

Isolation, identification and characterization of endophytes from

Cannabis sativa* L. and *Radula marginata

Zur Erlangung des akademischen Grades eines

Dr. rer. nat.

von der Fakultät Bio- und Chemieingenieurwesen
der Technischen Universität Dortmund
genehmigte Dissertation

vorgelegt von

M.Sc. Parijat Kusari

aus

Kolkata, Indien

Tag der mündlichen Prüfung: 15.12.2014

1. Gutachter: Prof. Dr. Oliver Kayser
2. Gutachter: Prof. Dr. Peter Proksch

Dortmund 2014

*“Learn from yesterday, live for today, hope for tomorrow.
The important thing is not to stop questioning”*

-Albert Einstein

Dedicated to my parents

ACKNOWLEDGEMENTS

I would like to take this opportunity to express my sincere appreciation and gratitude to all the people who accompanied and supported me in accomplishing my thesis.

I express my deepest gratitude to my supervisor **Prof. Dr. Oliver Kayser** (Department of Biochemical and Chemical Engineering, Chair of Technical Biochemistry, TU Dortmund) for his credence and impeccable support towards my scientific endeavors. His scientific discussions, constant guidance and remarkable motivation propelled me to overcome the challenges and move ahead with new vision. There was always a lot to learn from his critical comments on manuscript and intensive ideas. I sincerely thank him for giving me the privilege to work under his exuberant supervision and guidance.

I express my heartfelt thanks to **Prof. Dr. Dr.h.c. Michael Spiteller** (Institute of Environmental Research, INFU, Department of Chemistry and Chemical Biology, Chair of Environmental Chemistry and Analytical Chemistry, TU Dortmund) for his incessant scientific thoughts and guidance on various aspects of my research work. His critical suggestions and generous help has always inspired and encouraged me to enliven my scientific intentions. I express my sincere gratitude to him for all his valuable time, discussions and generosity.

My deepest appreciation and thanks to my brother **Dr. Souvik Kusari** (INFU) for his unconditional support and encouragement in taking my research forward. His critical guidance on different aspects of my research work and valuable comments on manuscript always motivated me to continuously counter the scientific obstacles.

I would like to thank my senior colleague **Dr. Marc Lamshöft** (Bayer CropScience, Monheim, Germany; formerly at INFU) for his valuable suggestions and mass spectrometric interpretations. My special thanks to **Selahaddin Sezgin** (INFU) for his support, assistance and discussion of MALDI work. I sincerely thank all my **colleagues at TB** (Chair of Technical Biochemistry) for their warm welcome, constant support and help.

I am thankful to **CLIB Graduate Cluster Industrial Biotechnology** for the PhD scholarship. I also thank the **Ministry of Innovation, Science and Research** of the **German Federal State North Rhine-Westphalia** (NRW) and **TU Dortmund** for the financial support. I acknowledge the **Federal Institute for Drugs and Medical Devices** (Bundesinstituts für Arzneimittel und Medizinprodukte, BfArM), Bonn, Germany and **Auckland Council, New Zealand Government** for granting the necessary permissions to work with *Cannabis sativa* L. plants and liverwort *Radula marginata*. I also sincerely thank **Bedrocan BV** (the Netherlands) for kindly providing the *Cannabis sativa* L. plants

Special thanks to my **parents** for their constant love and blessings throughout my education. I express my deep respect towards them for perpetual solace and unconditional affection.

TABLE OF CONTENTS

| | Page |
|--|-------------|
| Abstract | VII |
| Zusammenfassung | VIII |
| Chapter 1: Scope of the thesis | 1 |
| 1.1. Aims and objectives | 2 |
| Chapter 2: Introduction | 3 |
| 2.1. Introduction | 4 |
| 2.1.1. Endophytic microorganisms | 5 |
| 2.1.2. Overview of phytocannabinoids in <i>C. sativa</i> L. and liverwort <i>Radula marginata</i> | 6 |
| 2.1.3. Necessity for discovering endophytes harbored in <i>C.</i> <i>sativa</i> L. conferring plant fitness benefits | 14 |
| 2.1.4. Rationale for biocontrol prospects of endophytes | 14 |
| 2.1.5. Rationale for alternate antivirulence strategies of endophytes with respect to quorum responses | 16 |
| 2.2. References | 17 |
| Chapter 3: Endophytic fungi harboured in <i>Cannabis sativa</i> L.: diversity and potential as biocontrol agents against host-plant specific phytopathogens | 24 |
| 3.1. Abstract | 25 |
| 3.2. Introduction | 26 |
| 3.3. Materials and Methods | 28 |
| 3.3.1. Collection, identification, and authentication of plant material | 28 |
| 3.3.2. Isolation of endophytic fungi and establishment of <i>in vitro</i> axenic cultures | 28 |
| 3.3.3. Maintenance and storage of the axenic endophytic fungal isolates | 29 |
| 3.3.4. Total genomic DNA extraction, PCR amplification and sequencing | 29 |

| | |
|---|-----------|
| 3.3.5. Identification of endophytic fungi and phylogenetic evaluation | 30 |
| 3.3.6. Evaluation and quantification of fungal diversity | 30 |
| 3.3.7. Pathogens used for antagonistic assays | 33 |
| 3.3.8. <i>In vitro</i> antagonistic activity of endophytes against host phytopathogens | 33 |
| 3.4. Results | 34 |
| 3.4.1. Identification and characterization of the endophytic fungi | 34 |
| 3.4.2. Phylogeny and fungal diversity analysis | 35 |
| 3.4.3. <i>In vitro</i> antagonism assay of endophytes as potential biocontrol agents | 38 |
| 3.5. Discussion | 40 |
| 3.6. References | 46 |
| Chapter 4: Quorum quenching is an antivirulence strategy employed by endophytic bacteria | 54 |
| 4.1. Abstract | 55 |
| 4.2. Introduction | 56 |
| 4.3. Materials and Methods | 58 |
| 4.3.1. Collection and authentication of plant material | 58 |
| 4.3.2. Isolation and establishment of <i>in vitro</i> axenic cultures of endophytic bacteria | 58 |
| 4.3.3. Genomic DNA extraction, amplification of 16S rRNA gene and sequencing | 59 |
| 4.3.4. Preparation of cell free supernatant (CFS) | 60 |
| 4.3.6. Sample preparation for analytical measurements | 61 |
| 4.3.7. Preparation of standards | 62 |
| 4.3.8. High-resolution mass spectrometry | 62 |
| 4.3.9. Sample preparation for AP-MALDI imaging | 62 |
| 4.3.10. AP-MALDI imaging high-resolution mass spectrometry | 63 |
| 4.3.11. Sample preparation for UV-MALDI imaging | 63 |
| 4.3.12. UV-MALDI imaging high-resolution mass spectrometry | 64 |

| | | |
|-------------------|---|-----------|
| 4.4. | Results | 64 |
| 4.4.1. | Identification and characterization of the endophytic bacterial isolates | 64 |
| 4.4.2. | Spectrophotometric quantitation of quorum quenching behaviour | 65 |
| 4.4.3. | Quantification of quorum quenching behavior using high performance liquid chromatography high-resolution mass spectrometry (HPLC-ESI-HRMSⁿ) | 65 |
| 4.4.4. | Visualization of spatial distribution of quorum sensing signals and their quenching by endophytes using MALDI-imaging-HRMS | 70 |
| 4.5. | Discussion | 70 |
| 4.6. | References | 74 |
| Chapter 5: | Biocontrol potential of endophytes harbored in <i>Radula marginata</i> (liverwort) from the New Zealand ecosystem | 78 |
| 5.1. | Abstract | 79 |
| 5.2. | Introduction | 80 |
| 5.3. | Materials and Methods | 82 |
| 5.3.1. | Collection of the plant material | 82 |
| 5.3.2. | Isolation of endophytic bacteria and fungi and establishment of axenic cultures | 82 |
| 5.3.3. | Genomic DNA isolation, PCR amplification and sequencing | 83 |
| 5.3.4. | Pathogenic strains used for antagonistic assays and biofilm formations | 84 |
| 5.3.5. | Dual-plate antagonism assay of endophytic isolates against phytopathogens | 84 |
| 5.3.6. | Biofilm and anti-biofilm assay of bacterial endophytes | 85 |
| 5.4. | Results | 86 |
| 5.4.1. | Identification and characterization of endophytic isolates | 88 |
| 5.4.2. | Biofilm formation and anti-biofilm activity of endophytic bacterial isolates | 89 |
| 5.4.3. | Antagonistic assay against phytopathogens | 89 |

| | | |
|-------------------|---|------------|
| 5.5. | Discussion | 101 |
| 5.6. | References | 104 |
| Chapter 6: | Discussion and Outlook | 108 |
| 6.1. | Endophytic biodiversity of <i>Cannabis sativa</i> L. and their antagonistic prospects against phytopathogens | 109 |
| 6.2. | Comparison and evaluation of endophytic efficacies and functional traits of liverwort <i>Radula marginata</i> to that of <i>Cannabis sativa</i> | 110 |
| 6.3. | Attenuation of quorum sensing signaling by endophytes as quantified and visualized by HPLC-ESI-HRMS ⁿ and MALDI-imaging-HRMS | 112 |
| 6.4. | Outlook | 114 |
| Appendix | Appendix I-V | 116 |
| I. | List of Abbreviations | 117 |
| II. | List of Tables | 119 |
| III. | List of Figures | 121 |
| IV. | List of Original Contributions | 125 |
| V. | Curriculum Vitae | 127 |

ABSTRACT

Endophytes are a group of microorganisms that infect the internal tissues of plant without causing any immediate visible symptom of infection and/or manifestation of disease, and live in mutualistic association with plants for at least a part of their life cycle. In the last decade, discovery and characterization of potent endophytes producing bioactive natural products have led to the possibility of exploring the potential benefits of these microorganisms in agricultural and pharmaceutical sectors.

The objective of this study was to isolate, identify and assess the biocontrol efficacies of fungal and bacterial endophytes harbored in *Cannabis sativa* L. plants and the liverwort *Radula marginata*. Despite significant production of cannabinoids, the major secondary metabolites of *C. sativa* L. plants, numerous phytopathogens are able to attack different parts of the plant leading to disease. Thus far, the host-specific phytopathogens were challenged with the endophytes by devising dual culture antagonistic assays resulting in varying degrees of pathogen inhibition concomitant to a plethora of endophyte-pathogen antagonistic interactions. The overall biodiversity of endophytes distributed among the tissues were further evaluated using detailed statistical calculations to correlate with their functional traits. Additionally, using the rationale that structurally similar cannabinoids are produced by phylogenetically unrelated *C. sativa* and *R. marginata*, similar and discrete functional traits of endophytic community were explored.

This study also provides fundamental insights into the antivirulence strategies used by bacterial endophytes of *C. sativa* L. A combination of HPLC-ESI-HRMSⁿ and MALDI-imaging-HRMS was used to quantify and visualize the spatial distribution and quenching of four different AHLs (*N*-acyl-L-homoserine lactones) used by *Chromobacterium violaceum* for violacein-mediated quorum sensing. MALDI-imaging-HRMS was further used for visualizing the spatial localization of each AHL by *C. violaceum* and the concomitant selective impediment of the AHLs by bacterial endophytes.

The results reported in this thesis underline the defensive functional traits of selected endophytes and opens new avenues towards further exploitation of endophytes harbored in *C. sativa* L. plants and *R. marginata*.

ZUSAMMENFASSUNG

Endophyten sind Mikroorganismen, welche sich im inneren Gewebe von Pflanzen infizieren, ohne dadurch sichtbare Symptome oder Krankheiten auszulösen. Sie leben in mutualistischer Gemeinschaft mit Pflanzen zumindest für einen Teil ihres Lebenszyklus. Im letzten Jahrzehnt hat die Entdeckung und Charakterisierung von Endophyten, die bioaktive Naturprodukte synthetisieren, dazu geführt, dass möglichen Potential dieser Mikroorganismen für Landwirtschaft und Pharmazie zu untersuchen.

Ziel dieser Arbeit war die Isolierung und Identifizierung bakterieller und pilzlicher Endophyten aus *Cannabis sativa* L. und dem Lebermoos *Radula marginata*, sowie Untersuchungen zu deren biologischer Wirksamkeit. Trotz der Produktion signifikanter Mengen von Cannabinoiden, den wichtigsten Sekundärmetaboliten von *C. sativa*, sind zahlreiche phytopathogene Mikroorganismen in der Lage, verschiedene Teile der Pflanze anzugreifen und Krankheiten auszulösen. Die Interaktion der wirtsspezifischen phytopathogenen Mikroorganismen mit den Endophyten wurde in Zwei-Kultur-Antagonismus-Assays untersucht; verschiedene Abstufungen der Inhibierung wurden beobachtet, welche mit einer Fülle von antagonistischen Wechselwirkungen von Endophyten und Pathogenen verbunden sind. Die Biodiversität der Endophyten in den Pflanzengeweben wurde mit statistischen Methoden genauer untersucht, um funktionelle Eigenschaften der Endophyten zu korrelieren. Ausgehend von der Tatsache, dass Cannabinoide mit ähnlicher chemischer Struktur in den phylogenetisch nicht verwandten Arten *C. sativa* und *R. marginata* synthetisiert werden, wurden Ähnlichkeiten und Unterschiede der Endophyten-Gemeinschaft in beiden Pflanzen untersucht.

Die Arbeit liefert zudem fundamentale Erkenntnisse über die Antivirulenz-Strategien der bakteriellen Endophyten von *C. sativa*. Eine Kombination aus HPLC-ESI-HRMSⁿ und MALDI-imaging-HRMS wurde zur Quantifizierung und Visualisierung der räumlichen Verteilung von vier verschiedenen AHLs (*N*-acyl-L-homoserin lactone), genutzt, welche *Chromobacterium violaceum* für Violacein-vermitteltes „Quorum sensing“ verwendet. MALDI-imaging-HRMS wurde auch eingesetzt, um die räumliche Verteilung jeder der vier AHLs durch *C. violaceum* und die damit einhergehende selektive Unterdrückung der AHLs durch bakterielle Endophyten zu untersuchen.

Die Ergebnisse dieser Arbeit zeigen die Möglichkeiten von ausgewählten Endophyten bei Verteidigungsstrategie und öffnen neue Wege zur weiteren Nutzung von Endophyten aus *C. sativa* und *R. marginata*.

Chapter 1

SCOPE OF THE THESIS

1.1. AIMS AND OBJECTIVES:

The aim of this work was to isolate, identify, evaluate and compare the biocontrol prospects of fungal and bacterial endophytes harbored in *Cannabis sativa* L. plants and liverwort *Radula marginata*. Furthermore, this study provides fundamental insights into the antivirulence strategies of bacterial endophytic community of *C. sativa* L. The goals of this cumulative thesis are addressed as individual chapters describing the following points:

A. **Chapter 2** provides an introduction to the thesis and an overview of the relevant literature. It also highlights the various rationales for bioprospecting endophytes of *C. sativa* L. and *R. marginata*

B. **Chapter 3** evaluates the incidence, diversity and phylogeny of endophytic fungi isolated from various tissues of *C. sativa* L. plants, and further assess the biocontrol efficacies against the two major phytopathogens of the plant namely, *Botrytis cinerea* and *Trichothecium roseum*. Based on the knowledge of OSMAC (One Strain Many Compounds) approach, the antagonistic effects are evaluated against the two phytopathogens under five different media conditions.

C. **Chapter 4** provides fundamental insights into the potential of endophytic bacteria as biocontrol- as well as antivirulence agents in disrupting the cell-to-cell quorum sensing signals in the biosensor strain, *Chromobacterium violaceum*. In this study, we have used a combination of high performance liquid chromatography high-resolution mass spectrometry (HPLC-ESI-HRMSⁿ) and matrix assisted laser desorption ionization imaging high-resolution mass spectrometry (MALDI-imaging-HRMS) to quantify and visualize the spatial distribution of cell-to-cell quorum sensing signals in the biosensor strain, *C. violaceum*. We further showed that the potent endophytic bacteria can selectively and differentially quench the quorum sensing molecules of *C. violaceum*.

D. **Chapter 5** demonstrates the isolation, identification, biocontrol prospects, biofilm and anti-biofilm magnitudes of fungal and bacterial endophytes harbored in liverwort *R. marginata*. Furthermore, this study compares and evaluates the ecological significance and antagonistic potential of bacterial endophytic community of *R. marginata* against the two phytopathogens, as compared to that of *C. sativa* L. Therefore, it underlines the similar and discrete traits of endophytic community of plants from different geographical niches with similar secondary metabolite (cannabinoid) production.

Chapter 2

INTRODUCTION

Parijat Kusari, Michael Spiteller, Oliver Kayser, Souvik Kusari

PK, SK, MS and OK jointly conceived, planned and designed the chapter; PK performed the literature review and summarized the structure of the manuscript; PK wrote the manuscript with inputs from all coauthors; SK supervised PK and maintained scientific surveillance; MS and OK oversaw the entire project as senior authors and supervisors of PK

Parts of introduction have been published in:

Recent advances in research on *Cannabis sativa* L. endophytes and their prospect for the pharmaceutical industry. In Kharwar, R. N. et al. (eds.) Microbial Diversity and Biotechnology in Food Security, *Springer* India, Part I, pp. 3-15

2.1. INTRODUCTION

Cannabis is an annual herbaceous plant genus in the Cannabaceae family, mainly from Central Asia. *Cannabis* and *Humulus* are the only two recognized genera in the Cannabaceae family (Fernald 1950; Flores-Sanchez and Verpoorte 2008). In *Cannabis*, mainly one species is famously recognized, namely *C. sativa* (Linnaeus 1753), however, three other species (*C. indica*, *C. ruderalis* and *C. afghanica*) have also been described recently (McPartland et al. 2000); only species *H. lupulus* is recognized in the genus *Humulus*. However, *Cannabis sativa* L. (Fig. 1) is the most rigorously studied plant that has been in use all over the planet since ages either in the form of narcotic or medicinal preparations or as a source of food and fiber (Wills et al. 1998; Jiang et al. 2006; Murray et al. 2007). It is also the most controversial plant in the human history with a strongly divided medical-, research- and political community with respect to its use. The secondary metabolites of this plant constitute more than 400 compounds (Turner et al. 1980), with the most emphasis being led on cannabinoids. Recent investigations on liverworts led to the identification of bibenzyl cannabinoids (namely perrottetinene and perrottetinenic acid), with structural similarity to tetrahydrocannabinol, the major psychoactive secondary metabolite of *Cannabis sativa* L. plants (Toyota et al. 2002). *Radula marginata* (Radulaceae) is a species of liverwort commonly found in the New Zealand. Species belonging to *Radula* (for example *R. perrottetti*, *R. complanata*, *R. kojana*, and others) have been reported to contain aromatic compounds and prenyl bibenzyls (Asakawa et al. 1991a; Toyota et al. 1994).

C. sativa L. is commonly called 'hemp', and it is said that "hemp has no enemies" (Dewey 1914). However, this misleading notion is far from the truth since this plant is beleaguered by a plethora of specific and generalist microbial pathogens (Kusari et al. 2012a). A couple of infrequent attempts have been made so far for the eradication of the fungal pathogens attacking this plant (Ungerlerder et al. 1982; Kurup et al. 1983; Levitz and Diamond 1991; Bush Doctor 1993). However, a holistic, cost-effective and environmentally friendly means to eradicate the pathogen-mediated diseases in *Cannabis* is essential. Therefore, it might be possible to efficiently utilize the unique *C. sativa*-associated microorganisms (called 'endophytes') to thwart the loss of these therapeutically significant plants and considerably reduce the expanse of vulnerabilities caused by phytopathogens. Additionally, with the rationale of production of structurally similar cannabinoids by *Cannabis* and *Radula*, it might also be promising to evaluate the efficacies of endophytic community of *Radula marginata*, and further compare the similar and discrete functional traits of endophytic community of phylogenetically unrelated plants with similar biosynthetic principles. Further, gaining deeper insights into fundamental functional traits of endophytes will enable a more holistic approach

towards understanding the biological role played by the endophytes in different ecological niches, not only in host plant defense but also in maintaining colonization and their own survival inside plants.

2.1.1. Endophytic microorganisms

In last decade, discovery and intensive investigation of plant-associated microorganisms, termed endophytic microorganisms (or endophytes) have led to the possibility of exploring the potential benefits of these promising organisms in agriculture, medicinal and pharmaceutical sectors. Endophytes can be defined, in a generalist manner, as a group of microorganisms that infect the internal tissues of plant without causing any immediate symptom of infection and/or visible manifestation of disease, and live in mutualistic association with plants for at least a part of their life cycle (Bacon and White 2000; Kusari and Spiteller 2012; Kusari et al. 2013). de Bary (1866) first coined the term 'endophyte' (*endon* meaning within; *phyton* meaning plant). Endophytes are ubiquitously existent in almost every plant tissue examined till date (Guerin et al. 1898; Redecker et al. 2000; Strobel 2002; Staniek et al. 2008). With the increasing enormity of global health problems, and the incidence of drug-resistant microorganisms and new diseases, it has become clear that faster and effective pursuits for drug discovery and sustainable production must be made. This cumulative crisis has already led to the discovery and characterization of potent endophytes which can produce bioactive natural products, occasionally mimetic to their associated host plants (Eyberger et al. 2006; Kour et al. 2008; Kusari et al. 2008, 2009a, b, c, 2012b; Shweta et al. 2010). Endophytes are also known to produce a diverse range of biologically active secondary metabolites (Strobel and Daisy 2003; Strobel et al. 2004; Zhang et al. 2006; Gunatilaka 2006; Staniek et al. 2008; Suryanarayananana et al. 2009; Aly et al. 2010; Kharwar et al. 2011) that are known to produce host plant tolerance against various environmental stress herbivory, heat, salt, disease and drought (Stone et al. 2000; Redman et al. 2002; Arnold et al. 2003; Rodriguez et al. 2004, 2008; Waller et al. 2005; Márquez et al. 2007; Rodriguez and Redman 2008; Porrás-Alfaro and Bayman, 2011). Even with such colossal amounts and breadth of successful discoveries of potentially beneficial endophytes, it has still not been possible to utilize them commercially for the 'sustained production' of the desired pharmaceutically valuable compounds (Kusari et al. 2014). Therefore, understanding of the multitude of endophyte relationships with host plants needs more attention and investigation in various related aspects such as the endophyte-plant interactions, multispecies crosstalk, and links with herbivores and predators.



Fig. 1 *Cannabis sativa* L. plants sampled from the Bedrocan BV Medicinal Cannabis (the Netherlands). (Photograph courtesy of S. Kusari)

2.1.2. Overview of phytocannabinoids in *C. sativa* L. and liverwort *Radula marginata*

The major secondary metabolites of *C. sativa* L. constitute cannabinoids, terpenoids, flavonoids, alkaloids and lignans (Flores-Sanchez and Verpoorte 2008). Among them, cannabinoids are the ones most extensively studied. Cannabinoids are terpenophenolics found in the Indian hemp (*C. sativa* L.) constituting a class of chemical compounds that include phytocannabinoids (i.e., oxygen-containing C₂₁ aromatic hydrocarbon compounds found in *Cannabis* plant) and related chemical compounds which mimic the actions of phytocannabinoids or have a similar structure (e.g. endocannabinoids). Cannabinoids are known to occur naturally in significant measure in the plant. In general, all plant parts are known to contain cannabinoids (Flemming et al. 2007). However, these phytochemicals are more concentrated in a viscous resin that is produced in glandular trichomes. Table 1 summarizes the major cannabinoids and related precursors that have been isolated from *Cannabis sativa*.

