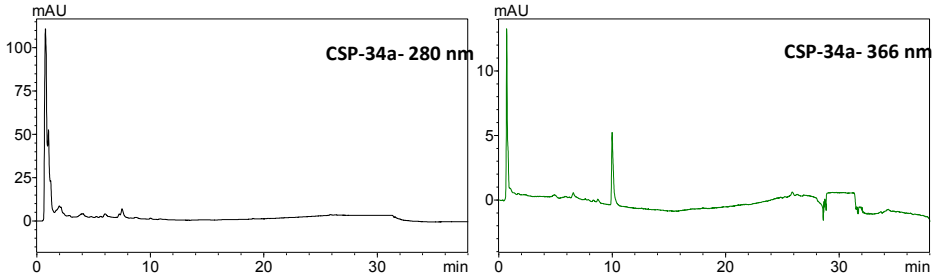


Figure A34: Chromatograms of EtOAc extract of CSP-34b after 21 days of cultivation – a) 280 nm and b) 366 nm.

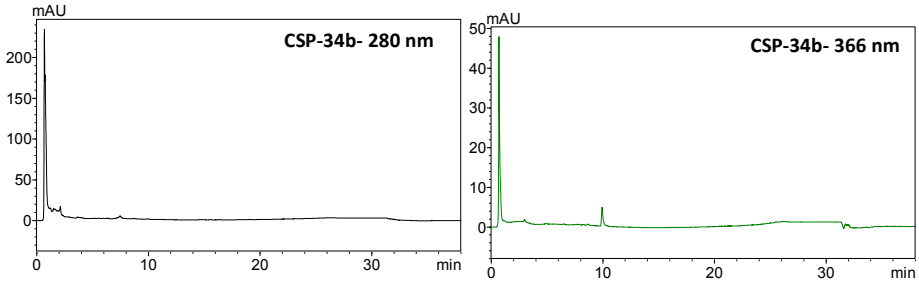


Figure A35: Chromatograms of EtOAc extract of CSP-37a after 21 days of cultivation – a) 280 nm and b) 366 nm.

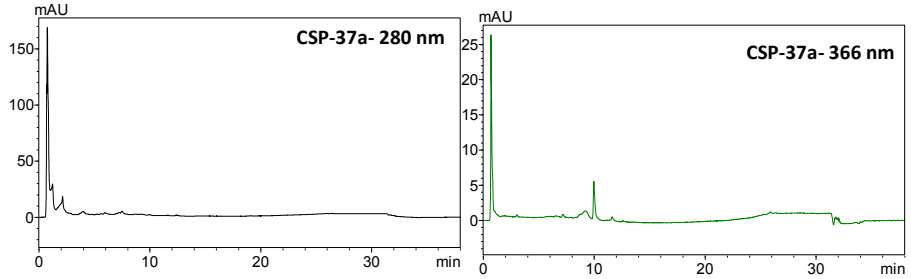


Figure A36: Chromatograms of EtOAc extract of CSP-57/R37 after 21 days of cultivation – a) 280 nm and b) 366 nm.

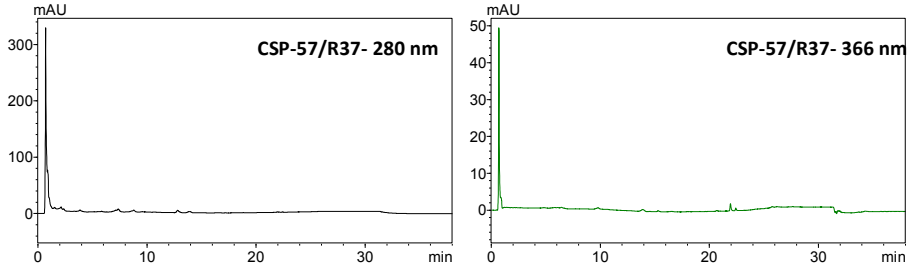


Figure A37: Chromatograms of EtOAc extract of CSP-R19 after 21 days of cultivation – a) 280 nm and b) 366 nm.

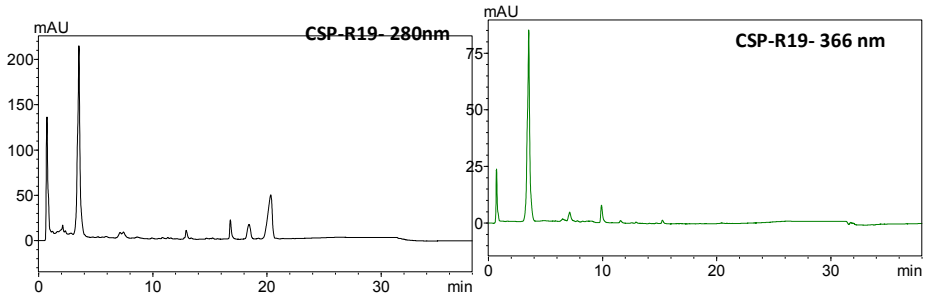


Figure A38: Chromatograms of EtOAc extract of CSP-R34 after 21 days of cultivation – a) 280 nm and b) 366 nm.

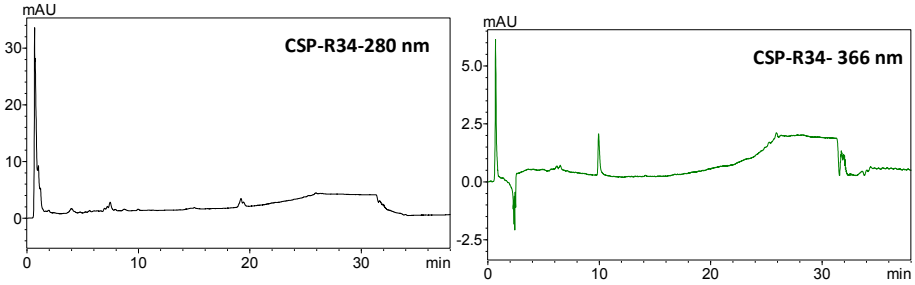


Figure A39: Chromatograms of EtOAc extract of CSP-SNB after 21 days of cultivation – a) 280 nm and b) 366 nm.

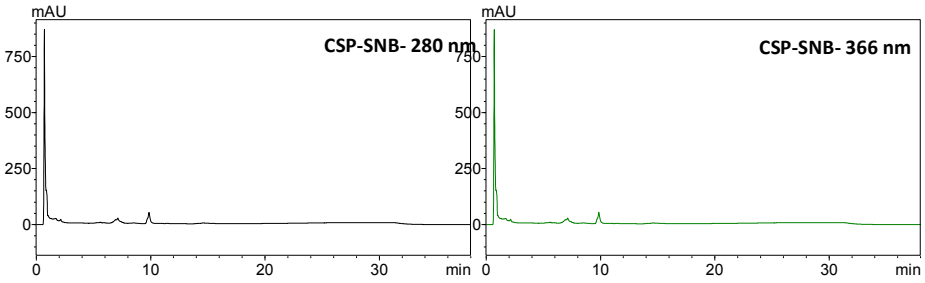


Figure A40: Chromatograms of EtOAc extract of czapek-dox medium after 21 days of cultivation – a) 280 nm and b) 366 nm.