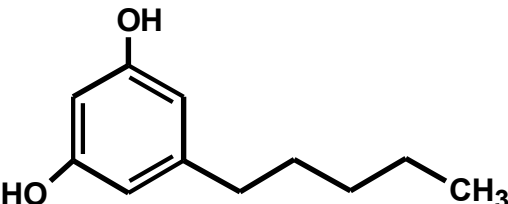
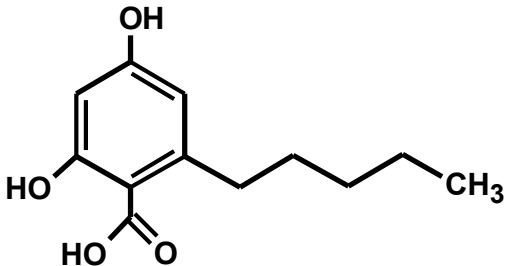
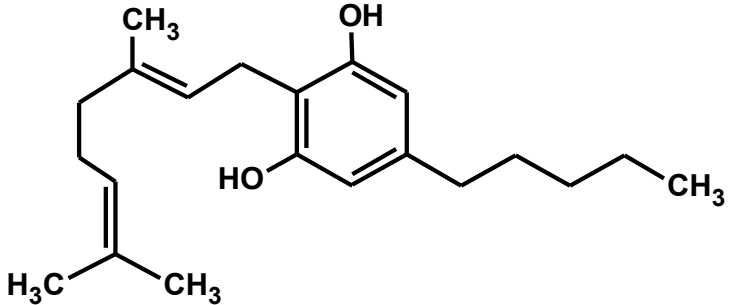
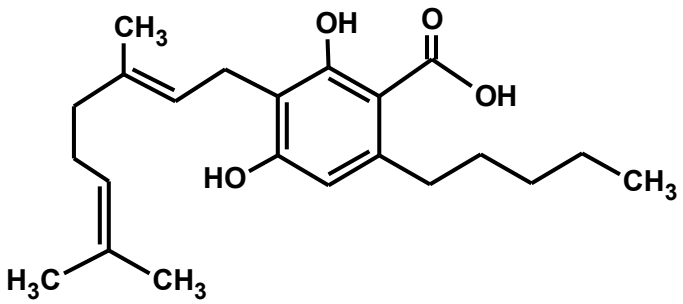
Although the plant is mainly regarded as drug of abuse due to high content of delta 9-tetrahydrocannabinol (Δ 9-THC), the main psychoactive compound, cannabinoids are known to have important therapeutic effects (Williamson and Evans 2000; Baker et al. 2003; Grotenhermen et al. 2002, 2012; Musty et al. 2004; Flores-Sanchez and Verpoorte 2008) such as analgesic, anti-spasmodic, anti-tremor, anti-inflammatory (Gomes et al. 2008), anti-

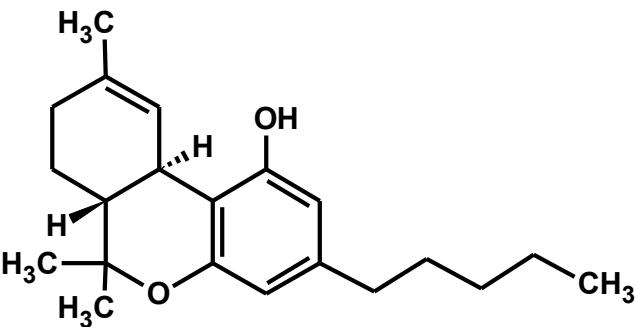
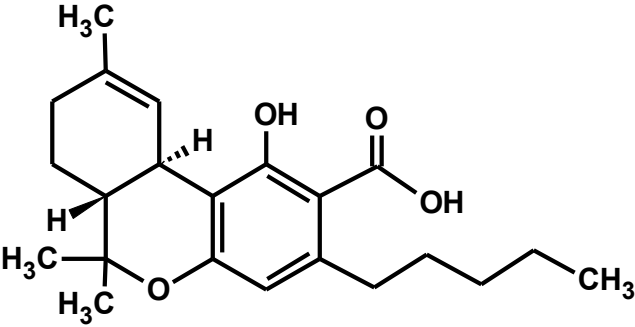
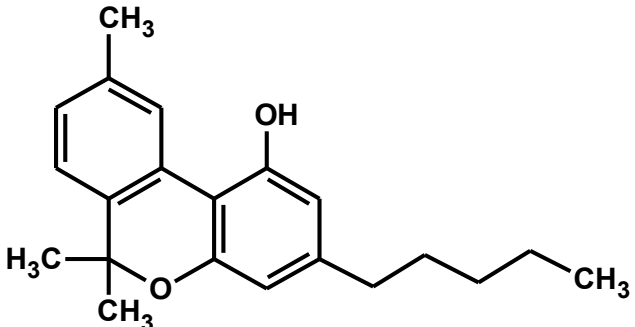
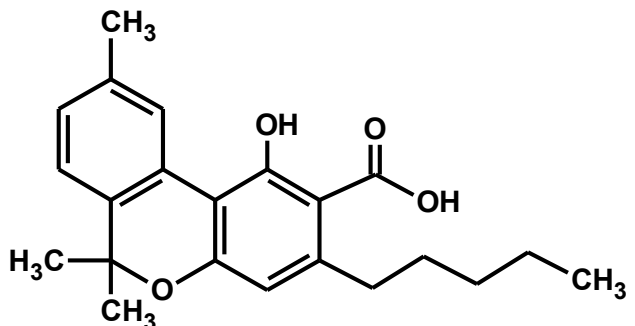
oxidant, antineoplastic (Carchman et al. 1976; Mojzisoava and Mojziso 2008), neuro-protective (Ameri et al. 1999), immunosuppressive, anti-nociceptive, antiepileptic, antidepressants and appetite stimulant. From 450 secondary natural product constituents in total (including 20 flavonoids, 15 polyketides), more than 108 cannabinoids have been discovered so far (Hazekamp et al. 2004, 2005; ElSohly and Slade 2005; Taura et al. 2007; Radwan et al. 2008; Ahmed et al. 2008; Fishedick et al. 2010; and refer to Natural Product Database, Nov. 2012). Due to such therapeutic potential of cannabinoids and the plant extracts themselves, several *Cannabis*-based medicines have already made their way to the pharmaceutical industries. Some prominent examples include Marinol® (Solvay Pharmaceuticals, Belgium), Sativex (GW Pharmaceuticals, UK), and Nabilone (Cesamet®, Veleant Pharmaceuticals International, USA). Although Δ^9 -THC is considered to be one of the major psychoactive compounds (Taura et al. 1995; Sirikantaramas et al. 2005; Pertwee 2006), other cannabinoids like cannabigerol, cannabidiol, cannabinol, olivetol, and cannabichromene prove to be therapeutically beneficial either alone or synergistically. These cannabinoids are also known to be effective against various pathogenic bacteria and fungi of clinical importance thereby signifying the antifungal and antibacterial potency of the compounds (ElSohly et al. 1982; Appendino et al. 2008; Pollastro et al. 2011). However, more studies are still required to confirm the potential benefits of whole plant extracts compared to that of pure cannabinoids (Williamson and Evans 2000; Wachtel et al. 2002; Russo and McPartland 2003; ElSohly et al. 2003). Δ^9 -THC and other cannabinoids are also subjected to directed biosynthesis, or in other words, induced in the medium by biotransformation of structurally related compounds using various fungal isolates or plant cell suspension cultures (Hartsel et al. 1983; McClanahan et al. 1985; Miyazawa et al. 1997; Tanaka et al. 1997; Toniazzo et al. 2005; Kawamoto et al. 2008; Saxena 2009; Flores-Sanchez et al. 2009; Happyana et al. 2013).

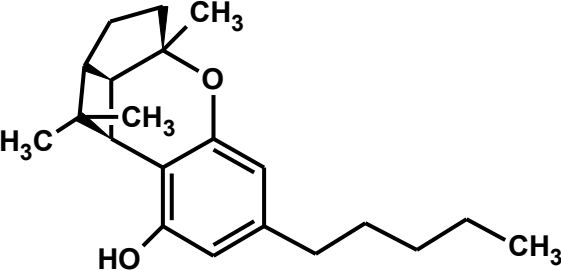
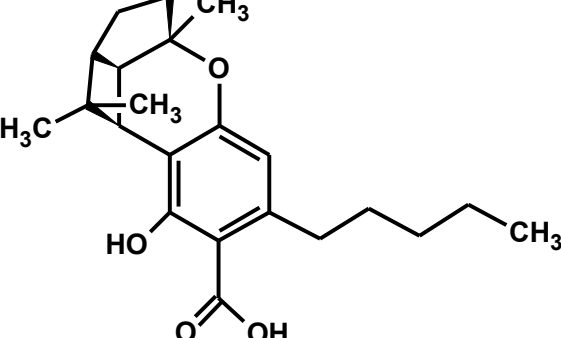
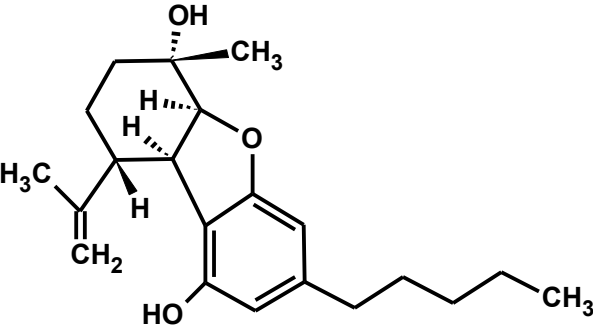
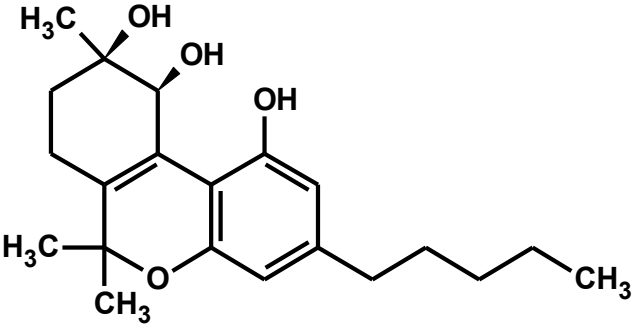
Liverworts are small, simple and non-vascular plants existing in almost all ecosystems, though they are abundant in the tropical niches. However, these small plants are highly rich in terpenoids and aromatic compounds. Some are also known to produce specific compounds with novel carbon skeleton that serve as significant markers of different genus of liverworts (Ludwiczuk and Asakawa 2008). *Radula marginata* (Radulaceae) is a species of liverwort commonly found in the New Zealand. Species belonging to *Radula* (for example *R. perrottetti*, *R. complanata*, *R. kojana*, and others) have been reported to contain aromatic compounds and prenyl bibenzyls (Asakawa et al. 1991a; Toyota et al. 1994). These compounds are known to have antimicrobial, antioxidant, antifungal, cytotoxic and other important biological activities (Ludwiczuk and Asakawa 2008). Recent research (Toyota et al

2002) led to the identification of new cannabinoids (namely perrottetinene and perrottetinenic acid) with structural similarity to tetrahydrocannabinol, the major psychoactive compound of *Cannabis sativa* L. plants. Table 2 summarizes the new cannabinoids identified in liverwort *Radula marginata*.

Table 1 Important natural cannabinoids and metabolic precursors found in the *Cannabis* plants

| Name of compound | Structure |
|--|--|
| Olivetol $C_{11}H_{16}O_2$ 180.2435 |  |
| Olivetolic acid $C_{12}H_{16}O_4$ 224.2530 |  |
| Cannabigerol $C_{21}H_{32}O_2$ 316.4775 |  |
| Cannabigerolic acid $C_{22}H_{32}O_4$ 360.4870 |  |

| | |
|---|--|
| <p>Δ^9-tetrahydrocannabinol</p> <p>$C_{21}H_{30}O_2$ 314.4617</p> |  |
| <p>Δ^9-tetrahydrocannabinolic acid</p> <p>$C_{22}H_{30}O_4$ 358.4712</p> |  |
| <p>Cannabinol</p> <p>$C_{21}H_{26}O_2$ 310.4299</p> |  |
| <p>Cannabinolic acid</p> <p>$C_{22}H_{26}O_4$ 354.4394</p> |  |

| | |
|---|---|
| <p>Cannabicyclol</p> <p>$C_{21}H_{30}O_2$ 314.4617</p> |  <p>The structure of Cannabicyclol features a bicyclic core consisting of a norbornane ring fused to a benzene ring. The norbornane ring has two methyl groups (CH₃) and a hydroxyl group (HO) attached. The benzene ring has a hydroxyl group (HO) at the 1-position, a pentyl group (CH₂)₅ at the 3-position, and a methyl group (CH₃) at the 4-position. The oxygen atom of the bicyclic system is also attached to the benzene ring.</p> |
| <p>Cannabicyclolic acid</p> <p>$C_{22}H_{30}O_4$ 358.482</p> |  <p>The structure of Cannabicyclolic acid is similar to Cannabicyclol, but with a carboxylic acid group (COOH) instead of a hydroxyl group at the 1-position of the benzene ring.</p> |
| <p>Cannabielsoin</p> <p>$C_{21}H_{30}O_3$ 330.4611</p> |  <p>The structure of Cannabielsoin features a bicyclic core consisting of a norbornane ring fused to a benzene ring. The norbornane ring has a hydroxyl group (OH) and a methyl group (CH₃) attached. The benzene ring has a hydroxyl group (HO) at the 1-position, a pentyl group (CH₂)₅ at the 3-position, and a methyl group (CH₃) at the 4-position. The oxygen atom of the bicyclic system is also attached to the benzene ring.</p> |
| <p>Cannabitriol</p> <p>$C_{21}H_{30}O_4$ 346.4605</p> |  <p>The structure of Cannabitriol features a bicyclic core consisting of a norbornane ring fused to a benzene ring. The norbornane ring has two methyl groups (CH₃) and a hydroxyl group (OH) attached. The benzene ring has a hydroxyl group (OH) at the 1-position, a pentyl group (CH₂)₅ at the 3-position, and a methyl group (CH₃) at the 4-position. The oxygen atom of the bicyclic system is also attached to the benzene ring.</p> |

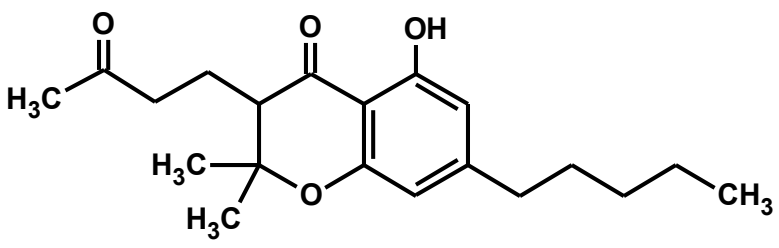
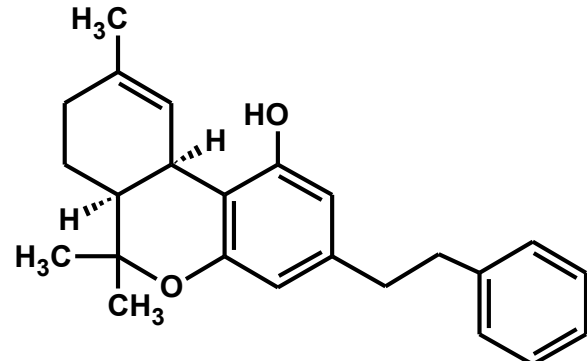
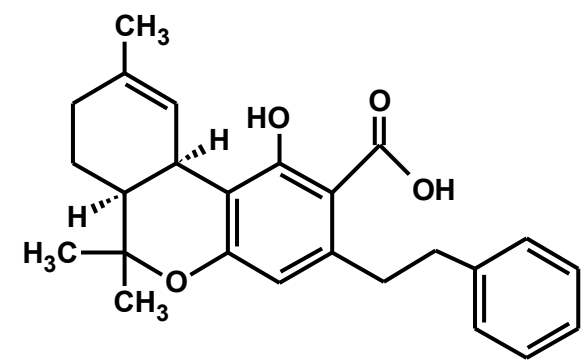
| | |
|---|---|
| <p>Cannabichromanone</p> <p>$C_{20}H_{28}O_4$ 332.4339</p> |  <p>The structure shows a central benzene ring with a hydroxyl group (OH) at the 1-position and a propyl chain at the 4-position. This benzene ring is fused to a six-membered ring containing an oxygen atom and a carbonyl group (C=O). The six-membered ring is further substituted with two methyl groups (H₃C) and a pentyl chain with a terminal methyl group (CH₃) and a carbonyl group (C=O).</p> |
|---|---|

Table 2 New cannabinoids found in liverwort *Radula marginata*

| Name of compound | Structure |
|--|---|
| <p>Perrottetinene</p> <p>$C_{24}H_{28}O_2$ 348.4779</p> |  <p>The structure features a central benzene ring with a hydroxyl group (HO) at the 1-position and a propyl chain at the 4-position. This benzene ring is fused to a six-membered ring containing an oxygen atom and two methyl groups (H₃C). The six-membered ring is also substituted with a hydrogen atom (H) and a cyclohexane ring with a methyl group (H₃C).</p> |
| <p>Perrottetinenic acid</p> <p>$C_{25}H_{28}O_4$ 392.4874</p> |  <p>The structure is similar to Perrottetinene, but the propyl chain at the 4-position of the benzene ring is replaced by a propyl chain with a terminal carboxylic acid group (COOH).</p> |

2.1.3. Necessity for discovering endophytes harbored in *C. sativa* L. conferring plant fitness benefits

Plants have been bioprospected for therapeutic potential since ages. Plants are known to contain various bioactive molecules with relevant biological functions such as chemical defense of the plant (Chen et al. 1924; Li et al. 2001; Lopez-Lazaro et al. 2003; Wink 2008; Holler et al. 2012). However, due to the continuous co-evolution of the attack-defense, counter-defense, and other forms of crosstalk between plants and interacting organisms (including microorganisms, herbivores, feeders, pests, etc.), plants alone are unable to defend themselves against parasites, pathogens and predators (Kusari et al. 2012d). For example, despite the significant quantity of cannabinoids in the *C. sativa* L. plant, there are still reports of numerous phytopathogens attacking the different organs of the plant starting from seedling to even a mature plant (McPartland 1996). A plethora of bacteria and fungi are known to be responsible for the devastating infections caused to the plant (Hockey 1927; McPartland 1991, 1993, 1994, 1995). As a case in point, the two major phytopathogens, namely *Botrytis cinerea* and *Trichothecium roseum*, are potent greenhouse threats for the *Cannabis* cultivars and are known to cause localized to (potentially) epidemic disasters (Barloy and Pelhate 1962; Bush Doctor 1985). Although elimination attempts against many pathogens have been made so far (Ungerlerder et al. 1982; Kurup et al. 1983; Levitz and Diamond 1991; Bush Doctor 1993), for total eradication of causative agents and/or prevention of their pathogenicity to *Cannabis* plants, future investigation is required.

2.1.4. Rationale for biocontrol prospects of endophytes

Plant-fungal associations are always accompanied by various physical and chemical interactions thereby establishing them either in localized and/or systemic manner (Kusari et al. 2014). It is immensely important to understand the reaction and stability of endophytes in any microbe-microbe interactions due to biotic selection pressures, outside the host environment. Thus, monitoring the magnitude of biocontrol efficacies under different media conditions not only provide information correlating to the well-known OSMAC (One Strain MAny Compounds) approach but also evaluates the probable contributions and capabilities of endophytes in aiding host fitness against the pathogens. The varying assortment of various bi-, tri- and multipartite interactions demonstrated by the endophytes against the host phytopathogens indicates that their efficacies are either due to production of secondary metabolites or the immediate intermediates in the biosynthetic pathway of those metabolites, triggered upon pathogen-challenge. This reveals that endophytes are capable of producing cryptic metabolites when elicited under certain selective interacting conditions apart from the normal metabolites produced under normal fermentation conditions (Kusari et al. 2012).

Nonetheless, there is still no known breakthrough in the biotechnological production of these bioactive natural products using endophytes. However, the pathogens encountered can serve as an inducer that might trigger the production of defense secondary metabolites enabling incessant discovery and sustained supply of bioactive pro-drugs against the current and emerging diseases of host plants.

Further, gaining a deeper insight of the various bi-, tri- and multipartite interactions of endophytes with associated host plants and neighboring microbial community like epiphytes, associated endophytes, endosymbiont and pathogens under various biotic selection pressures will enable a holistic approach towards production and co-evolution of bioactive natural products. Thus underlining the similar and discrete traits of endophytic community of plants from different ecological niches like *Cannabis sativa* and *Radula marginata*, with similar secondary metabolite (cannabinoids) production can provide a hypothesis that host plants containing similar phytochemicals might harbor same and/or similar or different endophytic microflora. Additionally, exhibiting an endophytic lifestyle in two different phylogenetically unrelated host plants with similar biosynthetic principles is noteworthy and can further explore their efficacies and magnitude in retaining certain defensive functional traits. However, whether the presence of similar species and their functional characteristic are attributed to similar biosynthetic principles of different host plants needs more plant survey from different geographical locations. Our work evaluates and compares the biocontrol efficacies of endophytic microbial community of *C. sativa* and *R. marginata* with respect to the varying assortment of antagonism against the phytopathogens. Fig. 2 shows representative plates demonstrating the emergence of endophytic fungal mycelia from surface-sterilized *Cannabis sativa* L. plant tissues on water agar media amended with antibiotic (streptomycin, 100 mg/L).



Fig. 2 Representative plates showing emergence of endophytic fungal mycelia from surface-sterilized *Cannabis sativa* L. plant tissues on water agar media amended with antibiotic (streptomycin, 100 mg/L)

2.1.5. Rationale for alternate antivirulence strategies of endophytes with respect to quorum responses

Quorum sensing is an important cell to cell communication system enabling microbe-microbe interaction, colonization, bacterial pathogenesis and invasion across populations, ranging from unicellular prokaryotes to multicellular eukaryotes (Hosni et al. 2011; Hartmann et al. 2014; Cornforth et al. 2014). *N*-acylated L-homoserine lactones (AHLs) of Gram-negative bacteria and oligopeptides of Gram-positive bacteria are released as autoinducers to facilitate quorum sensing (LaSarre and Federle 2013). These in turn coordinate responses across a population to establish crosstalk, the most important being able to thwart chemical defenses (e.g. production of antibiotic compounds) of other organisms (Teplitski et al. 2011). Over the last decades, quorum sensing has progressively received attention in clinical studies owing to an increasing drug resistance in pathogenic bacteria that is a dreaded challenge in curing current and emerging life threatening diseases. Therefore, alternate

“antivirulence” strategies are being sought to target quorum sensing in pathogenic bacteria (Amara et al. 2011; Claessen et al. 2014).

“Antivirulence strategy” comprises of interference with bacterial virulence and/or cell-to-cell signaling pathways without killing bacteria or preventing their growth. The overall strategy is to inhibit specific mechanisms that promote infection and are essential to persistence in a pathogenic cascade (for example, binding, invasion, subversion of host defenses and chemical signaling), and/or cause disease symptoms but without affecting the growth (Clatworthy et al. 2007; Rasko and Sperandio 2010; LaSarre and Federle 2013). Therefore, targeting quorum sensing in a pathogenic bacterial population mitigates virulence as opposed to suppressing bacterial growth. Inhibition of quorum sensing in pathogenic bacteria, a process known as “quorum quenching”, by endophytes has a fundamental advantage over other disease-management strategies (such as antimicrobial therapies) and opens new approaches to tackle drug-resistant bacteria. One important portion of our work encircle around the fundamental insights into the potential of endophytic bacteria harbored in *Cannabis sativa* L. plants, as antivirulence agents suppressing the virulence factors like quorum sensing molecules.

2.2. REFERENCES

- Ahmed SA, Ross SA, Slade D, Radwan MM, Zulfiqar F, ElSohly MA (2008) Cannabinoid ester constituents from high-potency *Cannabis sativa*. *J Nat Prod* 71:536–542
- Aly AH, Debbab A, Kjer J, Proksch P (2010) Fungal endophytes from higher plants: a prolific source of phytochemicals and other bioactive natural products. *Fungal Divers* 41:1-16
- Amara N, Krom BP, Kaufmann GF, Meijler MM (2011) Macromolecular inhibition of quorum sensing: enzymes, antibodies, and beyond. *Chem Rev* 111:195-208
- Ameri A (1999) The effects of cannabinoids on the brain. *Prog Neurobiol* 158:315-348
- Appendino G, Gibbons S, Giana A, Pagani A, Grassi G, Stavri M, Smith E, Rahman MM (2008) Antibacterial cannabinoids from *Cannabis sativa*: a structure-activity study. *J Nat Prod* 71:1427-1430
- Arnold AE, Mejia LC, Kylo D, Rojas EI, Maynard Z, Robbins N (2003) Fungal endophytes limit pathogen damage in a tropical tree. *Proc Natl Acad Sci USA* 100:15649-15654
- Asakawa Y, Hashimoto T, Takikawa K, Tori M, Ogawa S (1991a) Prenyl bibenzyls from the liverworts *Radula perrottetti* and *Radula complanata*. *Phytochemistry* 30:235-251
- Bacon CW, White JF (2000) *Microbial endophytes*. Marcel Dekker Inc New York
- Baker D, Pryce G, Giovannoni G, Thompson AJ (2003) The therapeutic potential of cannabis. *Lancet Neurol* 2:291-298

- Barloy J, Pelhate J (1962) Premières observations phytopathologiques relatives aux cultures de chanvre en Anjou. *Ann Epiphyties* 13:117-149
- Bush Doctor (1985) Damping Off. *Sinsemilla Tips* 5:35-39
- Carchman RA, Harris LS, Munson AE (1976) The inhibition of DNA synthesis by cannabinoids. *Cancer Res* 36:95-100
- Chen KK, Schmidt CF (1924) The action of ephedrine, the active principle of the Chinese drug ma huang. *J Pharmacol Exp Ther* 24:339-357
- Claessen D, Rozen DE, Kuipers OP, Søggaard-Andersen L, van Wezel GP (2014) Bacterial solutions to multicellularity: a tale of biofilms, filaments and fruiting bodies. *Nat Rev Microbiol* doi: 10.1038/nrmicro3178
- Clatworthy AE, Pierson E, Hung DT (2007) Targeting virulence: a new paradigm for antimicrobial therapy. *Nat Chem Biol* 3:541-548
- Cornforth DM, Popat R, McNally L, Gurney J, Scott-Phillips TC, Ivens A, Diggle SP, Brown SP (2014) Combinatorial quorum sensing allows bacteria to resolve their social and physical environment. *Proc Natl Acad Sci USA* 111:4280-4284
- de Bary A (1866) *Morphologie und Physiologie der Pilze, Flechten, und Myxomyceten*. Homeister's handbook of physiological botany, Vol II, Leipzig, Germany
- Dewey LH (1914) "Hemp". In: U.S.D.A. yearbook 1913 United States Department of Agriculture, Washington DC, pp. 283-347
- Elsohly HN, Turner CE, Clark AM, Elsohly MA (1982) Synthesis and antimicrobial activities of certain cannabichromene and cannabigerol related compounds. *J Pharm Sci* 71:1319-1323
- Elsohly MA, Slade D (2005) Chemical constituents of marijuana: the complex mixture of natural cannabinoids. *Life Sci* 78:539-548
- Elsohly MA, Wachtel SR, de Wit H (2003) *Cannabis* versus THC: response to Russo and McPartland. *Psychopharmacology* 165: 433-434
- Eyberger AL, Dondapati R, Porter JR (2006) Endophyte fungal isolates from *Podophyllum peltatum* produce podophyllotoxin. *J Nat Prod* 69:1121-1124
- Fernald ML (1950) *Gray's Manual of Botany*, 4th ed. American Book Company, New York, pp. 556
- Flemming T, Muntendam R, Steup C, Kayser O (2007) Chemistry and biological activity of tetrahydrocannabinol and its derivatives. In Khan MTH (ed) *Topics in Heterocyclic Chemistry*, Springer-Verlag Berlin/Heidelberg, Vol 10, pp. 1-42
- Flores-Sanchez IJ, Pec J, Fei J, Choi YH, Dusek J, Verpoorte R (2009) Elicitation studies in cell suspension cultures of *Cannabis sativa* L. *J Biotechnol* 143:157-168

- Grotenhermen F, Müller-Vahl K (2012) The therapeutic potential of *Cannabis* and cannabinoids. *Medicine Dtsch Arztebl Int* 109:495-501
- Guerin P (1898) Sur la presence d'un champignon dans l'ivraie. *J Botanique* 12:230-238
- Gunatilaka AAL (2006) Natural products from plant-associated microorganisms: distribution, structural diversity, bioactivity, and implications of their occurrence. *J Nat Prod* 69:509–526
- Happyana N, Agnolet S, Muntendam R, Van Dam A, Schneider B, Kayser O (2012) Cannabinoid analysis of laser-microdissected trichomes of *Cannabis sativa* L. by LC-MS and cryogenic NMR. *Phytochemistry* 87:51-59
- Hartmann A, Rothballer M, Hense BA, Schroder P (2014) Bacterial quorum sensing compounds are important modulators of microbe-plant interactions. *Front Plant Sci* 5:131
- Hartsel SC, Loh WH, Robertson LW (1983) Biotransformation of cannabidiol to cannabielsoin by suspension cultures of *Cannabis sativa* and *Saccharum officinarum*. *Planta Med* 48:17-19
- Hazekamp A, Choi YH, Verpoorte R (2004) Quantitative analysis of cannabinoids from *Cannabis sativa* using ¹H-NMR. *Chem Pharm Bull* 52:718-721
- Hazekamp A, Giroud C, Peltenburg A, Verpoorte R (2005) Spectroscopic and chromatographic data of cannabinoids from *Cannabis sativa*. *J Liq Chrom Rel Technol* 28:2361-2382
- Holler JG, Sondergaard K, Slotved HC, Guzman A, Molgaard P (2012) Evaluation of the antibacterial activity of Chilean plants traditionally used for wound healing therapy against multidrug-resistant *Staphylococcus aureus*. *Planta Med* 78:200-205
- Hosni T, Moretti C, Devescovi G, Suarez-Moreno ZR, Fatmi MB, Guarnaccia C, Pongor S, Onofri A, Buonauro R, Venturi V (2011) Sharing of quorum sensing signals and role of interspecies communities in a bacterial plant disease. *ISME J* 5:1857-1870
- Jiang HE, Li X, Zhao YX, Ferguson DK, Hueber F, Bera S, Wang YF, Zhao LC, Liu CJ, Li CS (2006) A new insight into *Cannabis sativa* (Cannabaceae) utilization from 2500-year-old Yanghai Tombs, Xinjiang, China. *J Ethnopharmacol* 108:414-422
- Kawamoto M, Utsukihara T, Abe C, Sato M, Saito M, Koshimura M, Kato N, Horiuchi CA (2008) Biotransformation of (±)-2-methylcyclohexanone by fungi. *Biotechnol Letts* 30:1655-1660
- Kharwar RN, Mishra A, Gond SK, Stierle D (2011) Anticancer compounds derived from fungal endophytes: their importance and future challenges. *Nat Prod Rep* 28:1208-1228

- Kour A, Shahl AS, Rehman S, Qazi PH, Sudan P, Khajuria RK, Sultan P, Verma V (2008) Isolation and identification of an endophytic strain of *Fusarium oxysporum* producing podophyllotoxin from *Juniperus recurva*. *World J Microbiol Biotechnol* 24:1115-1121
- Kusari P, Kusari S, Spiteller M, Kayser O (2013) Endophytic fungi harbored in *Cannabis sativa* L.: Diversity and potential as biocontrol agents against host plant-specific phytopathogens. *Fungal Divers* 60:137-151
- Kusari S, Singh S, Jayabaskaran C (2014) Biotechnological potential of plant-associated endophytic fungi: hope versus hype. *Trends Biotechnol* 32:297-303
- Kusari S, Spiteller M (2011) Are we ready for industrial production of bioactive plant secondary metabolites utilizing endophytes? *Nat Prod Rep* 28:1203-1207
- Kusari S, Spiteller M (2012) Metabolomics of endophytic fungi producing associated plant secondary metabolites: progress, challenges and opportunities. In *Metabolomics U. Roessner (ed) InTech ISBN 978-953-51-0046-1*, pp. 241-266
- Kusari S, Verma VC, Lamshöft M, Spiteller M (2012b) An endophytic fungus from *Azadirachta indica* A. Juss. that produces azadirachtin. *World J Microbiol Biotechnol* 28:1287-1294
- Kusari S, Zuehlke S, Spiteller M (2009a) An endophytic fungi from *Camptotheca acuminata* that produces camptothecin and analogues. *J Nat Prod* 72:2-7
- Kusari S, Zühlke S, Kosuth J, Cellarova E, Spiteller M (2009c) Light-independent metabolomics of endophytic *Thielavia subthermophila* provides insight into microbial hypericin biosynthesis. *J Nat Prod* 72:1825-1835
- Kusari S, Zühlke S, Spiteller M (2011) Effect of artificial reconstitution of the interaction between the plant *Camptotheca acuminata* and the fungal endophyte *Fusarium solani* on camptothecin biosynthesis. *J Nat Prod* 74:764-775
- LaSarre B, Federle MJ (2013) Exploiting quorum sensing to confuse bacterial pathogens. *Microbiol Mol Biol Rev* 77:73-111
- Li SH, Zhang HJ, Yao P, Sun HD, Fong HHS (2001) Taxane diterpenoids from the bark of *Taxus yunnanensis*. *Phytochemistry* 58:369-374
- Linnaeus C (1753) *Species plantarum*. T. I-II
- Lopez-Lazaro M, de la Pena NP, Pastor N, Martin-Cordero C, Navarro E, Cortes F, Ayuso MJ, Toro MV (2003) Anti-tumour activity of *Digitalis purpurea* L. subsp. *heywoodii*. *Planta Med* 69:701-704
- Ludwiczuk A and Asakawa Y (2008) Distribution of terpenoids and aromatic compounds in selected southern hemispheric liverworts. In: *Fieldiana Botany Number 47*. Field Museum of Natural History, Chicago, pp. 37-58

- Márquez LM, Redman RS, Rodriguez RJ, Roossinck MJ (2007) A virus in a fungus in a plant: three-way symbiosis required for thermal tolerance. *Science* 315:513-515
- McClanahan RH, Robertson LW (1985) Microbial transformation of olivetol by *Fusarium roseum*. *J Nat Prod* 48:660-663
- McPartland JM (1991) Common names for diseases of *Cannabis sativa* L. *Plant Dis* 75:226-227
- McPartland JM (1994) Microbiological contaminants of marijuana. *J Int Hemp Assoc* 1:41-44
- McPartland JM (1995) *Cannabis* pathogens X: *Phoma*, *Ascochyta* and *Didymella* species. *Mycologia* 86:870-878
- McPartland JM (1996) A review of *Cannabis* diseases. *J Int Hemp Assoc* 3:19-23
- McPartland JM, Clarke RC, Watson DP (2000) Hemp diseases and pests: management and biological control. CABI Publishing, Wallingford, UK
- Miyazawa M, Nankai H, Kameoka H (1997) Enantioselective cyclization of (±)-lavandulol to (-)-(2S, 4S)-1,5-epoxy-5-methyl-2-(1-methylethenyl)-4-hexanol by *Glomerella cingulata*. *Nat Prod Lett* 9:249-252
- Mojzisoava G, Mojzisz J (2008) Flavonoids and their potential health benefits: relation to heart diseases and cancer. *Recent Prog Med Plants* 21:105-129
- Murray RM, Morrison PD, Henquet C, Di Forti M (2007) *Cannabis*, the mind and society: the hash realities. *Nat Rev Neurosci* 8:885-895
- Musty RE (2004) Natural cannabinoids: interactions and effects. In: Guy GW, Whittle BA, Robson PJ (eds) *The medicinal uses of cannabis and cannabinoids*. Pharmaceutical Press, London, UK, pp. 165-204
- Pollastro F, Tagliatalata-Scafati O, Allar M, Munoz E, Marzo VD, Petrocellis LD, Appendino G (2011) Bioactive prenylogous cannabinoid from fiber hemp (*Cannabis sativa*). *J Nat Prod* 74:2019-2022
- Porrás-Alfaro A, Bayman P (2011) Hidden fungi, emergent properties: endophytes and microbiomes. *Annu Rev Phytopathol* 49:291-315
- Radwan MM, Ross SA, Slade D, Ahmed SA, Zulfiqar F, ElSohly MA (2008) Isolation and characterization of new *Cannabis* constituents from a high potency variety. *Planta Med* 74:267-272
- Rasko DA, Sperandio V (2010) Anti-virulence strategies to combat bacteria-mediated disease. *Nat Rev Drug Discov* 9:117-128
- Redecker D, Kodner R, Graham LE (2000) Glomalean fungi from the Ordovician. *Science* 289:1920-1921
- Redman RS, Sheehan KB, Stout RG, Rodriguez RJ, Henson JM (2002) Thermotolerance conferred to plant host and fungal endophyte during mutualistic symbiosis. *Science*

298:1581

- Rodriguez R, Redman R (2008) More than 400 million years of evolution and some plants still can't make it on their own: plant stress tolerance via fungal symbiosis. *J Exp Bot* 59:1109-1114
- Rodriguez RJ, Henson J, Van Volkenburgh E, Hoy M, Wright L, Beckwith F et al. (2008) Stress tolerance in plants via habitat-adapted symbiosis. *ISME J* 2:404-416
- Rodriguez RJ, Redman RS, Henson JM (2004) The role of fungal symbioses in the adaptation of plants to high stress environments. *Mitig Adapt Strateg Glob Chang* 9:261-272
- Russo EB, McPartland JM (2003) *Cannabis* is more than simply delta(9)-tetrahydrocannabinol. *Psychopharmacology (Berl)* 165:431-432
- Saxena S (2009) Fungal biotransformations of cannabinoids: Potential for new effective drugs. *Curr Opin Drug Discov Develop* 12:305-312
- Shweta S, Zühlke S, Ramesha BT, Priti V, Kumar PM, Ravikanth G, Spiteller M, Vasudeva R, Shaanker RU (2010) Endophytic fungal strains of *Fusarium solani*, from *Apodytes dimidiata* E. Mey. ex Arn (Icacinaceae) produce camptothecin, 10-hydroxycamptothecin and 9-methoxycamptothecin. *Phytochemistry* 71:117-122
- Staniek A, Woerdenbag HJ, Kayser O (2008) Endophytes: exploiting biodiversity for the improvement of natural product-based drug discovery. *J Plant Interact* 3:75-93 11
- Stone JK, Bacon CW, White JF (2000) An overview of endophytic microbes: endophytism. In: Bacon CW, White JF (ed) *Microbial endophytes*, New York Marcel Dekker Inc., pp. 3-30
- Strobel GA (2002) Microbial gifts from rain forests. *Can J Plant Pathol* 24:14-20
- Strobel GA, Daisy B (2003) Bioprospecting for microbial endophytes and their natural products. *Microbial Mol Biol Rev* 67:491-502
- Suryanarayana TS, Thirunavukkarasu N, Govindarajulu MB, Sasse F, Jansend, R, Murali TS (2009) Fungal endophytes and bioprospecting. *Fungal Biol Rev* 23:9-19
- Tanaka H, Takahashi R, Morimoto S, Shoyama YA (1997) New cannabinoid, Δ^6 -tetrahydrocannabinol 2 Δ -O- β -d-glucopyranoside, biotransformed by plant tissue. *J Nat Prod* 60:168-170
- Taura F, Sirikantaramas S, Shoyama Y, Shoyama Y, Morimoto S (2007) Phytocannabinoids in *Cannabis sativa*: Recent Studies on Biosynthetic Enzymes. *Chem Biodivers* 4:1649-1663
- Teplitski M, Mathesius U, Rumbaugh KP (2011) Perception and degradation of *N*-acyl homoserine lactone quorum sensing signals by mammalian and plant cells. *Chem Rev* 111:100-116

- Toniazzo G, de Oliveira D, Dariva C, Oestreicher EG, Antunes OA (2005) Biotransformation of (-)- β -pinene by *Aspergillus niger* ATCC 9642. *Appl Biochem Biotechnol* 121-124:837-844
- Toyota M, Kinugawa T, Asakawa Y (1994) Bibenzyl cannabinoid and bisbibenzyl derivative from the liverwort *Radula perrottetti*. *Phytochemistry* 37:859-862
- Toyota M, Shimamura T, Ishii H, Renner M, Braggins J, Asakawa Y (2002) New bibenzyl cannabinoid from the New Zealand liverwort *Radula marginata*. *Chem Pharm Bull* 50:1390-1392
- Turner CE, Elsohly MA, Boeren EG (1980) Constituents of *Cannabis sativa* L. XVII a review of the natural constituents. *J Nat Prod* 43:169-234
- Wachtel SR, ElSohly MA, Ross SA, Ambre J, de Wit H (2002) Comparison of the subjective effects of D9-tetrahydrocannabinol and marijuana in humans. *Psychopharmacology* 161:331-339
- Waller F, Achatz B, Baltruschat H, Fodor J, Becker K, Fischer M et al. (2005) The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance and higher yield. *Proc Natl Acad Sci USA* 102:13386-13391
- Wink M (2008) Plant secondary metabolism: Diversity, function and its evolution. *Nat Prod Commun* 3:1205-1216
- Zhang HW, Song YC, Tan RX (2006) Biology and chemistry of endophytes. *Nat Prod Rep* 23:753-771

Chapter 3

ENDOPHYTIC FUNGI HARBORED IN *CANNABIS SATIVA* L.: DIVERSITY AND POTENTIAL AS BIOCONTROL AGENTS AGAINST HOST-PLANT SPECIFIC PHYTOPATHOGENS

Parijat Kusari, Souvik Kusari, Michael Spiteller, Oliver Kayser

PK, SK, MS and OK jointly conceived, planned and designed the study; PK performed all the experiments and analyzed the data; PK and SK performed the bioinformatics analyses and interpreted the data; PK wrote the manuscript with inputs from all coauthors; SK supervised PK and maintained scientific surveillance; MS and OK oversaw the entire project as senior authors and supervisors of PK

Fungal Diversity (2013) 60:137-151

3.1. ABSTRACT

The objective of the present work was isolation, phylogenetic characterization, and assessment of biocontrol potential of endophytic fungi harbored in various tissues (leaves, twigs, and apical and lateral buds) of the medicinal plant, *Cannabis sativa* L. A total of 30 different fungal endophytes were isolated from all the plant tissues which were authenticated by molecular identification based on rDNA ITS sequence analysis (ITS1, 5.8S and ITS2 regions). The Menhinick's index revealed that the buds were immensely rich in fungal species, and Camargo's index showed the highest tissue-specific fungal dominance for the twigs. The most dominant species was *Penicillium copticola* that could be isolated from the twigs, leaves, and apical and lateral buds. A detailed calculation of Fisher's log series index, Shannon diversity index, Simpson's index, Simpson's diversity index, and Margalef's richness revealed moderate overall biodiversity of *C. sativa* endophytes distributed among its tissues. The fungal endophytes were challenged by two host phytopathogens, *Botrytis cinerea* and *Trichothecium roseum*, devising a dual culture antagonistic assay on five different media. We observed eleven distinct types of pathogen inhibition encompassing a variable degree of antagonism (%) on changing the media. This revealed the potential chemodiversity of the isolated fungal endophytes not only as promising resources of biocontrol agents against the known and emerging phytopathogens of *Cannabis* plants, but also as sustainable resources of biologically active and defensive secondary metabolites.

Keywords: *Cannabis sativa*; endophytic fungi; fungal diversity; antagonism; *Botrytis cinerea*; *Trichothecium roseum*

3.2. INTRODUCTION

Cannabis sativa L. (Cannabaceae) is an annual herbaceous plant, native mainly to Central Asia, that has been in use all over the planet either in the form of narcotic or medicinal preparations or as a source of food and fiber (Jiang et al. 2006). The secondary metabolites of this plant constitute more than 400 compounds (Turner et al. 1980), with the most emphasis being led on cannabinoids. More than 108 cannabinoids have already been discovered (Hazekamp et al. 2004, 2005; ElSohly and Slade 2005; Radwan et al. 2008; Ahmed et al. 2008; Fishedick et al. 2010). Although *Cannabis* is regarded as mainly a drug of abuse at present, cannabinoids are known to have important therapeutic effects such as analgesic, anti-spasmodic, anti-tremor, anti-inflammatory, antioxidant, neuro-protective, and appetite stimulant (Baker et al. 2003; Gomes et al. 2008; Mojzisoava and Mojzis 2008). Such pronounced efficacies of cannabinoids have led to the development of various *Cannabis*-based medicines, namely dronabinol (Marinol®, Solvay Pharmaceuticals, Belgium), Sativex (GW Pharmaceuticals, UK), and nabilone (Cesamet®, Valeant Pharmaceuticals International, USA). Although Δ^9 -tetrahydrocannabinol (Δ^9 -THC) is considered to be the major psychoactive compound (Taura et al. 1995; Sirikantaramas et al. 2005; Pertwee 2006), there is still a lot of intensive investigation to verify if pure cannabinoids provide better therapeutic effect over the whole plant extracts, and the worth of other compounds in *Cannabis*-based medicinal use (Wachtel et al. 2002; ElSohly et al. 2003; Russo and McPartland 2003; Grotenhermen and Müller-Vahl 2012).

C. sativa is commonly known as 'hemp'. Owing to the potent phytochemical constituents and diverse use of this plant by humans, an overall fallacy that "hemp has no enemies" (Dewey 1914) has developed. Unfortunately, this plant is attacked by a plethora of phytopathogens leading to a number of diseases (McPartland 1996) prevalent in every organ (such as leaf, flower, stem and root) and growth stage (seedling to mature plant). A number of specific and non-specific bacteria and fungi have been found to be associated with the plant as pathogens, and responsible for different stress symptoms and diseases (Taylor et al. 1982; Kurup et al. 1983; McPartland 1983, 1994; Schwartz 1985; Grotenhermen and Müller-Vahl 2012). In particular, more than 80 different fungal species have been discovered so far that poses some form of threat to *Cannabis* plants (Hockey 1927; McPartland 1995). However, two of the most threatening diseases of *C. sativa* have been shown to be caused by the phytopathogens *Botrytis cinerea* and *Trichothecium roseum* (McPartland 1996). On the one hand, *B. cinerea* attacks the leaves, flowers, stems and branches of this plant leading to the disease known as 'gray mold', which can completely destroy the plant within 1 week (Barloy and Pelhate 1962). This fungal pathogen forms a grey brown mat and encircles leaves,

stems and flowers and can even spread epidemic disasters in the field (van der Werf and van Geel 1994; van der Werf et al. 1995). *B. cinerea* also causes another disease called 'damping off' where it weakens the seeds or seedlings before or after they germinate, or even kill the seedlings (Bush Doctor 1985). On the other hand, *T. roseum* attacks the leaves and flowers of *C. sativa* plants causing the dreaded 'pink rot' disease, which is a greenhouse threat for cultivars (McPartland 1991). Although some sporadic attempts have been made for the elimination of the fungal pathogens from this plant (Ungerlerder et al. 1982; Kurup et al. 1983; Levitz and Diamond 1991; Bush Doctor 1993), a more comprehensive, practical and ecologically relevant means to eradicate the pathogen-mediated diseases in *Cannabis* is necessary. It is, thus, highly desirable to effectively address these threats to prevent the loss of these medicinally relevant plants and drastically reduce the amount of hazards caused by these specific and/or other opportunistic pathogens.

Plant associated bacterial and fungal communities play an important role in balancing the ecosystem. Endophytic microorganisms ('endophytes') are a group of highly assorted organisms that internally infect living plant tissues without instigating any noticeable symptom of infection or visible manifestation of disease, and live in mutualistic association with plants for at least a part of their life cycle (Hyde and Soyong 2008; Botella and Diez 2011; Purahong and Hyde 2011; Vesterlund et al. 2011; Kusari and Spitteller 2012; Kusari et al. 2012b). Endophytes, mainly represented by fungi but also by bacteria, have great promise with diverse potential for exploitation (Staniek et al. 2008; Li et al. 2012). A plethora of competent endophytic fungi have already been discovered that are capable of providing different forms of fitness benefits to their associated host plants (Hamilton et al. 2012; Hamilton and Bauerle 2012). For example, these organisms have demonstrated the capacity to produce a diverse range of biologically active secondary metabolites (Strobel and Daisy 2003; Strobel et al. 2004; Gunatilaka 2006; Zhang et al. 2006; Staniek et al. 2008; Suryanarayana et al. 2009; Aly et al. 2010; Kharwar et al. 2011; Debbab et al. 2012), occasionally including those similar to their associated host plants (Eyberger et al. 2006; Kusari et al. 2008, 2009a, b, c, 2011, 2012a), and induce host plant tolerance to environmental stress, herbivory, heat, salt, disease and drought (Stone et al. 2000; Redman et al. 2002; Arnold et al. 2003; Rodriguez et al. 2004, 2008; Waller et al. 2005; Márquez et al. 2007; Rodriguez and Redman 2008; Porras-Alfaro and Bayman 2011).

The objective of the work reported in this manuscript was to evaluate the diversity of endophytic fungi isolated from different tissues of *Cannabis sativa* L., and further screen them as potential biocontrol agents against two major fungal pathogens of the plant, namely *Botrytis cinerea* and *Trichothecium roseum*. Based on the knowledge that the biosynthesis of

secondary metabolites in endophytes are dependent on the culture parameters and available nutrition (OSMAC, One Strain MAny Compounds) (Bode et al. 2002), we further evaluated the antagonistic effects of isolated endophytes against the two host-specific pathogens on five different media. To the best of our knowledge, this is the first report of the incidence, diversity, phylogeny, and assessment of biocontrol potential of endophytic fungi harbored in *C. sativa*.

3.3. MATERIALS AND METHODS

3.3.1. Collection, identification, and authentication of plant material

As part of an effort to identify endophytic fungi that provide fitness benefits to their host plants, *Cannabis sativa* plants were sampled from the Bedrocan BV Medicinal Cannabis (the Netherlands). The plants were identified and authenticated as *C. sativa* by experienced botanists at the Bedrocan BV. Plants specimens are under deposit at Bedrocan BV with voucher numbers (A1)05.41.050710. These plants were then transported to the TU Dortmund, Germany immediately, and processed within 6 h of collection. Import of the plant material was allowed according to the permission of the Federal Institute for Drugs and Medical Devices (*Bundesinstituts für Arzneimittel und Medizinprodukte*, BfArM), Bonn, Germany under the license number 458 49 89. Different parts of the plants such as fresh leaves, twigs and apical lateral buds were carefully excised from the live host plant (roots were unavailable due to legislative restrictions). The excised tissues were washed thoroughly in running tap water followed by deionized (DI) water to remove any dirt sticking to them and stored at 4 °C until the isolation procedure of endophytic fungi was commenced (≤ 10 min).

3.3.2. Isolation of endophytic fungi and establishment of *in vitro* axenic cultures

The surface sterilization and isolation of endophytes was done following previously established procedures (Kusari et al. 2009a). The explants were thoroughly washed in running tap water, and small fragments of leaves, twigs, and buds of approximately 10 mm (length) by 5 mm (breadth) were cut with the aid of a flame-sterilized razor blade (same number of fragments for each tissue type). Then, the small tissue fragments were surface-sterilized by sequential immersion in 70% ethanol for 1 min, 1.3 M sodium hypochlorite (3-5% available chlorine) for 3 min, and 70% ethanol for 30 s. Finally, these surface-sterilized tissue pieces were rinsed thoroughly in sterile, double-distilled water for a couple of minutes, to remove excess surface sterilants. The excess moisture was blotted on a sterile filter paper. The surface-sterilized tissue fragments, thus obtained, were evenly placed (four

fragments in each plate) in petri dishes (Diagonal GmbH & Co. KG, Germany) containing water agar (WA) medium (Roth, cat. no. 5210.2) amended with streptomycin (100 mg/L) to eliminate any bacterial growth. The petri dishes were sealed using Parafilm (Diagonal GmbH & Co. KG, Germany). The petri dishes were incubated at $28\pm 2^{\circ}\text{C}$ until fungal growth started. To ensure proper surface sterilization and isolation of fungal endophytes, unsterilized tissue fragments (only washed thoroughly in water) were prepared simultaneously, placed in both WA and Sabouraud agar (SA; Roth, cat. no. X932), and incubated under the same conditions in parallel, to isolate the surface-contaminating fungi (differentiated morphologically by both macroscopic and microscopic evaluation) (Kusari et al. 2009b). The cultures were monitored every day to check the growth of endophytic fungi. The endophytic organisms, which grew out from the sample segments over 4-6 weeks were isolated and subcultured onto a rich mycological medium, SA, and brought into pure culture. To ensure proper surface sterilization, surface-sterilized tissue fragments were imprinted simultaneously in WA as well as SA and incubated under the same conditions in parallel (secondary protocol, 'imprint technique') (Schulz et al. 1998; Sánchez Márquez et al. 2007).

3.3.3. Maintenance and storage of the axenic endophytic fungal isolates

The axenic cultures, obtained above, were coded according to their host tissue origin (L1, L2, etc. from leaves, T1, T2, T3, etc. from twigs, and A1, A2, A3, etc. from apical/lateral buds), and were routinely maintained on PDA, SA and CDA (Czapek-Dox Agar; Merck, Darmstadt, Germany) in active form. For long-term storage, the colonies were preserved in the form of spores (those which readily sporulated in axenic cultures) as well as vegetative form in 15% (v/v) glycerol at -80°C . Agar blocks impregnated with mycelia were used directly for storage of the vegetative forms. For the isolation of the genomic DNA of the endophytes, a set of conical flasks of 500 mL capacity each with 100 mL Sabouraud broth (SB; Roth, cat. no. AE23.1) was used with proper autoclaving. The endophytic fungi were inoculated in the respective flasks from the parent axenic cultures. The flasks were incubated at $28\pm 2^{\circ}\text{C}$ with proper shaking (150 rpm) on a rotary shaker (Heidolph UNIMAX 2010, Germany) over 4-6 weeks.

3.3.4. Total genomic DNA extraction, PCR amplification and sequencing

The total genomic DNA (gDNA) was extracted from the *in vitro* cultures using peqGOLD fungal DNA mini kit (Peqlab Biotechnologie GmbH, Germany, cat. no. 12-3490-02) strictly following the manufacturer's guidelines. The DNA was then subjected to PCR amplification using primers ITS4 and ITS5 according to White et al. (1990). The amplified fragment consisted of ITS1, 5.8S and ITS2 regions of the rDNA. The PCR reaction was performed in

50 µL reaction mixture containing 10 µL Phusion HF buffer (5X), 1 µL dNTPs (10 mM), 0.5 µL forward primer (100 µM), 0.5 µL reverse primer (100 µM), 3 µL of template DNA, 1 µL of Phusion polymerase (2U/µL), and 34 µL of sterile double-distilled water. The PCR cycling protocol consisted of an initial denaturation at 98°C for 3 min, 30 cycles of denaturation, annealing and elongation at 98°C for 10 sec, 58°C for 30 sec and 72°C for 45 sec. This was followed by a final elongation step of 72°C for 10 min. As a negative control, the template DNA was replaced by sterile double-distilled water. The PCR amplified products were checked by gel electrophoresis spanning approximately 500-600 bp (base pairs). The PCR products were further purified using peqGOLD micro spin cycle pure kit (Peqlab, cat. no. 12-6293-01) according to the manufacturer's instructions. The amplified products were then sequenced on ABI 3730xl DNA analyzer at GATC Biotech (Cologne, Germany).

3.3.5. Identification of endophytic fungi and phylogenetic evaluation

For strain identification, the sequences were matched against the nucleotide database using the Basic Local Alignment Search Tool (BLASTn) of the US National Centre for Biotechnology Information (NCBI) for the final identification of the endophytes. The sequences were aligned using ClustalW-Pairwise Sequence Alignment of the EMBL Nucleotide Sequence Database. The sequence alignments were trimmed and verified by the MUSCLE (UPGMA) algorithm (Edgar 2004) using MEGA5 software (Tamura et al. 2011). When the similarity between a particular problem-sequence and a phylogenetically associated reference-sequence was $\geq 99\%$, only then the sequences were considered to be conspecific (Yuan et al. 2010). The phylogenetic tree was reconstructed and the evolutionary history inferred using the Neighbor-Joining method (Saitou and Nei 1987). The robustness of the internal branches was also assessed with 1000 bootstrap replications (Felsenstein 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and were calculated in the units of the number of base substitutions per site. The sequences of this study were deposited at the EMBL-Bank. The accession numbers are detailed in Table 1.

3.3.6. Evaluation and quantification of fungal diversity

Species richness among the isolated endophytic fungi was determined by calculating the Menhinick's index (D_{mn}) (Whittaker 1977) using the following equation:

$$D_{mn} = \frac{s}{\sqrt{N}}$$

Therein, s is the number of different endophytic species in a sample (in this case, plant tissue) and N is the total number of isolated endophytic fungi in a given sample. The fungal dominance was then determined by Camargo's index ($1/D_{mn}$), where D_m represents species richness. A species was defined as dominant if $P_i > 1/D_{mn}$ (Camargo 1992), where P_i is the relative abundance of a species, i defined as the number of competing species present in the community. The species diversity was also evaluated comparing the whole community of isolated endophytic fungi from all tissues of the plant to understand whether these organisms were distributed randomly through the tissues, aggregated, or uniformly distributed (Lamshead and Hodda 1994). Furthermore, to quantify the endophytic fungal diversity of *C. sativa* in different tissues, Fisher's log series index (α), the Shannon diversity index (H'), Simpson's index (D) and Simpson's diversity index ($1-D$), and Margalef's richness (D_{mg}) were calculated (Fisher et al. 1943; Simpson 1949; Margalef 1958; Lamshead et al. 1983; Suryanarayana 2000; Hoffman 2008; Tao 2008) using the following equations, respectively:

$$\frac{N(1-x)}{x}$$

Where, x was calculated by

$$\frac{S}{N} = \frac{(1-x)}{x} \ln \frac{1}{(1-x)}$$

$$H' = - \sum_i P_i \ln(P_i)$$

Where, H' values could start from 0 (only one species present with no uncertainty as to what species each individual will be) and go higher revealing high uncertainty as species are relatively evenly distributed.

$$D = \sum_i \frac{n_i(n_i - 1)}{N(N - 1)}$$

Where, D could range between 0 (infinite diversity) and 1 (no diversity).

$$D_{mg} = \frac{(S - 1)}{\ln(N)}$$

Therein, N is the number of individuals (defined by numbers of endophytic fungal isolates), S is the number of taxa (ITS genotype), n is the total number of endophytic microorganisms of

a particular species, and i is the proportion of species relative to the total number of species (P_i). Taxon accumulation curves and bootstrap estimates of total species richness based on recovered fungal isolates were generated using the software BioDiversity Pro (McAleece et al. 1997).

Table 1 Summary of the fungal endophytes isolated from various tissues of *C. sativa* with their respective strain codes, EMBL-Bank accession numbers, and closest affiliations of the representative isolates in the GenBank according to rDNA ITS analysis

| Strain number (endophyte) | Part (tissue) of the plant | EMBL-Bank accession number | Most closely related strain (accession number) | Reference | Maximum identity (%) |
|---------------------------|----------------------------|----------------------------|--|-----------------------|----------------------|
| L1 | Leaf | HE962579 | <i>Penicillium copticola</i> (JN617685.1) | Houbraken et al. 2011 | 98 |
| L2 | | HE962580 | <i>Penicillium copticola</i> (JN617685.1) | Houbraken et al. 2011 | 99 |
| L3 | | HE962581 | <i>Penicillium copticola</i> (JN617685.1) | Houbraken et al. 2011 | 99 |
| L4 | | HE962582 | <i>Penicillium copticola</i> (JN617685.1) | Houbraken et al. 2011 | 99 |
| L5 | | HE962482 | <i>Chaetomium globosum</i> (HQ914911.1) | NA | 99 |
| L6 | | HE962576 | <i>Chaetomium globosum</i> (JF773585.1) | NA | 99 |
| L7 | | HE962577 | <i>Eupenicillium rubidurum</i> (HQ608058.1) | Rodrigues et al. 2011 | 99 |
| L8 | | HE962578 | <i>Eupenicillium rubidurum</i> (HQ608058.1) | Rodrigues et al. 2011 | 99 |
| T1 | Twig | HE962583 | <i>Penicillium copticola</i> (JN617685.1) | Houbraken et al. 2011 | 99 |
| T2 | | HE962584 | <i>Penicillium copticola</i> (JN617685.1) | Houbraken et al. 2011 | 99 |
| T3 | | HE962585 | <i>Penicillium copticola</i> (JN617685.1) | Houbraken et al. 2011 | 99 |
| T4 | | HE962586 | <i>Penicillium copticola</i> (JN617685.1) | Houbraken et al. 2011 | 98 |
| T5 | | HE962587 | <i>Penicillium copticola</i> (JN617685.1) | Houbraken et al. 2011 | 99 |
| T6 | | HE962588 | <i>Penicillium sp.</i> (JF439496.1) | Han et al. 2011 | 99 |
| A1 | Apical/lateral buds | HE962589 | <i>Penicillium copticola</i> (JN617685.1) | Houbraken et al. 2011 | 99 |
| A2 | | HE962590 | <i>Penicillium copticola</i> (JN617685.1) | Houbraken et al. 2011 | 99 |
| A3 | | HE962591 | <i>Penicillium copticola</i> (JN617685.1) | Houbraken et al. 2011 | 99 |
| A4 | | HE962592 | <i>Paecilomyces lilacinus</i> | NA | 99 |

| | | | | |
|-----|----------|--|------------------------|----|
| | | (GU980015.1) [syn. <i>Purpureocillium lilacinum</i>] | | |
| A5 | HE962593 | <i>Penicillium copticola</i> (JN617685.1) | Houbraken et al. 2011 | 99 |
| A6 | HE962594 | <i>Penicillium sumatrense</i> (AY213677.1) | Rakeman et al. 2005 | 99 |
| A7 | HE962595 | <i>Penicillium meleagrinum</i> var. <i>viridiflavum</i> (HM469412.1) | Jang et al. 2011 | 99 |
| A8 | HE962596 | <i>Penicillium copticola</i> (JN617685.1) | Houbraken et al. 2011 | 99 |
| A9 | HE962597 | <i>Penicillium copticola</i> (JN617685.1) | Houbraken et al. 2011 | 99 |
| A10 | HE962598 | <i>Penicillium copticola</i> (JN617685.1) | Houbraken et al. 2011 | 99 |
| A11 | HE962599 | <i>Penicillium copticola</i> (JN617685.1) | Houbraken et al. 2011 | 99 |
| A12 | HE962600 | <i>Aspergillus versicolor</i> (FJ878627.1) | Arabatzis et al. 2011 | 99 |
| A13 | HE962601 | <i>Penicillium copticola</i> (JN617685.1) | Houbraken et al., 2011 | 99 |
| A14 | HE962602 | <i>Penicillium copticola</i> (JN617685.1) | Houbraken et al., 2011 | 99 |
| A15 | HE962603 | <i>Penicillium sumatrense</i> (AY213677.1) | Rakeman et al., 2005 | 99 |
| A16 | HE962604 | <i>Penicillium copticola</i> (JN617685.1) | Houbraken et al., 2011 | 99 |

NA not available (not published or not yet published)

3.3.7. Pathogens used for antagonistic assays

The endophytic fungi were tested against the known pathogens of the *Cannabis* plant, which were obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. The fungi *Botrytis cinerea* (accession number DSM 5145) and *Trichothecium roseum* (accession number DSM 63066) were employed. The medium used for the activation of the microorganisms were malt extract agar (MEA; Roth, cat. no. X923.1) and potato dextrose agar (PDA; Roth, cat. no. X931.1). Activation was performed strictly according to the DSMZ guidelines. The activated strains were routinely maintained on PDA, MEA, and SA respectively. All procedures were carried out under aseptic conditions.

3.3.8. *In vitro* antagonistic activity of endophytes against host phytopathogens

The *in vitro* antagonistic behavior of all endophytes was tested against the host plant-specific pathogens *B. cinerea* and *T. roseum* using the dual culture plate antagonism assay method established earlier (Trejo-Estrada et al. 1998; Chamberlain and Crawford 1999; Miles et al.

2012) suitably modified. Five different kinds of media were used for the bioassay namely SA, MEA, PDA, WA and Nutrient agar (NA; Difco, cat. no. 234000) respectively. The plates were prepared in 90 mm sterile petri dishes (Diagonal GmbH & Co. KG, Germany) with approximately 22 mL of the media, yielding a final depth of 4mm. Then, 5 mm plugs of each endophyte and pathogen were co-cultured in the five different media mentioned above and incubated at $28\pm 2^{\circ}\text{C}$. The plugs were placed at the two opposite edge of the petri dishes facing each other. The pathogens alone were inoculated as controls. The diameter of growth of both endophyte and pathogen were monitored daily and recorded at 5, 10 and 15 days, respectively. All control and test plates were run in duplicates. Relative growth inhibitions (% antagonism) were calculated against the control plates for each of the endophyte-pathogen combinations, in each of the five medium used in the bioassay. Percentage antagonism was calculated by using a modified equation mentioned below (Chamberlain and Crawford 1999):

Radial growth of pathogen in presence of endophyte (RG)

$$= \frac{\text{total growth of pathogen} - \text{fungal plug inoculum of pathogen}}{2}$$

Radial growth of pathogen in absence of endophyte (control)

$$= \frac{\text{total growth of pathogen in control plate} - \text{fungal plug inoculum of pathogen}}{2}$$

$$\% \text{ Antagonism} = 1 - \frac{RG}{\text{control}} \times 100$$

3.4. RESULTS

3.4.1. Identification and characterization of the endophytic fungi

A plethora of fungal endophytes were isolated from the various tissues of *C. sativa* L. such as leaves, twigs, and apical and lateral buds. A total of 30 endophytic fungal isolates were isolated from various tissues, whereby the buds hosted the largest number of endophytes (16 isolates) followed by the leaves (8 isolates) and finally the twigs (6 isolates) (Table 1). The selective media supporting the pure culture of fungi was noted, and the isolates were preserved in our microbial library. The endophytic fungi were authenticated by molecular identification based on rDNA ITS sequence analysis. The amplified ITS sequences of the genomic DNA (ITS1, intervening 5.8S, and ITS2) spanning around 500-600 bp were used for the identification of the fungal endophytes. All the sequences were matched against the

nucleotide database using the Basic Local Alignment Search Tool (BLASTn) of the US National Centre for Biotechnology Information (NCBI), which revealed the most homologous sequences. The detailed description of the fungal endophytes with respective codes, EMBL-Bank accession numbers, and closest sequence homologs are summarized in Table 1. The identities of the endophytes were considered conspecific only at a minimum threshold identity of $\geq 99\%$ compared to the most closely related strains (Yuan et al. 2010), with the exception of only two sequences (for isolates L1 and T4) which revealed at least 98% similarity to known reported sequences. All the endophytic fungal isolates belonged to phylum Ascomycota. Most of the isolates belonged to *Penicillium* which could, thus, be assigned as the major genus harbored in the leaves, twigs as well as buds. Other isolated endophytic fungal genus included *Chaetomium*, *Aspergillus*, and *Paecilomyces*.

3.4.2. Phylogeny and fungal diversity analysis

The phylogenetic tree gave a more detailed idea about the relationship between the different species of fungal endophytes obtained from different parts of *C. sativa* L. (Fig. 1). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches in the figure (bootstrap values $>50\%$). The tree has been drawn to scale, with branch lengths in the same units as those of the evolutionary distance used to infer the phylogenetic tree. The number of isolates obtained from different tissues of *C. sativa* ranged from 6 to 16 for twigs and buds, respectively. The species richness determined by calculating the Menhinick's index (D_{mn}) revealed that the buds were rich in endophytic fungal species ($D_{mn} = 1.25$), followed by the leaves ($D_{mn} = 1.06$), and finally the twigs ($D_{mn} = 0.81$). Camargo's index depicting the tissue-specific fungal dominance was 1.23 for the twigs (highest), followed by that of leaves (0.94) and buds (0.8). The dominant species was *Penicillium copticola*, isolated from the twigs, leaves, and apical and lateral buds, with a relative proportion of $P_i = 0.66$. The next dominant species were *Chaetomium globosum* (leaves), *Eupenicillium rubidurum* (syn. *E. meridianum*) (leaves), and *Penicillium sumatrense* (buds) with their $P_i = 0.06$. The rest of the species were less dominant ($P_i = 0.03$). Whole community analysis revealed that the endophytic fungal species were dispersed randomly within the host plant tissues ($\chi^2(k) = 22.29$, with k at 24).

To characterize the biodiversity of our samples, we calculated Fisher's log series index (α), the Shannon diversity index (H'), Simpson's index (D) and Simpson's diversity index ($1-D$), and Margalef's richness (D_{mg}), respectively. The values obtained by these tests (for leaves 1.74, 0.42, 0.28, 0.71, 7.75; for twigs 1.98, 0.34, 0.47, 0.52, 8.28; for buds 2.49, 0.45,

0.46, 0.53, 5.81) indicate that the biodiversity of fungal endophytes in *C. sativa* is not too high. The Shannon index revealed higher certainty of endophytic fungal species consistency in the twigs compared to that of the leaves and buds. Furthermore, the Simpson's index clearly showed that the leaves harbored highly diverse fungal endophytes compared to those harbored by either the twigs or the buds. Finally, Margalef's index revealed that the twigs had high taxonomic richness compared to the leaves or buds.

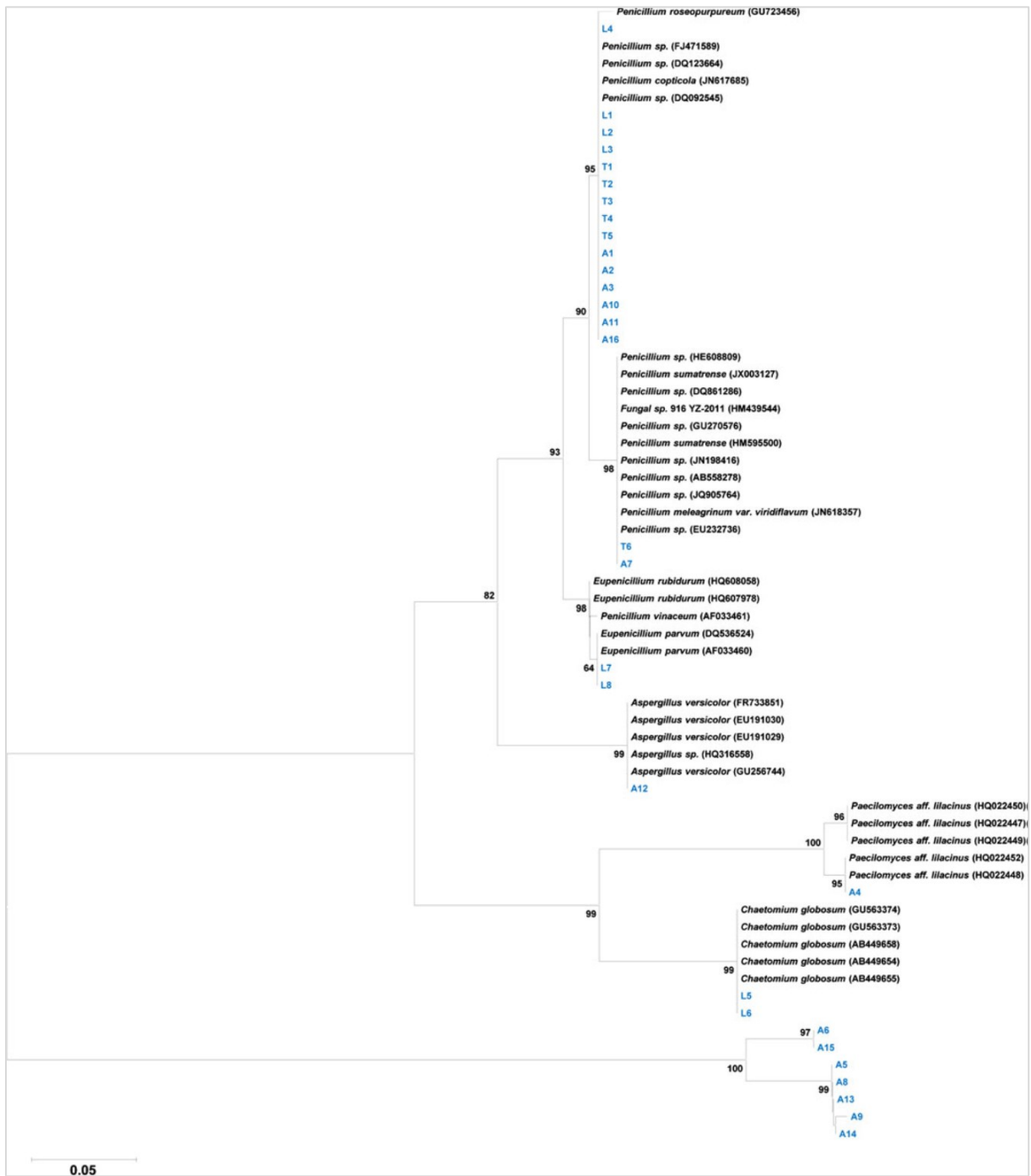


Fig. 1 Phylogenetic tree based on neighbor-joining analysis of the rDNA ITS sequences of the endophytic fungal isolates obtained from various tissues of *C. sativa*. The endophytic fungal codes are shown in blue. For the closely related species, the taxonomic names are followed by their respective accession numbers in brackets. Significant bootstrap values (>50%) are indicated at the branching points. The tree has been drawn to scale.

3.4.3. In vitro antagonism assay of endophytes as potential biocontrol agents

From the in vitro plate bioassay of different fungal endophytes with each of the host plant pathogen in five different types of media gave a clear idea about various types of interactions that can exist between them. Understanding endophyte-pathogen interaction is a vital for understanding the biodiversity of the plant tissue microflora compared to their chemodiversity. By macroscopic evaluation of the interaction and consulting with earlier reports on various endophyte-pathogen interaction types (Trejo-Estrada et al. 1998; Miles et al. 2012), we could assign the interactions of the isolated endophytic fungi with the two *Cannabis* pathogens on five different media into eleven types (Table 2 and Fig. 2).

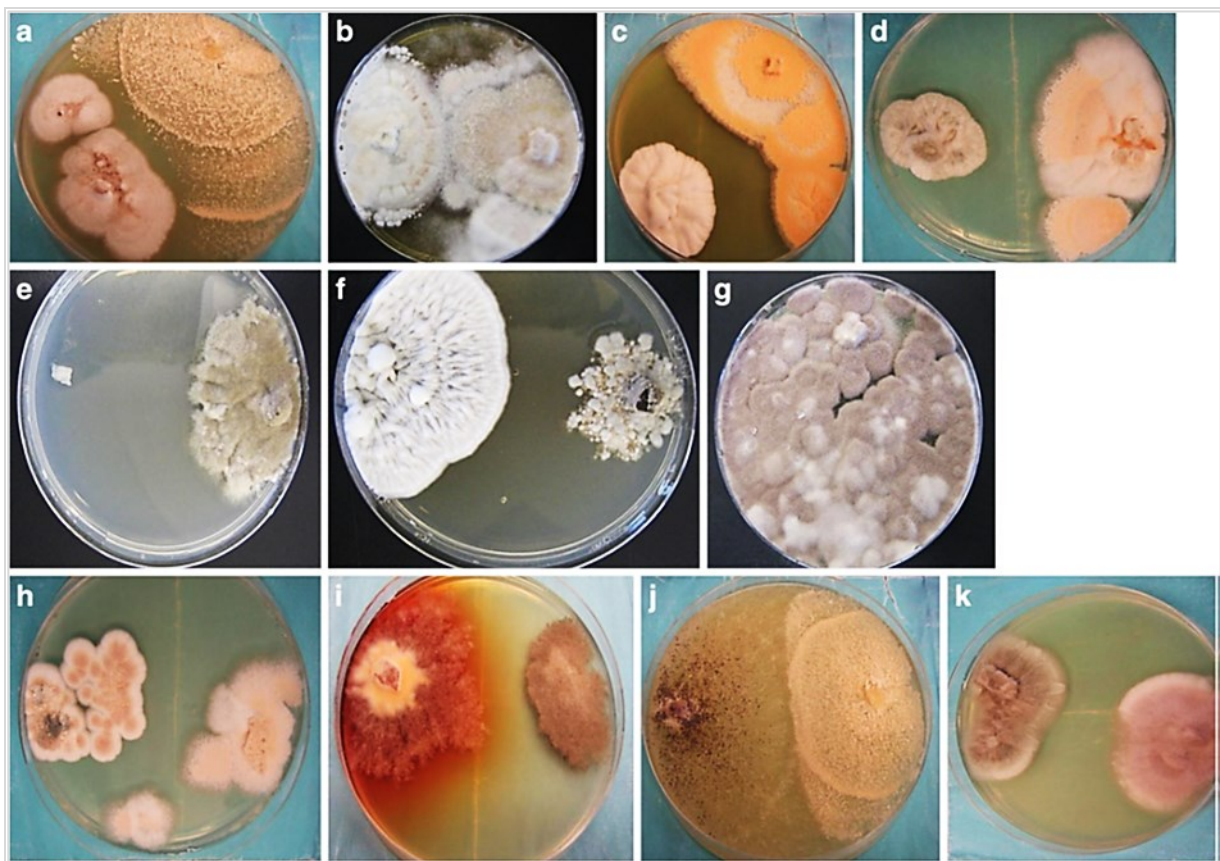


Fig. 2 Types of endophyte-host pathogen interactions observed in dual culture antagonistic assay. (a-k) Interaction types I-XI, where endophytes are shown on the left and challenging pathogen on the right of the representative Petri plates.

Table 2 Different types of dual culture interactions between isolated endophytic fungi and the two host pathogens (*B. cinerea* and *T. roseum*) on five different solid media

| Type code | Interaction descriptions |
|-----------|---|
| I | Both endophyte and pathogen grow towards each other, but growth stopped as their mycelia came in physical contact; no overgrowth after mycelia contact; no inhibition zone (no halo); no color alteration of mycelia; no sporulation of endophytic fungus |
| II | Both endophyte and pathogen grow towards each other followed by slight overgrowth of endophyte on pathogen after their mycelia came in physical contact; no inhibition zone (no halo); no color alteration of mycelia; no sporulation of endophytic fungus |
| III | Both endophyte and pathogen grow towards each other, but growth stopped before their mycelia came in physical contact; no inhibition zone (no halo); no color alteration of mycelia; no sporulation of endophytic fungus |
| IV | Both endophyte and pathogen grow towards each other, but growth stopped before their mycelia came in physical contact and clear halo (inhibition zone) produced by the endophyte around its biomass; no halo by the pathogen; no color alteration of mycelia; no sporulation of endophytic fungus |
| V | Both endophyte and pathogen grow towards each other, but growth stopped before their mycelia came in physical contact and clear halo (inhibition zone) produced by the pathogen around its biomass; no halo by the endophyte; no color alteration of mycelia; no sporulation of endophytic fungus |
| VI | Both endophyte and pathogen grow towards each other, but growth stopped before their mycelia came in physical contact and respective clear halo (inhibition zone) produced by both the endophyte and the pathogen around their biomass; no color alteration of mycelia; no sporulation of endophytic fungus |
| VII | Both endophyte and pathogen grow towards each other followed by complete overgrowth of endophyte on pathogen after their mycelia came in physical contact; no color alteration of mycelia; no sporulation of endophytic fungus |
| VIII | Both endophyte and pathogen grow towards each other, but growth stopped before their mycelia came in physical contact and endophyte releasing visible exudates from its entire mycelial biomass; no color alteration of mycelia; no sporulation of endophytic fungus |
| IX | Both endophyte and pathogen grow towards each other, but growth stopped before their mycelia came in physical contact and endophyte releasing visible (colored) pigments (secondary metabolites) from the point of contact leading to complete color change of the media; no color alteration of mycelia; no sporulation of endophytic fungus |
| X | Both endophyte and pathogen grow towards each other, but growth stopped as their mycelia came in physical contact and endophyte sporulating profusely; no color alteration of mycelia |
| XI(E/P) | Both endophyte and pathogen grow towards each other, but growth stopped as their mycelia came in physical contact; color alteration of mycelia either by endophyte (E) or pathogen (P) or both (E/P); no sporulation of endophytic fungus |

The percentage antagonism (growth inhibition percentage) of each fungal endophyte was calculated against each of the two phytopathogens (Chamberlain and Crawford 1999). All the growth inhibition percentages along with their respective endophyte-pathogen interaction types are summarized in Tables 3 (against *B. cinerea*) and 4 (against *T. roseum*). As expected from the OSMAC concept (Bode et al. 2002; Kusari et al. 2012b), the growth inhibition varied largely among the different fungal isolates in different media. Further, not only were diverse types of interactions between individual fungal endophyte and pathogen observed in different media, but such interactions also resulted in different degrees of growth inhibition. Almost all the endophytic isolates were capable of inhibiting, to a varying extent on different media, one or both of the host-specific pathogens with a higher extent of antagonism against *T. roseum*. The inhibition efficacies of the endophytes were least against *B. cinerea* on WA medium, on which mainly one type of endophyte-pathogen interaction could be observed (type III). Here, both the endophyte and pathogen grew towards each other, but their growth stopped before their mycelia came in physical contact without any visible zone of inhibition or halo, the color of mycelia remain unaltered, and no sporulation of endophytic fungus could be seen. On the same WA medium, however, the endophytes demonstrated visible antagonistic inhibition against *T. roseum*, with the endophytes isolated from the apical and lateral buds of the plant demonstrating high inhibition effects. This pattern was similar on NA medium, where the endophytes more prominently inhibited *T. roseum* than *B. cinerea*. Interestingly, most of the fungal endophytes started sporulating copiously on NA when challenged with either one of the pathogenic strains (mainly against *T. roseum*), revealing in a typical fashion the unfavorable conditions for countering the confronting pathogen. When the endophytes were challenged by the pathogenic strains on PDA and MEA media, a capricious type of interacting features could be observed that accompanied the inhibitions. The visible interaction types between the endophytes and the pathogens on SA were similar to that on WA, but the antagonistic effect on both *B. cinerea* and *T. roseum* were more pronounced. Interestingly, the endophytic fungal strain A4 (*Paecilomyces lilacinus*) could completely inhibit the growth of the phytopathogen *B. cinerea* on all tested media, and of *T. roseum* on PDA and MEA along with prominent inhibition on SA, NA and WA. The endophyte strain T6 (*Penicillium sp.*) and L3 (*Penicillium copticola*) were also dominant antagonists of the tested pathogens on one or more media.

3.5. DISCUSSION

Over the last decades, endophytic microorganisms have garnered immense importance as valuable natural resources for imminent utilization in diverse areas such as agriculture and biotechnology (Aly et al. 2011; Rajulu et al. 2011; Kusari and Spiteller 2011; Li et al. 2012). A

number of bioprospecting strategies could be engaged in order to discover competent endophytes with desirable traits. For instance, endophytes could be isolated from randomly sampled plants from different population, or initially performing a detailed investigation of an ecosystem in order to determine its features with regard to its natural population of plant species, their relationship with the environment, soil composition, and biogeochemical cycles, followed by endophyte isolation and characterization (Debbab et al. 2012; Kusari and Spiteller 2012). Another approach could be to evaluate the evolutionary relatedness among groups of plants at a particular sampling site, correlating to species, genus, and populations, through morphological data matrices and molecular sequencing, followed by isolation of endophytes from the desired plants. Medicinal plants could also be bioprospected for endophytes, especially those plants capable of producing phytotherapeutic secondary metabolites (Aly et al. 2011; Debbab et al. 2012).

Herein we report for the first time, the isolation and incidence of endophytic fungi harbored in different tissues of *Cannabis sativa* L. plants. We used the bioprospecting rationale that *C. sativa* which contains a number of therapeutically relevant compounds including cannabinoids, might also harbor competent endophytes capable of providing fitness benefits to the host plant. Such benefits could encompass the endophytes producing a plethora of bioactive compounds, even the ones exclusive to the associated plant, thereby assisting in the chemical defense of the host against invading pathogens (Strobel and Daisy 2003; Strobel et al. 2004; Arnold et al. 2003; Rodriguez et al. 2004, 2008; Zhang et al. 2006; Gunatilaka 2006; Staniek et al. 2008; Suryanarayanan et al. 2009; Aly et al. 2010; Kharwar et al. 2011; Porras-Alfaro and Bayman, 2011; Debbab et al. 2012;).

However, random screening of endophytes in axenic cultures often leads to rediscovery of known natural products, with a very high possibility of the 'cryptic' bioactive molecules not produced under normal lab conditions (Bode et al. 2002; Scherlach and Hertweck 2009). Thus, in order to screen for the most promising endophytes, we estimated the potential of the isolated endophytic fungi as biocontrol agents by challenging them with two major fungal pathogens of the host plant, *Botrytis cinerea* and *Trichothecium roseum*. The isolated endophytic fungi were challenged by the host-specific phytopathogens on five different media, namely SA, MEA, PDA, WA and NA. The distinct types of inhibition representing the different types of antagonism (Trejo- Estrada et al. 1998; Chamberlain and Crawford 1999; Miles et al. 2012) we observed in our study revealed both the endophytic biodiversity of *C. sativa* and their potential chemodiversity in the form of producing a wide range (and/or number) of natural products with varying inhibitory activities under different media conditions. It has been well established that even slight variations in the *in vitro* cultivation conditions can

Table 3 Growth inhibition (% antagonism) of the phytopathogen *Botrytis cinerea* by isolated fungal endophytes of *C. sativa* on five different media after 15 days, and the respective endophyte-pathogen interaction types

| Endophyte strain number | Growth inhibition (% antagonism) on different media | | | | | Interaction type on different media (type code) | | | | |
|-------------------------|---|--------------------|----------------------------|-------------------------|-----------------|---|--------------------|----------------------------|-------------------------|-----------------|
| | Sabou raud agar (SA) | Nutrient agar (NA) | Potato dextrose agar (PDA) | Malt extract agar (MEA) | Water agar (WA) | Sabou raud agar (SA) | Nutrient agar (NA) | Potato dextrose agar (PDA) | Malt extract agar (MEA) | Water agar (WA) |
| L1 | 33 | 15 | 50 | 93 | 20 | IV | III,X | VI | III,VIII | III |
| L2 | 33 | 29 | 63 | 67 | 20 | I | III,X | VI,VIII | III,VIII | III |
| L3 | 100 | 100 | 38 | 100 | 60 | VII | VII,X | V,VIII | VII,VIII | III |
| L4 | 17 | 43 | 25 | 87 | 0 | I,VIII | III,X | VI | I,VIII | III |
| L5 | NI | 57 | 0 | 67 | NI | NA | II, X | I,IX | III,IX | NA |
| L6 | 67 | 57 | NI | 67 | 0 | III | VII,X | I,IX | III,IX | III |
| L7 | 17 | -29 | 25 | 47 | 0 | III | III | V,VIII | III, | III |
| L8 | -33 | 29 | 8 | 47 | 0 | III | III,XI(E) | I,VIII | I | III |
| T1 | 50 | 15 | 50 | 32 | 40 | III | III,X | VI | III,VIII | III |
| T2 | 20 | 0 | 50 | 73 | 20 | III | III | VI | III,VIII | III |
| T3 | 30 | 57 | 55 | 67 | -20 | III | III,X | VI | III,VIII | III |
| T4 | -3 | 29 | 88 | 87 | 0 | III | III | VI | III,VIII | III |
| T5 | -17 | 15 | 38 | 67 | 20 | III | III | VI | III,VIII | III |
| T6 | 67 | 100 | 50 | 100 | 100 | I,VIII | I,VII | V,VIII | VII,VIII | VII |
| A1 | 67 | 0 | 38 | 60 | 0 | III | III,XI(P) | IV,VIII, XI(P) | III, XI(P) | III |
| A2 | 67 | -43 | 48 | 73 | 0 | I | I,VIII, XI(P) | VI,VIII, XI(P) | I,XI(P) | III |
| A3 | NI | NI | NI | NI | NI | NA | NA | NA | NA | NA |
| A4 | 100 | 100 | 100 | 100 | 100 | VII | VII,X | VII | VII,VIII | VII |
| A5 | 33 | 40 | 63 | 67 | 40 | III | I,X | VI,VIII | IV | III |
| A6 | 50 | 29 | 50 | 67 | 0 | I | I | IV | I,VIII | III |
| A7 | 17 | 29 | 63 | 73 | -40 | III | I,X, XI(P) | V,VIII | I,VIII | III |
| A8 | 33 | 43 | 25 | 47 | 0 | III | III | VI,VIII | III,VIII | III |
| A9 | 17 | 29 | 38 | NI | 0 | III | III,VIII | IV,VIII | NA | iii |
| A10 | 33 | 29 | 38 | 67 | 40 | III,VIII | I | IV,VIII | I,VIII | III |
| A11 | 0 | 43 | 38 | 67 | 40 | III,VIII | I | VI,VIII | III,VIII | III |
| A12 | 17 | 15 | 0 | 47 | 0 | III,XI(E) | I | V,VIII | I | III,X |
| A13 | 17 | -20 | 13 | 53 | -20 | I | III,X | VI,VIII | I | III |
| A14 | 100 | -57 | 38 | 67 | 0 | VII | I | VI,VIII | III,VIII | III,X |
| A15 | 17 | NI | 38 | 80 | 40 | I | NA | V | III | I |
| A16 | 17 | 15 | 75 | 87 | 20 | I | III,XI(P) | I,VIII | III,VIII | III |

NI, pathogen not inhibited

NA, not applicable

Negative values represent endophyte inhibited by pathogen (%)

Table 4 Growth inhibition (% antagonism) of the phytopathogen *Trichothecium roseum* by isolated fungal endophytes of *C. sativa* on five different media after 15 days, and the respective endophyte-pathogen interaction types

| Endophyte strain number | Growth inhibition (% antagonism) on different media | | | | | Interaction type on different media (type code) | | | | |
|-------------------------|---|--------------------|----------------------------|-------------------------|-----------------|---|--------------------|----------------------------|-------------------------|-----------------|
| | Sabou raud agar (SA) | Nutrient agar (NA) | Potato dextrose agar (PDA) | Malt extract agar (MEA) | Water agar (WA) | Sabou raud agar (SA) | Nutrient agar (NA) | Potato dextrose agar (PDA) | Malt extract agar (MEA) | Water agar (WA) |
| L1 | 33 | 15 | 50 | 93 | 20 | IV | III,X | VI | III,VIII | III |
| L2 | 33 | 29 | 63 | 67 | 20 | I | III,X | VI,VIII | III,VIII | III |
| L3 | 100 | 100 | 38 | 100 | 60 | VII | VII,X | V,VIII | VII,VIII | III |
| L4 | 17 | 43 | 25 | 87 | 0 | I,VIII | III,X | VI | I,VIII | III |
| L5 | NI | 57 | 0 | 67 | NI | NA | II, X | I,IX | III,IX | NA |
| L6 | 67 | 57 | NI | 67 | 0 | III | VII,X | I,IX | III,IX | III |
| L7 | 17 | -29 | 25 | 47 | 0 | III | III | V,VIII | III, | III |
| L8 | -33 | 29 | 8 | 47 | 0 | III | III,XI(E) | I,VIII | I | III |
| T1 | 50 | 15 | 50 | 32 | 40 | III | III,X | VI | III,VIII | III |
| T2 | 20 | 0 | 50 | 73 | 20 | III | III | VI | III,VIII | III |
| T3 | 30 | 57 | 55 | 67 | -20 | III | III,X | VI | III,VIII | III |
| T4 | -3 | 29 | 88 | 87 | 0 | III | III | VI | III,VIII | III |
| T5 | -17 | 15 | 38 | 67 | 20 | III | III | VI | III,VIII | III |
| T6 | 67 | 100 | 50 | 100 | 100 | I,VIII | I,VII | V,VIII | VII,VIII | VII |
| A1 | 67 | 0 | 38 | 60 | 0 | III | III,XI(P) | IV,VIII, XI(P) | III, XI(P) | III |
| A2 | 67 | -43 | 48 | 73 | 0 | I | I,VIII, XI(P) | VI,VIII, XI(P) | I,XI(P) | III |
| A3 | NI | NI | NI | NI | NI | NA | NA | NA | NA | NA |
| A4 | 100 | 100 | 100 | 100 | 100 | VII | VII,X | VII | VII,VIII | VII |
| A5 | 33 | 40 | 63 | 67 | 40 | III | I,X | VI,VIII | IV | III |
| A6 | 50 | 29 | 50 | 67 | 0 | I | I | IV | I,VIII | III |
| A7 | 17 | 29 | 63 | 73 | -40 | III | I,X, XI(P) | V,VIII | I,VIII | III |
| A8 | 33 | 43 | 25 | 47 | 0 | III | III | VI,VIII | III,VIII | III |
| A9 | 17 | 29 | 38 | NI | 0 | III | III,VIII | IV,VIII | NA | iii |
| A10 | 33 | 29 | 38 | 67 | 40 | III,VIII | I | IV,VIII | I,VIII | III |
| A11 | 0 | 43 | 38 | 67 | 40 | III,VIII | I | VI,VIII | III,VIII | III |
| A12 | 17 | 15 | 0 | 47 | 0 | III,XI(E) | I | V,VIII | I | III,X |
| A13 | 17 | -20 | 13 | 53 | -20 | I | III,X | VI,VIII | I | III |
| A14 | 100 | -57 | 38 | 67 | 0 | VII | I | VI,VIII | III,VIII | III,X |
| A15 | 17 | NI | 38 | 80 | 40 | I | NA | V | III | I |
| A16 | 17 | 15 | 75 | 87 | 20 | I | III,XI(P) | I,VIII | III,VIII | III |

NI, pathogen not inhibited

NA, not applicable

impact the kind and range of secondary metabolites endophytes produce (Scherlach and Hertweck 2009; Kusari et al. 2012b). Recently for example, it was shown that the plant-associated *Paraphaeosphaeria quadrisepata* could start producing six new secondary metabolites when only the water used to make the media was changed from tap water to distilled water (Paranagama et al. 2007). Further, changing the medium from solid to liquid resulted in the production of radicicol instead of chaetochromin A by *Chaetomium chiversii* (Paranagama et al. 2007). Therefore, in order to verify this concept, known as OSMAC (Bode et al. 2002; Paranagama et al. 2007; Kusari et al. 2012b), we evaluated the different strategies that isolated endophytes employ against the competing pathogens on five different media. As expected, we observed a varying degree of antagonistic behavior and eleven distinct kinds of endophyte-pathogen interactions when the assays were performed on five different media. The results revealed that varying the media conditions indeed might have triggered the production of the 'cryptic' metabolites by the endophytes when challenged by the pathogens. Nevertheless, the different types and efficacies of pathogen inhibition might also be due to instability of the secondary metabolites or their reactive intermediates, a volatile nature of the compounds produced, or the compounds being produced in quantities below the minimum inhibitory concentrations (MIC) for counteracting the pathogens.

It is imperative that any plant-fungal interaction is always preceded by a physical encounter between a plant and a fungus, followed by several physical and chemical barriers that must be overcome to efficaciously establish a plant-endophyte association (Kusari et al. 2012b). It is mostly by chance encounters that particular fungi establish as endophytes for a particular ecological niche, or plant population, or plant tissue, either in a localized and/or systemic manner (Hyde and Soyong 2008). Thus, even a fungus that is pathogenic in one ecological niche can be endophytic to plant hosts in another ecosystem. It has been established for a plethora of fungi that pathogenic-endophytic lifestyles are interchangeable and are due to a number of environmental, chemical and/or molecular triggers (Schulz et al. 1999; Hyde and Soyong 2008; Eaton et al. 2011). Furthermore, groups of fungi containing large numbers of plant pathogenic species also contain large numbers of endophytic taxa. A vast majority of endophytes discovered so far are filamentous Ascomycota; this phylum comprises more than 3000 genera of mostly plant pathogens (Berbee 2001; Heckman et al. 2001; Mueller and Schmit 2007). Therefore, it is compelling that the diverse fungal isolates obtained from the tested *C. sativa* plants in the present work are selected towards coexistence with the hosts as endophytes. Interestingly for example, we found a number of *Penicillium* species exhibiting endophytic lifestyle in the associated *C. sativa* host plants (Table 1). Admittedly, only the 'cultivable' endophytic fungi could be isolated in this study and do not represent the non-culturable endophytic microorganisms of the sampled *C. sativa* plants. It should also be

mentioned here that 5.8S-ITS analysis can sometimes underestimate the endophytic fungal 'species diversity' (Gazis et al. 2011), and additional parameters should be coupled to ITS rDNA sequence data before fungal isolates can be referred at the 'species' level. Further, it is highly desirable to compare the obtained ITS sequences with those from type species, when available, in order to authenticate the tentative species identification (Ko et al. 2011). Thus, the ITS-based species identification concept may not be in full agreement with the current classical concepts of *Trichocomaceae*. Nevertheless, this work can serve as the handle for further studies (both ITS-based and different other methods) on endophytes of *Cannabis* bioprospected from different other populations, different collection centers, and wild populations (when accessible) for a landscape or global scale diversity analysis.

Taken together, our results firmly revealed that the endophytic fungi harbored in different tissues of the investigated *C. sativa* plants have great promise not only as biocontrol agents against the known and emerging phytopathogens of *Cannabis* plants, but also as a sustainable resource of biologically active novel secondary metabolites. Further, it would be interesting to compare our results (which were performed using *C. sativa* L. plants from Bedrocan BV) to those of *Cannabis* plants sampled from different wild and/or agricultural populations from different parts of the world. Using the cues from the results of the present work, we have now initiated the fermentation of the endophytes in the selective media, both under axenic conditions as well as in suitably devised cocultures with the challenging pathogens, for the discovery and structural elucidation of the bioactive compounds produced by the endophytes of this plant. This would then lead us towards further mass-balance studies and gene discovery, to cross-reference the biodiversity of these endophytic fungi to their actual biochemical potential. It would, thus, be possible to completely elucidate the chemical ecology of production of target and/or non-target molecules (quantitative) by these endophytes leading to the aforementioned 'interaction types' (qualitative) with the host-specific pathogens.

3.6. REFERENCES

- Ahmed SA, Ross SA, Slade D, Radwan MM, Zulfiqar F, ElSohly MA (2008) Cannabinoid ester constituents from high-potency *Cannabis sativa*. *J Nat Prod* 71:536–542
- Aly AH, Debbab A, Kjer J, Proksch P (2010) Fungal endophytes from higher plants: a prolific source of phytochemicals and other bioactive natural products. *Fungal Divers* 41:1-16
- Aly AH, Debbab A, Proksch P (2011) Fifty years of drug discovery from fungi. *Fungal Divers* 50:3-19
- Arabatzis M, Kambouris M, Kyprianou M, Chrysaiki A, Foustoukou M, Kanellopoulou M, Kondyli L, Kouppari G, Koutsia-Karouzou C, Lebessi E, Pangalis A, Petinaki E, Stathi A, Triikka-Graphakos E, Vartzioti E, Vogiatzi A, Vyzantiadis TA, Zerva L, Velegraki A (2011) Polyphasic identification and susceptibility to seven antifungals of 102 *Aspergillus* isolates recovered from immunocompromised hosts in Greece. *Antimicrob Agents Chemother* 55:3025-3030
- Arnold AE, Mejia LC, Kylo D, Rojas EI, Maynard Z, Robbins N (2003) Fungal endophytes limit pathogen damage in a tropical tree. *Proc Natl Acad Sci USA* 100:15649-15654
- Baker D, Pryce G, Giovannoni G, Thompson AJ (2003) The therapeutic potential of cannabis. *Lancet Neurol* 2:291-298
- Barloy J, Pelhate J (1962) Premières observations phytopathologiques relatives aux cultures de chanvre en Anjou. *Ann. Epiphyties* 13:117-149
- Berbee ML (2001) The phylogeny of plant and animal pathogens in the Ascomycota. *Physiol Mol Plant Pathol* 59:165-187
- Bode HB, Bethe B, Höfs R, Zeeck A (2002) Big effects from small changes: possible ways to explore nature's chemical diversity. *ChemBioChem* 3:619-627
- Botella L, Diez JJ (2011) Phylogenetic diversity of fungal endophytes in Spanish stands of *Pinus halepensis*. *Fungal Divers* 47:9-18
- Bush Doctor, The (1985) Damping Off. *Sinsemilla Tips* 5:35-39
- Bush Doctor, The (1993) How to preserve pot potency. *High Times* No 213: 75, 77-78
- Camargo JA (1992) Can dominance influence stability in competitive interactions? *Oikos* 64:605–609
- Chamberlain K, Crawford DL (1999) In vitro and in vivo antagonism of pathogenic turfgrass fungi by *Streptomyces hygroscopicus* strains YCED9 and WYE53. *J Ind Microbiol Biotechnol* 23:641-646
- Debbab A, Aly AH, Proksch P (2012) Endophytes and associated marine derived fungi-ecological and chemical perspectives. *Fungal Divers* 57:45-83
- Dewey LH (1914) "Hemp." in U.S.D.A. Yearbook 1913 United States Department of

- Agriculture, Washington DC: 283-347
- Eaton CJ, Cox MP, Scott B (2011) What triggers grass endophytes to switch from mutualism to pathogenism? *Plant Sci* 180:190-195
- Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acid Res* 32:1792-97.
- EISOhly MA, Slade D (2005) Chemical constituents of marijuana: the complex mixture of natural cannabinoids. *Life Sci* 78:539–548
- EISOhly MA, Wachtel SR, de Wit H (2003) *Cannabis* versus THC: response to Russo and McPartland. *Psychopharmacology* 165:433–434
- Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39:783-791
- Fischedick JT, Hazekamp A, Erkelens T, Choi YH, Verpoorte R (2010) Metabolic fingerprinting of *Cannabis sativa* L., cannabinoids and terpenoids for chemotaxonomic and drug standardization purposes *Phytochemistry* 71:2058–2073
- Fisher RA, Corbet AS, Williams CB (1943) The relation between the number of species and the number of individuals in a random sample of an animal population. *J Anim Ecol* 12:42-58
- Gazis R, Rehner S, Chaverri P (2011) Species delimitation in fungal endophyte diversity studies and its implications in ecological and biogeographic inferences. *Mol Ecol* 20:3001-3013
- Gomes A, Fernandes E, Lima JLFC, Mira L, Corvo ML (2008) Molecular mechanisms of anti-inflammatory activity mediated by flavonoids. *Curr Med Chem* 15:1586–1605
- Grotenhermen F, Müller-Vahl K (2012) The therapeutic potential of cannabis and cannabinoids. *Dtsch Arztebl Int* 109:495–501
- Gunatilaka AAL (2006) Natural products from plant-associated microorganisms: distribution, structural diversity, bioactivity, and implications of their occurrence. *J Nat Prod* 69:509–526
- Hamilton CE, Bauerle TL (2012) A new currency for mutualism? Fungal endophytes alter antioxidant activity in hosts responding to drought. *Fungal Divers* 54:39-49
- Hamilton CE, Gundel PE, Helander M, Saikkonen K (2012) Endophytic mediation of reactive oxygen species and antioxidant activity in plants: a review. *Fungal Divers* 54:1-10
- Han G, Feng X, Tian X (2011) Isolation and evaluation of terrestrial fungi with algicidal ability from Zijin Mountain, Nanjing, China. *Microbiology (Reading, Engl)*, in press
- Hazekamp A, Choi YH, Verpoorte R (2004b) Quantitative analysis of cannabinoids from *Cannabis sativa* using ¹H-NMR. *Chem Pharm Bull* 52:718-721

- Hazekamp A, Giroud C, Peltenburg A, Verpoorte R (2005) Spectroscopic and chromatographic data of cannabinoids from *Cannabis sativa*. J Liq Chrom Rel Technol 28: 2361-2382
- Heckman DS, Geiser DM, Eidell BR, Stauffer RL, Kardos NL, Hedges SB (2001) Molecular evidence for the early colonization of land by fungi and plants. Science 293:1129-1133
- Hockey JF (1927) Report of the Dominion field laboratory of plant pathology, Kentville Nova Scotia. Canada Department of Agriculture: 28-36
- Hoffman M, Gunatilaka M, Ong J, Shimabukuro M, Arnold AE (2008) Molecular analysis reveals a distinctive fungal endophyte community associated with foliage of Montane oaks in southeastern Arizona. J Arizona- Nevada Acad Sci 40:91–100
- Houbraken J, Frisvad JC, Samson RA (2011) Taxonomy of *Penicillium* section Citrina. Stud Mycol 70:53-138
- Hyde KD, Soyong K (2008) The fungal endophyte dilemma. Fungal Divers 33:163-173
- Jang Y, Huh N, Lee J, Lee JS, Kim GH, Kim JJ (2011) Phylogenetic analysis of major molds inhabiting woods and their discoloration characteristics Part 2. Genus *Penicillium* Holzforschung 65:265-270
- Jiang HE, Li X, Zhao YX, Ferguson DK, Hueber F, Bera S, Wang YF, Zhao LC, Liu CJ, Li CS (2006) A new insight into *Cannabis sativa* (Cannabaceae) utilization from 2500-year-old Yanghai Tombs, Xinjiang, China. J Ethnopharmacol 108:414-422
- Kharwar RN, Mishra A, Gond SK, Stierle D (2011) Anticancer compounds derived from fungal endophytes: their importance and future challenges. Nat Prod Rep 28:1208-1228
- Ko TWK, Stephenson SL, Bahkali AH, Hyde KD (2011) From morphology to molecular biology: can we use sequence data to identify fungal endophytes? Fungal Divers 50:113-120
- Kurup VP, Resnick A, Kagen SL, Cohen SH, Fink JN (1983) Allergenic fungi and actinomycetes in smoking materials and their health implications. Mycopathologia 82:61-64
- Kusari S, Hertweck C, Spiteller M (2012b) Chemical ecology of endophytic fungi: origins of secondary metabolites. Chem Biol 19:792-798
- Kusari S, Lamshöft M, Spiteller M (2009b) *Aspergillus fumigatus* Fresenius, an endophytic fungus from *Juniperus communis* L. Horstmann as a novel source of the anticancer pro-drug deoxypodophyllotoxin. J Appl Microbiol 107:1019-1030
- Kusari S, Lamshöft M, Zühlke S, Spiteller M (2008) An endophytic fungus from *Hypericum perforatum* that produces hypericin. J Nat Prod 71:159-162

- Kusari S, Spiteller M (2011) Are we ready for industrial production of bioactive plant secondary metabolites utilizing endophytes? *Nat Prod Rep* 28:1203-1207
- Kusari S, Spiteller M (2012) Metabolomics of endophytic fungi producing associated plant secondary metabolites: progress, challenges and opportunities. In *Metabolomics U*. Roessner ed. (InTech ISBN 978-953-51-0046-1):241-266
- Kusari S, Verma VC, Lamshöft M, Spiteller M (2012a) An endophytic fungus from *Azadirachta indica* A. Juss. that produces azadirachtin. *World J Microbiol Biotechnol* 28:1287-1294
- Kusari S, Zuehlke S, Spiteller M (2009a) An endophytic fungus from *Camptotheca acuminata* that produces camptothecin and analogues. *J Nat Prod* 72:2-7
- Kusari S, Zühlke S, Kosuth J, Cellarova E, Spiteller M (2009c) Light-independent metabolomics of endophytic *Thielavia subthermophila* provides insight into microbial hypericin biosynthesis. *J Nat Prod* 72:1825-1835
- Kusari S, Zühlke S, Spiteller M (2011) Effect of artificial reconstitution of the interaction between the plant *Camptotheca acuminata* and the fungal endophyte *Fusarium solani* on camptothecin biosynthesis. *J Nat Prod* 74:764-775
- Lamshead PJD, Hodda M (1994) The impact of disturbance on measurements of variability in marine nematode populations. *Vie et Milieu* 44: 21-27
- Lamshead PJD, Platt HM, Shaw KM (1983) Detection of differences among assemblages of marine benthic species based on an assessment of dominance and diversity. *J Nat Hist* 17:859-874
- Levitz SM, Diamond RD (1991) Aspergillosis and marijuana. *Ann Int Med* 115:578-579
- Li H-Y, Wei D-Q, Shen M, Zhou J-P (2012) Endophytes and their role in phytoremediation. *Fungal Divers* 54:11-18
- Margalef R (1958) Information theory in ecology. *Gen Syst* 3:36-71
- Márquez LM, Redman RS, Rodriguez RJ, Roossinck MJ (2007) A virus in a fungus in a plant: three-way symbiosis required for thermal tolerance. *Science* 315:513-515
- McAleece N, Gage JDG, Lamshead PJD, Paterson GLJ (1997) BioDiversity Professional statistics analysis software Jointly developed by the Scottish Association for Marine Science and the Natural History Museum London
- McPartland JM (1983) Fungal pathogens of *Cannabis sativa* in Illinois. *Phytopathology* 72: 797
- McPartland JM (1991) Common names for diseases of *Cannabis sativa* L. *Plant Dis* 75:226-7
- McPartland JM (1994) Microbiological contaminants of marijuana. *J Int Hemp Assoc* 1:41-44

- McPartland JM (1995c) *Cannabis* pathogens X: *Phoma*, *Ascochyta* and *Didymella* species. *Mycologia* 86:870-878
- McPartland JM (1996) A review of *Cannabis* diseases. *J Int Hemp Assoc* 3:19-23
- Miles LA, Lopera CA, Gonzá'lez S, Cepero de Garcí'a MC, Franco AE, Restrepo S (2012) Exploring the biocontrol potential of fungal endophytes from an Andean Colombian Paramo ecosystem. *BioControl*, in press, doi: 10.1007/s10526-012-9442-6
- Mojziso'va G, Mojzis J (2008) Flavonoids and their potential health benefits: relation to heart diseases and cancer. *Recent Prog Med Plants* 21:105–129
- Mueller GM, Schmit JP (2007) Fungal biodiversity: what do we know? What can we predict? *Biodivers Conserv* 16:1-5
- Paranagama PA, Wijeratne EMK, Gunatilaka AAL (2007) Uncovering biosynthetic potential of plant-associated fungi: effect of culture conditions on metabolite production by *Paraphaeosphaeria quadriseptata* and *Chaetomium chiversii*. *J Nat Prod* 70:1939–1945
- Pertwee RG (2006) Cannabinoid pharmacology: the first 66 years. *Br J Pharmacol* 147:163–171
- Porrás-Alfaro A, Bayman P (2011) Hidden fungi, emergent properties: Endophytes and microbiomes. *Annu Rev Phytopathol* 49:291-315
- Purahong W, Hyde KD (2011) Effects of fungal endophytes on grass and non-grass litter decomposition rates. *Fungal Divers* 47:1-7
- Radwan MM, Ross SA, Slade D, Ahmed SA, Zulfiqar F, ElSohly MA (2008) Isolation and characterization of new *cannabis* constituents from a high potency variety. *Planta Med* 74:267–272
- Rajulu MBG, Thirunavukkarasu N, Suryanarayanan TS, Ravishankar JP, Gueddari NEE, Moerschbacher BM (2011) Chitinolytic enzymes from endophytic fungi. *Fungal Divers* 47:43-53
- Rakeman JL, Bui U, Lafe K, Chen YC, Honeycutt RJ, Cookson BT (2005) Multilocus DNA sequence comparisons rapidly identify pathogenic molds. *J Clin Microbiol* 43:3324-3333
- Redman RS, Sheehan KB, Stout RG, Rodriguez RJ, Henson JM (2002) Thermotolerance conferred to plant host and fungal endophyte during mutualistic symbiosis. *Science* 298:1581
- Rodrigues A, Mueller UG, Ishak HD, Bacci MJr, Pagnocca FC (2011) Ecology of microfungal communities in gardens of fungus-growing ants (Hymenoptera: Formicidae): a year-long survey of three species of attine ants in Central Texas. *FEMS Microbiol Ecol* 78:244-255

- Rodriguez R, Redman R. (2008) More than 400 million years of evolution and some plants still can't make it on their own: plant stress tolerance via fungal symbiosis. *J Exp Bot* 59:1109-14
- Rodriguez RJ, Henson J, Van Volkenburgh E, Hoy M, Wright L, Beckwith F, Kim YO, Redman RS (2008) Stress tolerance in plants via habitat-adapted symbiosis. *ISME J* 2:404-16
- Rodriguez RJ, Redman RS, Henson JM (2004) The role of fungal symbioses in the adaptation of plants to high stress environments. *Mitig Adapt Strat Global Change* 9:261-72
- Russo EB, McPartland JM (2003) *Cannabis* is more than simply delta(9)-tetrahydrocannabinol. *Psychopharmacology (Berl)* 165:431-432
- Saitou N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406-425
- Sánchez Márquez S, Bills GF, Zabalgoitia I (2007) The endophytic mycobiota of the grass *Dactylis glomerata*. *Fungal Divers* 27:171-195
- Scherlach K, Hertweck C (2009) Triggering cryptic natural product biosynthesis in microorganisms. *Org Biomol Chem* 7:1753-1760
- Schulz B, Guske S, Dammann U, Boyle, C (1998) Endophyte-host interactions II. Defining symbiosis of the endophyte-host interaction. *Symbiosis* 25:213-227
- Schulz B, Roemmert, AK, Dammann U, Aust HJ, Strack, D (1999) The endophyte-host interaction: a balanced antagonism. *Mycol Res* 103:1275-1283
- Schwartz IS (1985) Marijuana and fungal infection. *Am J Clin Path* 84:256
- Simpson EH (1949) Measurement of diversity. *Nature* 163:688
- Sirikantaramas S, Taura F, Tanaka Y, Ishikawa Y, Morimoto S, Shoyama Y (2005) Tetrahydrocannabinolic acid synthase, the enzyme controlling marijuana psychoactivity, is secreted into the storage cavity of the glandular trichomes. *Plant Cell Physiol* 46:1578-1582
- Staniek A, Woerdenbag HJ, Kayser O (2008) Endophytes: Exploiting biodiversity for the improvement of natural product-based drug discovery. *J Plant Interact* 3:75-93
- Stone JK, Bacon CW, White JF (2000) An overview of endophytic microbes: endophytism. In: Bacon CW, White JF (ed) *Microbial endophytes*, New York Marcel Dekker Inc 2000: pp 3-30
- Strobel GA, Daisy B (2003) Bioprospecting for microbial endophytes and their natural products. *Microbiol Mol Biol Rev* 67:491-502

- Strobel GA, Daisy B, Castillo U, Harper J (2004) Natural products from endophytic microorganisms. *J Nat Prod* 67:257–268
- Suryanarayanan TS, Kumaresan V (2000) Endophytic fungi of some halophytes from an estuarine mangrove forest. *Mycol Res* 104:1465–1467
- Suryanarayananana TS, Thirunavukkarasub N, Govindarajulub MB, Sassecc F, Jansend, R, Murali TS (2009) Fungal endophytes and bioprospecting. *Fungal Biol Rev* 23:9–19
- Tamura K, Nei M, Kumar S (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc Natl Acad Sci USA* 101:11030–11035
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28:2731–2739
- Tao G, Liu ZY, Hyde KD, Liu XZ, Yu XN (2008) Whole rDNA analysis reveals novel and endophytic fungi in *Bletilla ochracea* (Orchidaceae). *Fungal Divers* 33:101–122
- Taura F, Morimoto S, Shoyama Y, Mechoulam R (1995b) First direct evidence for the mechanism of Δ^1 -tetrahydrocannabinolic acid biosynthesis. *J Am Chem Soc* 117:9766–9767
- Taylor DN, Wachsmuth IK, Shangkuan YH, Schmidt EV, Barrett TJ, Schrader JS, Scherach CS, McGee HB, Feldman RA, Brenner DJ (1982) Salmonellosis associated with marijuana. *New England J Med* 306:1249–1253
- Trejo-Estrada SR, Sepulveda IR, Crawford DL (1998) In vitro and in vivo antagonism of *Streptomyces violaceusniger* YCED9 against fungal pathogens of turfgrass. *World J Microbiol Biotechnol* 14:865–872
- Turner CE, Elsohly MA, Boeren EG (1980a) Constituents of *Cannabis sativa* L. XVII A review of the natural constituents. *J Nat Prod* 43:169–234
- Ungerlerder JT, Andrysiak T, Tashkin DP, Gale RP (1982) Contamination of marijuana cigarettes with pathogenic bacteria. *Cancer Treatment Rep* 66:589–590
- van der Werf HMG, van Geel WCA (1994) Vezelhenneep als papiergrondstof, teeltonderzoek 1987-1993 Fiber hemp as a raw material for paper, crop research 1987-1993. Report nr. 177, PAGV, Lelystad, the Netherlands, pp. 62
- van der Werf HMG, van Geel WCA, Wijlhuizen M (1995) Agronomic research on hemp (*Cannabis sativa* L.) in the Netherlands 1987-1993. *J Int Hemp Assoc* 2:14–17
- Vesterlund S-R, Helander M, Faeth SH, Hyvönen T, Saikkonen K (2011) Environmental conditions and host plant origin override endophyte effects on invertebrate communities structure and guilds. *Fungal Divers* 47:109–118

- Wachtel SR, ElSohly MA, Ross SA, Ambre J, de Wit H (2002) Comparison of the subjective effects of D9-tetrahydrocannabinol and marijuana in humans. *Psychopharmacology* 161:331–339
- Waller F, Achatz B, Baltruschat H, Fodor J, Becker K, Fischer M, Heier T, Hückelhoven R, Neumann C, von Wettstein D, Franken P, Kogel KH (2005) The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance and higher yield. *Proc Natl Acad Sci USA* 102:13386- 13391
- White TJ, Bruns TD, Lee S, Taylor JW (1990) Amplification and direct sequencing of fungal rRNA genes for phylogenetics In *Protocols: A guide to methods and applications*. PCR Academic press, San Diego pp. 315-322
- Whittaker RH (1977) Evolution of species diversity in land communities. *Evol Biol* 10:1-67
- Williamson EM, Evans FJ (2000) Cannabinoids in clinical practice. *Drugs* 60:1303–1314
- Yuan ZL, Zhang CL, Lin FC, Kubicek CP (2010) Identity, diversity, and molecular phylogeny of the endophytic mycobiota in the roots of rare wild rice (*Oryza granulate*) from a nature reserve in Yunnan, China. *Appl Environ Microbiol* 76:1642-1652
- Zhang HW, Song YC, Tan RX (2006) Biology and chemistry of endophytes. *Nat Prod Rep* 23:753–771

Chapter 4

QUORUM QUENCHING IS AN ANTIVIRULENCE STRATEGY EMPLOYED BY ENDOPHYTIC BACTERIA

Parijat Kusari, Souvik Kusari, Marc Lamshöft, Selahaddin Sezgin, Michael Spitteller, Oliver Kayser

PK, SK, MS and OK jointly conceived, planned and designed the study; PK performed all the experiments and analyzed the data; PK, ML and SS performed the analytical experiments, analyzed and interpreted the data; PK wrote the manuscript with inputs from all coauthors; SK supervised PK and maintained scientific surveillance; MS and OK oversaw the entire project as senior authors and supervisors of PK

Applied Microbiology and Biotechnology (2014) 98:7173-7183

4.1. ABSTRACT

Bacteria predominantly use quorum sensing to regulate a plethora of physiological activities such as cell-cell crosstalk, mutualism, virulence, competence, biofilm formation, and antibiotic resistance. In this study, we investigated how certain potent endophytic bacteria harbored in *Cannabis sativa* L. plants use quorum quenching as an antivirulence strategy to disrupt the cell-to-cell quorum sensing signals in the biosensor strain, *Chromobacterium violaceum*. We used a combination of high performance liquid chromatography high-resolution mass spectrometry (HPLC-ESI-HRMSⁿ) and matrix assisted laser desorption ionization imaging high-resolution mass spectrometry (MALDI-imaging-HRMS) to first quantify and visualize the spatial distribution of the quorum sensing molecules in the biosensor strain, *C. violaceum*. We then showed, both quantitatively and visually in high spatial resolution, how selected endophytic bacteria of *C. sativa* can selectively and differentially quench the quorum sensing molecules of *C. violaceum*. This study provides fundamental insights into the antivirulence strategies used by endophytes in order to survive in their ecological niches. Such defense mechanisms are evolved in order to thwart the plethora of pathogens invading associated host plants in a manner that prevents the pathogens from developing resistance against the plant/endophyte bioactive secondary metabolites. This work also provides evidence towards utilizing endophytes as tools for biological control of bacterial phytopathogens. In continuation, such insights would even afford new concepts and strategies in the future for combating drug resistant bacteria by quorum-inhibiting clinical therapies.

Keywords: Bacterial endophytes; quorum quenching; *N*-acylated homoserine lactones; *Cannabis sativa* L.; High-resolution mass spectrometry; MALDI imaging high-resolution mass spectrometry; microbe-microbe interaction; microbe-plant interaction; phytopathology

4.2. INTRODUCTION

Quorum sensing is one of the intrinsic chemical cell-to-cell signaling cascades in bacteria that facilitate invasion, colonization of particular niches, and pathogenesis of a plethora of organisms, ranging from unicellular prokaryotes to multicellular eukaryotes (Hosni et al. 2011). *N*-acylated L-homoserine lactones (AHLs) of Gram-negative bacteria and oligopeptides of Gram-positive bacteria are released as autoinducers to facilitate quorum sensing (LaSarre and Federle 2013). These in turn coordinate responses across a population to establish crosstalk, the most important being able to thwart chemical defenses (e.g. production of antibiotic compounds) of other organisms (Teplitski et al. 2011). Over the last decades, quorum sensing has progressively received attention in clinical studies owing to an increasing drug resistance in pathogenic bacteria that is a dreaded challenge in curing current and emerging life-threatening diseases. Therefore, alternate ‘antivirulence’ strategies are being sought to target quorum sensing in pathogenic bacteria (Burmolle et al. 2010; Amara et al. 2011; Claessen et al. 2014). Inhibition of quorum sensing in pathogenic bacteria, a process known as ‘quorum quenching’, has a fundamental advantage over other disease-management strategies (such as antimicrobial therapies) and opens new approaches to tackle drug resistant bacteria. Targeting quorum sensing in a pathogenic bacterial population mitigates virulence as opposed to suppressing bacterial growth and therefore, does not introduce any selective pressure for developing drug (or antibiotic) resistance (Clatworthy et al. 2007; Rasko and Sperandio 2010; LaSarre and Federle 2013).

Beyond the clinical setting, quorum sensing is also crucially relevant in microbe-microbe and plant-microbe crosstalk in almost all ecological niches (Safari et al. 2014). In the recent years, a promising group of microorganisms called ‘endophytes’ have garnered attention owing to their potential utility in the pharmaceutical and agricultural sectors (Porrás-Alfaro and Bayman 2011; Kusari and Spiteller 2011; Kharwar et al. 2011; Aly et al. 2013; Kusari et al. 2014). These diverse groups of microorganisms inhabit the internal tissues of plant without any manifestation of disease for a part of their life cycle and engage in multipartite interactions with other organisms (host plant, associated endophytes, invading pathogens, pests, and feeders). Such interactions lead to the development of different functional traits endophytes such as synthesizing bioactive secondary metabolites, controlling plant diseases by quorum quenching, and even aiding in plant tolerance towards environmental stress like drought and salinity (Kusari et al. 2012, 2013, 2014).

In this study, we quantified and visualized the distribution and modulation of cell-to-cell signaling communication system of the biosensor strain, *Chromobacterium violaceum*, and further quenching of the quorum coordination by potent endophytic bacteria harbored in

Cannabis sativa L. plants. *C. sativa* is a medicinal plant that contains pharmaceutically-relevant cannabinoids such as delta 9-tetrahydrocannabinol (Taura et al. 2007; Grotenhermen et al. 2012). Our earlier investigation of the endophytic fungal community of this plant with regard to their attack-defense ecological strategies against host plant-specific phytopathogens revealed their potential as biocontrol agents (Kusari et al. 2013). This further prompted us to investigate the endophytic bacterial community harbored in the same plant and their ecological roles in host-plant fitness. Using high performance liquid chromatography coupled to high-resolution mass spectrometry with electrospray ionization (HPLC-ESI-HRMSⁿ), we have proved that potent endophytic bacteria target and quench four different AHLs [*N*-hexanoyl-L-homoserine lactone (C6-HSL), *N*-octanoyl-L-homoserine lactone (C8-HSL), *N*-decanoyl-L-homoserine lactone (C10-HSL), and *N*-(3-oxo-decanoyl)-L-homoserine lactone (3-oxo-C10-HSL)] used by *C. violaceum* for violacein-mediated quorum sensing. We further used matrix assisted laser desorption ionization imaging high-resolution mass spectrometry (MALDI-imaging-HRMS) to show the spatial localization of each AHL by *C. violaceum* and the concomitant selective impediment of the AHLs by bacterial endophytes. MALDI-imaging mass spectrometry has gained impetus in natural products research for elucidating organismal interactions by visualizing the exact location of biosynthesis and distribution of target and non-target compounds in vitro and in vivo (Rompp et al. 2013; Shih et al. 2014; Bjarnholt et al. 2014). To the best of our knowledge, this is the first report of ‘visualizing’ quorum sensing and quorum quenching using MALDI-imaging high-resolution mass spectrometry.

4.3. MATERIALS AND METHODS

4.3.1. Collection and authentication of plant material

C. sativa L. plants were sampled from the Bedrocan BV Medicinal Cannabis (Veendam, Netherlands). The plants were identified and authenticated as *C. sativa* L. by experienced botanists at the Bedrocan BV. Plants specimens are under deposit at Bedrocan BV with voucher numbers (A1)05.41.050710. Import of the plant material was allowed according to the permission of the Federal Institute for Drugs and Medical Devices (Bundesinstituts für Arzneimittel und Medizinprodukte, BfArM), Bonn, Germany under the license number 458 49 89. The plant material was transported to TU Dortmund, Germany in sealed plastic zip-lock bags at 4°C and processed for the isolation of endophytes within 6 hours of plant collection.

4.3.2. Isolation and establishment of in vitro axenic cultures of endophytic bacteria

The plant materials were excised in small fragments (approx. 20 mm length) with the aid of flame-sterilized razor blade. The excised explants were washed thoroughly under running tap water followed by deionized water (DI) to remove any dirt attached to them. The explants were then surface sterilized following previously established procedures (Kusari et al. 2009a). Briefly, the small fragments were surface sterilized by sequential immersion in 70% ethanol for 1 min, 1.3 M sodium hypochlorite (3-5% available chlorine) for 3 min, and 70% ethanol for 30 s. Finally, these surface-sterilized tissue pieces were rinsed thoroughly in sterile, double-distilled water for a couple of minutes, to remove excess surface sterilants. The excess moisture was blotted on sterile filter paper. The surface-sterilized tissue fragments were placed in sterile mortar-pestle and crushed with the addition of sterile double-distilled water. The macerated tissues, thus obtained, were carefully plated on petri dishes containing Nutrient agar (NA). The petri dishes were sealed using parafilm and incubated at $28 \pm 2^\circ\text{C}$. To ensure proper surface sterilization, three different techniques were implemented. Firstly, the sterile double-distilled water of the final rinse were plated on NA and incubated in parallel under similar conditions. Secondly, the surface-sterilized tissue fragments were imprinted simultaneously in NA and incubated under similar conditions (secondary protocol, 'imprint technique') (Schulz et al. 1998; Sánchez Márquez et al. 2007). Finally, the unsterilized fragments (only washed in tap water followed by deionized water) were prepared simultaneously and incubated in parallel to isolate the surface-contaminating bacterial isolates and further differentiated by both macroscopic and microscopic evaluation (Kusari et al. 2009b). The cultures were monitored every day to check the growth of bacterial colonies. The bacterial colonies which grew on the plates after few days were subcultured

successively onto fresh NA plates and incubated at $28 \pm 2^\circ\text{C}$, and finally isolated as axenic strains. The endophytic bacterial isolates were routinely maintained on NA plates in active form and preserved in 15% (v/v) glycerol at -80°C for long-term storage. The endophytic bacterial isolates were assigned suitable strain designations (see Table 1) and were deposited in the internal culture collection of Technical Biochemistry, TU Dortmund, Germany.

4.3.3. Genomic DNA extraction, amplification of 16S rRNA gene and sequencing

A set of 500 mL capacity conical flasks, each with 100 mL autoclaved Nutrient broth (NB; Roth, Karlsruhe, Germany), were used for the isolation of genomic DNA of bacterial endophytes. A loop full of each bacterial isolate from the parent axenic culture was inoculated in the respective flasks containing NB and incubated at $28 \pm 2^\circ\text{C}$ with proper shaking (150 rpm) on a rotary shaker (INFORS HT Multitron 2, Einsbach, Germany) for 24-36 h depending on the growth kinetics of each endophytic bacterium. The growth kinetics was monitored by measuring the optical density at 600 nm (OD_{600}). The bacterial isolates were grown till the mid-log phase ('steady state') for extraction of genomic DNA. The total genomic DNA was extracted using peqGOLD bacterial DNA kit (Peqlab Biotechnologie GmbH, Erlangen, Germany) strictly following the manufacturer's guidelines. The DNA was then subjected to PCR amplification using the primers 27f and 1492r (Lane 1991).

The PCR amplification was performed in a 50 μL reaction mixture containing 10 μL Phusion HF buffer (5X), 1 μL dNTPs (10 mM), 0.5 μL forward primer (100 μM), 0.5 μL reverse primer (100 μM), 3 μL of template DNA, 1 μL of Phusion polymerase (2U/ μL ; Fermentas, Thermo Scientific, Schwerte, Germany), and 34 μL of sterile double-distilled water. The PCR cycling conditions consisted of an initial denaturation at 98°C for 3 min, 30 cycles of denaturation, annealing and elongation at 98°C for 10 sec, 60°C for 30 sec and 72°C for 45 sec. This was followed by a final elongation step of 72°C for 10 min. As a negative control, the template DNA was replaced by sterile double-distilled water. The PCR amplified products obtained were approximately around 1500 bp (base pairs) and were visualized by gel electrophoresis. The products were further purified using peqGOLD micro spin cycle pure kit (Peqlab Biotechnologie GmbH, Erlangen, Germany) strictly following manufacturer's instructions. The amplified products were then sequenced from both directions at GATC Biotech (Cologne, Germany) using the above mentioned primers. The sequences of all the endophytic bacteria under this study have been deposited at the EMBL-Bank under the accession numbers HG424705 to HG424717 (see Table 1).

Table 1 16S rRNA based identification of bacterial endophytes isolated from *Cannabis sativa* L. plants with their respective strain codes and the EMBL-Bank accession numbers

| Strain number (endophyte) | EMBL-Bank Accession number | Most closely related species (accession number) | Maximum identity |
|---------------------------|----------------------------|---|------------------|
| B1 | HG424705 | <i>Bacillus licheniformis</i> (AB055006.1) | 99% |
| B2 | HG424706 | <i>Bacillus licheniformis</i> (KF040981.1) | 99% |
| B3 | HG424707 | <i>Bacillus</i> sp. (JQ808527.1) | 99% |
| B4 | HG424708 | <i>Bacillus megaterium</i> (KC443085.1) | 99% |
| B5 | HG424709 | <i>Bacillus pumilus</i> (EU500930.1) | 99% |
| B6 | HG424710 | <i>Bacillus licheniformis</i> (KF148636.1) | 97% |
| B7 | HG424711 | <i>Bacillus pumilus</i> (JQ798393.1) | 98% |
| B8 | HG424712 | <i>Brevibacillus borstelensis</i> (JQ229800.1) | 98% |
| B9 | HG424713 | <i>Bacillus</i> sp. (JQ678041.1) | 99% |
| B10 | HG424714 | <i>Bacillus subtilis</i> (JX123316.1) | 99% |
| B11 | HG424715 | <i>Bacillus</i> sp. (FJ908092.1) | 99% |
| B12 | HG424716 | <i>Mycobacterium peregrinum</i> (JX266704.1) | 99% |
| B13 | HG424717 | <i>Mycobacterium</i> sp. (HE575961.1) | 99% |

4.3.4. Preparation of cell free supernatant (CFS)

The CFS was prepared according to previously established methods, suitably modified (Ou et al. 2009; Rishi et al. 2011; Zhao et al. 2011). A set of conical flasks of 300 mL capacity, each with 50 mL autoclaved NB, was used. Each bacterial isolate was inoculated in respective flasks from their parent axenic cultures and incubated at $28 \pm 2^\circ\text{C}$ with proper shaking (150 rpm) on a rotary shaker (INFORS HT Multitron 2, Einsbach, Germany) till the mid-log phase. The bacterial cultures were centrifuged at 10,000 rpm for 30 min at 4°C . For the preparation of CFS, the supernatant was separated from the cell pellets and filtered twice

through sterile 0.22 µm Rotilabo®-Spritzenfilter (Carl Roth GmbH, Karlsruhe, Germany) for the complete removal of cells. The CFS of each endophytic bacterial isolate was serially diluted to a factor of 10^{-4} to 10^{-8} , and 100 µL were spread on NA plates to check for any bacterial contamination.

4.3.5. Quorum sensing/quenching activity of the endophytic isolates

The type strain *C. violaceum* (accession number DSM 30191) was obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. The activation of the bacterial strain was performed according to DSMZ guidelines. The medium used for the activation was Nutrient broth (NB; Roth, Karlsruhe, Germany). *C. violaceum* was routinely maintained on NA and cultured aerobically in NB at 30°C with proper shaking (150 rpm). To determine the quorum sensing and/or quenching responses of the endophytic bacterial isolates, spectrophotometric quantitation of violacein was analyzed according to previously established procedure, suitably modified (Limsuwan et al. 2008; Thenmozhi et al. 2009). *C. violaceum* was grown overnight in NB till OD_{600} of 0.1 was achieved. A set of conical flasks of 300 mL capacity, each with 10 mL NB seeded with 1 mL overnight culture (OD_{600} of 0.1) of *C. violaceum*, was used. The conical flasks contained 1 mL, 2 mL, 4 mL and 8 mL of CFS, respectively. As a control, equal volumes of NB were added to the respective control flasks. All the flasks were incubated overnight at 30°C with proper shaking (150 rpm). 1 mL culture from each of the flasks was centrifuged at 13,000 rpm for 10 min for precipitation of insoluble violacein. 1 mL dimethyl sulfoxide (DMSO) was added to the cell pellets and vortexed vigorously for 1 min to solubilize the violacein completely. The solution was centrifuged again at 13,000 rpm for 5 min to remove the cell debris. The supernatant was then quantified spectrophotometrically at OD_{585} . Each setup was prepared in triplicates (biological replicates) and the quantitation was repeated thrice (technical replicates). 1 mL culture from each of the setups was serially diluted to a factor of 10^{-4} to 10^{-8} , and 100 µL was spread on NA plates to check for any bacterial contamination.

Furthermore, a suitably modified colony forming units (CFU/mL) assay (Choo et al. 2006) was also performed to confirm that the CFS extracts significantly reduced only the violacein production but did not have any effect on the growth of *C. violaceum*. Briefly, 1 mL culture from each of the experimental setups (CFS treated) and control (untreated) was serially diluted from 10^{-4} to 10^{-8} . 10 µL (at dilution of 10^{-8}) was spread on NA plates and incubated overnight at 30°C. Each cell count was repeated twice.

4.3.6. Sample preparation for analytical measurements

The endophytic isolates showing quorum quenching responses in the flask assay were further analyzed via high performance liquid chromatography high-resolution mass spectrometry (LC-FTMSⁿ). A set of conical flasks of 500 mL capacity, each with 200 mL NB and 1 mL overnight culture (OD₆₀₀ of 0.1) of *C. violaceum*, was used. CFS of the isolates showing quorum quenching responses was added to the respective flasks. NB was used as a control. The flasks were incubated overnight at 30°C with proper shaking (150 rpm). The cultures were centrifuged at 13,000 rpm for 30 min. 10 µL of internal standard d₃-C6-HSL (0.1 mg/mL) was added to the spent supernatant and extracted twice with an equal volume of ethyl acetate. The organic extracts were evaporated to vacuum and reconstituted in 1 mL HPLC-grade methanol. The methanolic extracts were subjected to mass spectrometry. The *C. violaceum* culture alone (with NB instead of CFS) was extracted as a positive control. The NB alone was extracted as a negative control. The mass spectrometric analysis was performed in duplicates.

4.3.7. Preparation of standards

All standard compounds C6-HSL, C8-HSL, 3-oxo-C10-HSL and C10-HSL and the internal standard d₃-C6-HSL were purchased from Sigma-Aldrich, Steinheim, Germany and were dissolved in HPLC-grade methanol at a concentration of 0.1 mg/mL. Calibration standards (2, 5, 20, 50, 200, 500, 2000 and 5000 ng/mL) were prepared by serial dilution of the stock solutions containing in addition, an absolute 100 ng of the internal standard in methanol/water (1:4).

3.8. High-resolution mass spectrometry

The high-resolution mass spectra were obtained with an LTQ-Orbitrap Spectrometer (Thermo Fisher, MA, USA) equipped with a HESI-II source. The spectrometer was operated in positive mode (1 spectrum s⁻¹; mass range: 180-600) with nominal mass resolving power of 60,000 at *m/z* 400 with a scan rate of 1 Hz) with automatic gain control to provide high-accuracy mass measurements within 2 ppm deviation using an internal standard; Bis (2-ethylhexyl) phthalate: *m/z* = 391.284286. The spectrometer was attached with an Agilent (Santa Clara, USA) 1200 HPLC system consisting of LC-pump, PDA detector (λ = 205 nm), auto sampler (injection volume 10 µL) and column oven (30 °C). Following parameters were used for experiments: spray voltage 5 kV, capillary temperature 260 °C, tube lens 65 V. Nitrogen was used as sheath gas (50 arbitrary units) and auxiliary gas (5 arbitrary units). MS/MS experiments were performed by CID (collision induced decay, 35 eV) mode. Helium served as the collision gas. The separations were performed by using a Macherey-Nagel

Nucleodur Gravity C18 column (50 x 2 mm, 1.8 μm particle size) with a H_2O (+ 0.1% HCOOH) (A) / acetonitrile (+ 0.1% HCOOH) (B) gradient (flow rate 300 $\mu\text{L min}^{-1}$). Samples were analyzed by using a gradient program as follows: 80% A isocratic for 2 min, linear gradient to 90% B over 12 min, after 100% B isocratic for 3.5 min, the system returned to its initial condition (80% A) within 0.5 min, and was equilibrated for 6 min. The quantitation of the compounds was achieved by extraction of accurate masses (max. deviation 2 ppm) of their quasi-molecular ions $[\text{M}+\text{H}]^+$. An internal standard was used for calibration, since matrix effects could significantly influence the results of the MS measurements. The calibration graph was linear from a concentration of 2 ng/mL to 5000 ng/mL. The quantitation was achieved by plotting the ratio of the analyte signal to the internal standard signal as a function of the analyte concentration of the standards.

4.3.9. Sample preparation for AP-MALDI imaging

The biosensor strain (*C. violaceum*) was inoculated onto a thin NA layer plated over glass slides. After 24 h incubation at 30°C, a DHB (2, 5-dihydroxybenzoic acid) matrix layer was applied on the biosensor colony through a new sprayer (TransMIT GmbH, Giessen - a brand name not relayed yet) for detection of C6-HSL. DHB (7 mg/mL in 50% acetone, 49.9% H_2O and 0.1% formic acid) was sprayed with the parameters 15.0 $\mu\text{L min}^{-1}$ matrix flow, 4.5 L min^{-1} nitrogen gas flow, 100 rpm sample platform drive with 40 min spray duration time. The sample was then subsequently measured. Images of the biosensor were taken with an optical microscope (Leica Microsystems, Wetzlar, Germany).

For measurement of C8-HSL, C10-HSL, and 3-oxo-C10-HSL, α -cyano-4-hydroxycinnamic acid (HCCA; 7 mg/mL in 50% acetonitrile, 49.8% H_2O and 0.2% trifluoroacetic acid) was sprayed with the parameters 15.0 $\mu\text{L min}^{-1}$ matrix flow, 3.5 L min^{-1} nitrogen gas flow, 100 rpm sample platform drive for 2 x 20 min spray duration time. The sample was then air-dried and subsequently measured. Images of the biosensor were taken with an optical microscope (Leica Microsystems, Wetzlar, Germany).

4.3.10. AP-MALDI imaging high-resolution mass spectrometry

Imaging experiments were performed with a Q-Exactive mass spectrometer (Thermo Fisher Scientific GmbH, Bremen, Germany) coupled to an atmospheric pressure (AP) MALDI ion source imagine10 (TransMIT GmbH, Giessen, Germany) using the Full-Scan mode (positive mode) within the mass range of m/z 100 – 400 ($R = 70000 @ m/z 200$) with the internal lock masses of 273.03936 $[\text{2M}-2\text{H}_2\text{O}+\text{H}]^+$ (DHB) and 379.09246 $[\text{2M}+\text{H}]^+$ (HCCA), respectively. Acceleration voltage was set at 4 kV. A pulsed nitrogen laser (λ : 337.1 nm) at a frequency of

60 Hz with pulse duration times of 500 ms was used for generating UV radiation. Laser attenuation was set at 20°C. Scan resolution was set at 40 μm and 100 μm .

Mirion (v.2.1.4.411) (TransMIT GmbH, Giessen, Germany) mass spectrometry imaging software was used to create images from the obtained data/pixels with mass information. False colors were attached to masses with the corresponding pixel.

4.3.11. Sample preparation for UV-MALDI imaging

The biosensor strain (*C. violaceum*) was inoculated onto a thin NA layer plated over glass slides supplemented with various endophyte CFS concentrations. After 24 h incubation at 30°C, a matrix layer (HCCA) was applied on the biosensor (colony) through a sprayer ImagePrep (Bruker Corporation, Bremen, Germany). HCCA (7 mg/mL in 50% acetonitrile, 49.8% H₂O and 0.2% trifluoroacetic acid) was sprayed 3 times. Sample wetness, matrix thickness and incubation time were set at 4 (medium) on a relative scale from 1 to 7. Spray duration time was approximately 40 min for each spray cycle. The sample was then queued for subsequent measurement. Images of the biosensor challenged by endophyte CFS were documented with an optical microscope (Leica Microsystems, Wetzlar, Germany).

4.3.12. UV-MALDI imaging high-resolution mass spectrometry

Imaging experiments were performed with a LTQ Orbitrap XL mass spectrometer coupled to a MALDI ion source (both from Thermo Fisher Scientific GmbH, Bremen, Germany) using the Full-Scan mode (positive mode) within the mass range of m/z 100 – 400 ($R = 60000 @ m/z$ 200) with the internal lock masses of 273.03936 $[2M-2H_2O+H]^+$ (DHB) and 379.09246 $[2M+H]^+$ (HCCA), respectively. A pulsed nitrogen laser (λ : 337.1 nm) was used for generating UV radiation. The sample was rastered with 1 microscan per step at a laser energy adjusted at 20 μJ . The scan resolution was set at 100 μm .

ImageQuest (v.1.0.1) (Thermo Fisher Scientific, Bremen, Germany) mass spectrometry imaging software was used to create images from the obtained data/pixels with mass information. False colors were attached to masses with the corresponding pixel.

4.4. RESULTS

4.4.1. Identification and characterization of the endophytic bacterial isolates

A total of 13 endophytic bacterial isolates were isolated from the *Cannabis sativa* L. plants sampled from Bedrocan BV Medicinal Cannabis (Veendam, the Netherlands). The isolates were identified and characterized by molecular identification based on 16S rRNA analysis.

The amplified sequences spanning around 1500 bp were used for the identification of the bacterial isolates. The PCR amplified sequences were matched against the nucleotide database of the US National Centre for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLASTn) for the identification of the bacterial isolates. The sequences were aligned using the EMBOSS-Pairwise Sequence Alignment of the EMBL Nucleotide Database. The sequences of the endophytic bacterial isolates have been deposited at EMBL-Bank. The identities of the endophytes were considered conspecific only at a minimum threshold identity of $\geq 98\%$ with the exception of only one isolate with 97% sequence similarity. The accessions numbers with sequence similarities are detailed in Table 1.

4.4.2. Spectrophotometric quantitation of quorum quenching behaviour

C. violaceum is a Gram-negative biosensor strain known to produce violacein, a purple pigment, as a result of quorum sensing utilizing the CviI/CviR synthase-receptor signaling (McClellan et al. 1997). Loss of this pigment is indicative of quorum quenching behavior. For the preliminary selection of endophytic bacterial strains capable of quorum quenching, spectrophotometric quantitation of violacein was performed (Choo et al. 2006) using the cell free supernatants (CFS) of the isolated endophytes. Four of the total isolated endophytic bacteria (*Bacillus* sp. strain B3, *Bacillus megaterium* strain B4, *Brevibacillus borstelensis* strain B8, and *Bacillus* sp. strain B11) exhibited significant potential in weakening the cell-to-cell quorum signals (C6-HSL, C8-HSL, C10-HSL, and 3-oxo-C10-HSL) of *C. violaceum* in a concentration-dependent manner (Fig. 1c-f). Furthermore, the CFU count assay confirmed that the CFS of isolates B3, B4, B8 and B11 did not impose any effect on the growth of *C. violaceum* but only reduced the violacein production (Fig. 1k). The logarithmic values of the bacterial count per mL (with replicates of each count) when treated with 8 mL CFS of each of the 4 bacterial isolates in comparison to control (untreated *C. violaceum*) is presented in Fig. 1k.

4.4.3. Quantification of quorum quenching behavior using high performance liquid chromatography high-resolution mass spectrometry (HPLC-ESI-HRMSⁿ)

The four strains (B3, B4, B8 and B11) were further analyzed in detail by LC-HRMS/MS using both external reference standards and an internal standard (d3-C6-HSL) to quantify the exact amount of each of the AHLs being selectively quenched (Fig. 1a,b). In particular, the quorum sensing in *C. violaceum* was quenched by all the four AHLs in different concentrations: the highest concentration was that of C10-HSL (2088 ng/mL), followed by C8-HSL (320 ng/mL), 3-oxo-C10-HSL (7.1 ng/mL) and finally, C6-HSL (10.4 ng/mL) (Fig. 1g-j). Although C10-HSL

was produced in higher concentration than C6-HSL signifying both CviR/C10-HSL- and CviR/C6-HSL-mediated regulation of signaling in this particular strain of *C. violaceum*, selective quorum quenching by the bacterial endophytes (Fig. 1g-j) corroborated earlier observations that C6-HSL is the primary and limiting autoinducer in *C. violaceum* (McClellan et al. 1997). For example, the endophyte strain B11 quenched all the AHLs produced by the biosensor except C10-HSL (Fig. 1g-j). Interestingly, C6-HSL was quenched to less than half the concentration produced in the biosensor strain under control conditions (Fig. 1g) and concomitantly, there was a striking increase in the production of C10-HSL by the biosensor when challenged by endophyte strain B11. The same trend was also observed for strains B3, B4 and B8 (Fig. 1g-j).

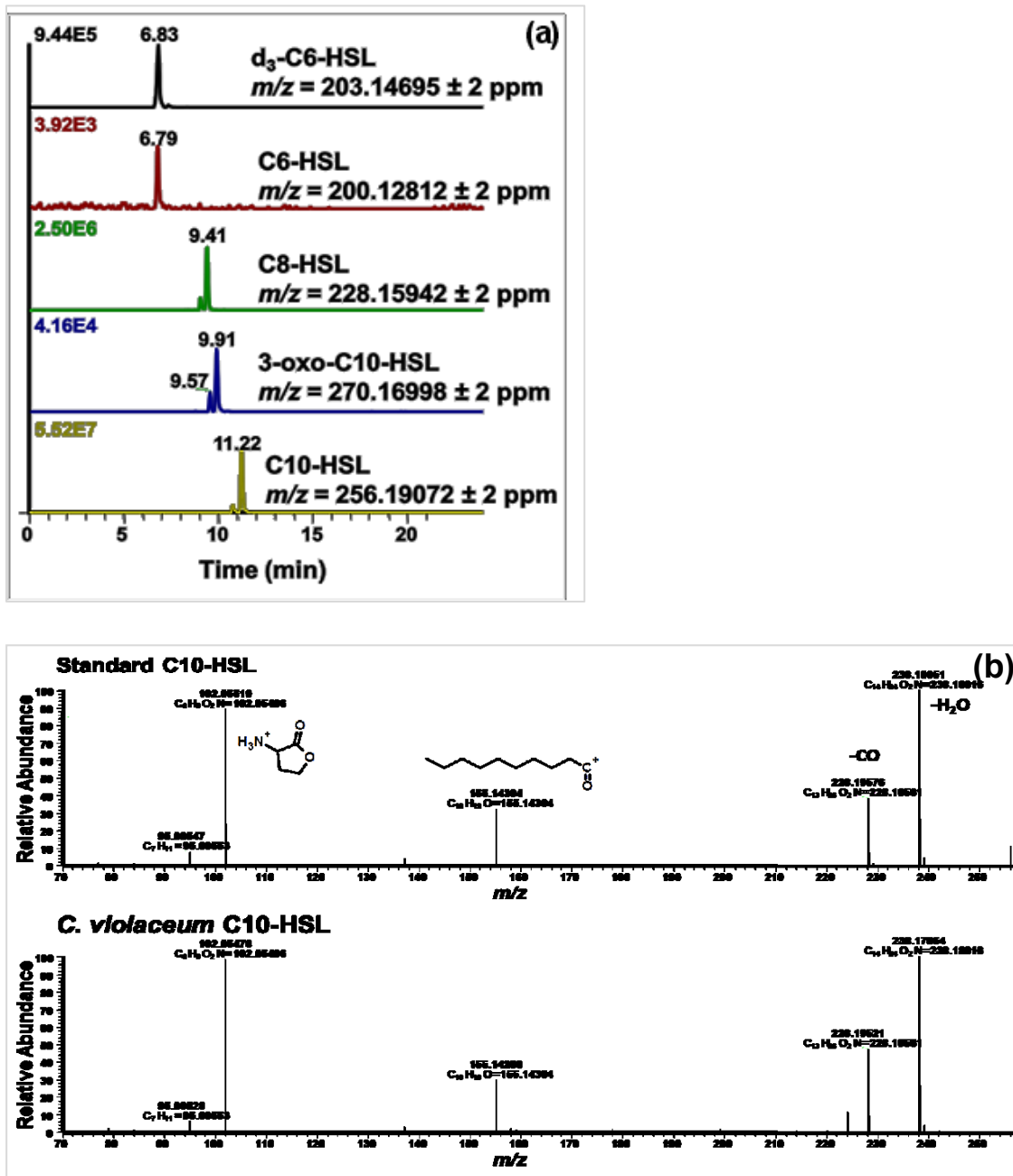


Fig. 1 Quorum quenching of AHLs produced by biosensor strain *C. violaceum* by potent bacterial endophytes of *C. sativa* L. plants. **(a)** Representative LC-FTMS extracted ion chromatograms of detected AHLs (of control) quenched by CFS of endophytic bacterial strains (shown for strain B4). The ions monitored are displayed in each trace and correspond to the most abundant protonated molecules $[M+H]^+$ using a maximum deviation of 2 ppm **(b)** Representative MS/MS spectra showing comparison of the main detected component, C10-HSL (m/z 256.1) in authenticated standard (top) and in the control biosensor strain (bottom).

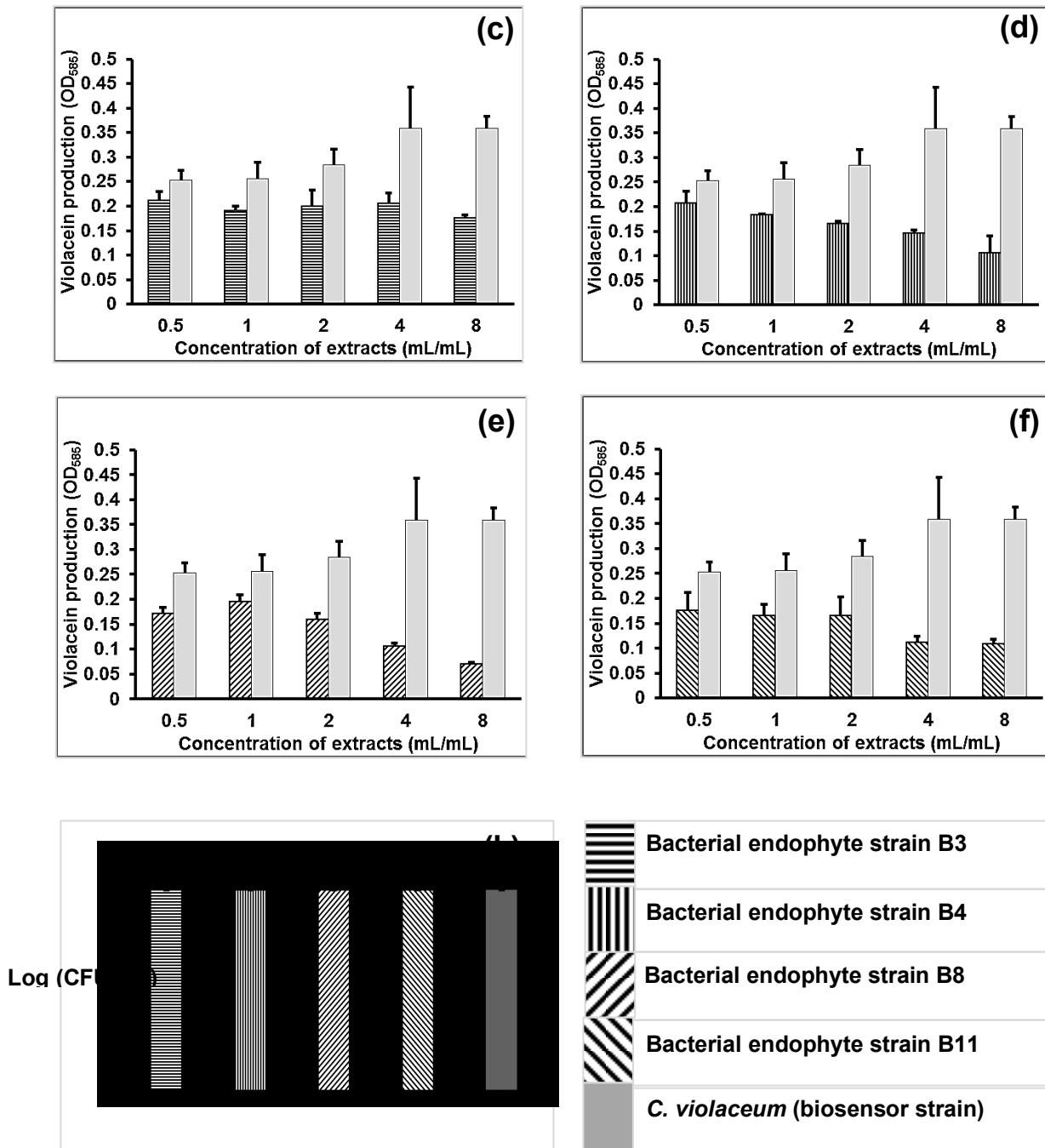


Fig. 1 Quorum quenching of AHLs produced by biosensor strain *C. violaceum* by potent bacterial endophytes of *C. sativa* L. plants. **(c-f)** Quorum quenching responses of the CFS of four endophytic bacterial strains (B3, B4, B8, and B11) against the biosensor strain *C. violaceum* (control). **(g)** Comparison of bacterial cell count of *C. violaceum* when treated with CFS extracts and when untreated (control). All the data represent the logarithmic value of CFU/mL (\pm SD).

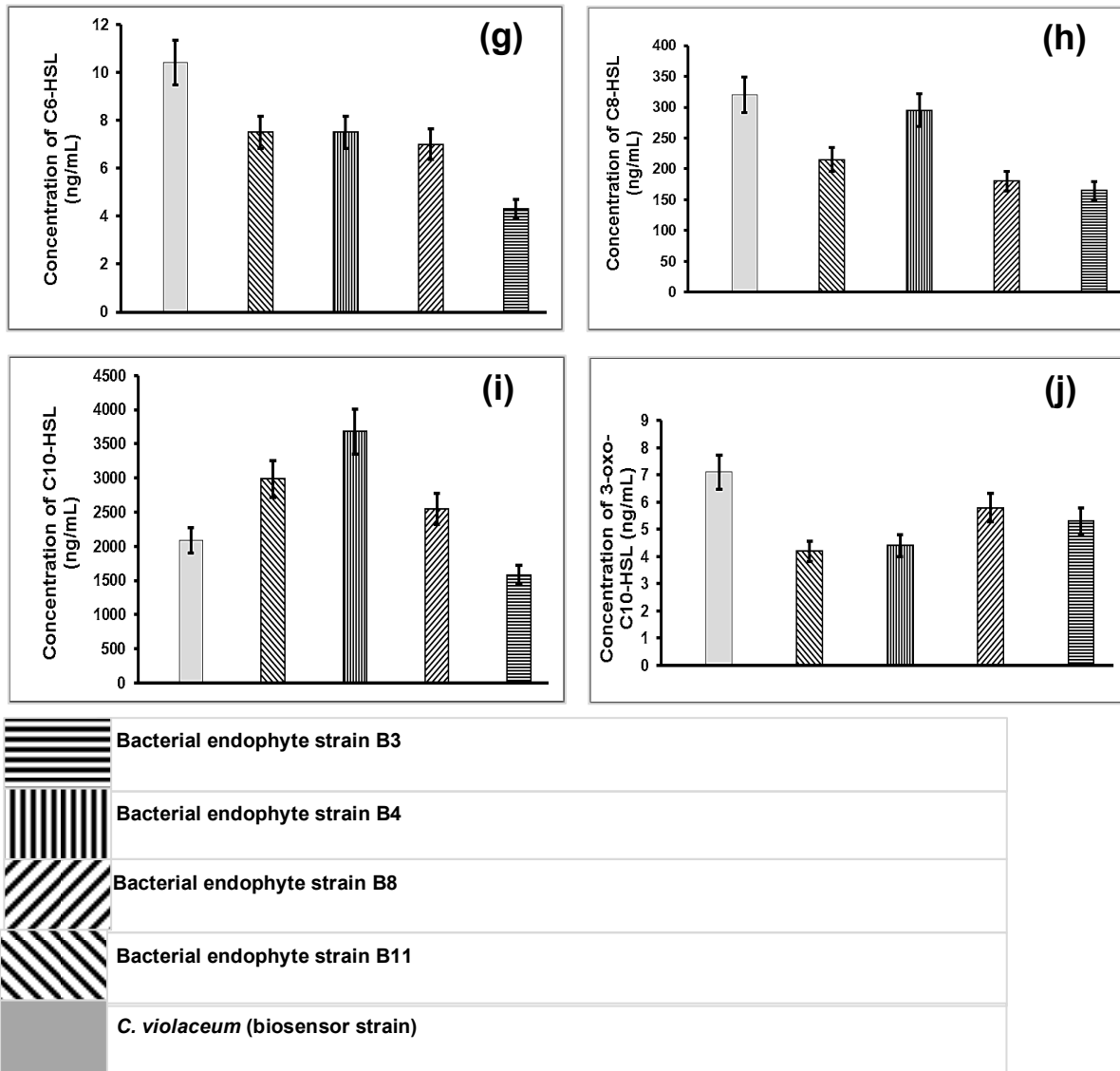


Fig. 1 Quorum quenching of AHLs produced by biosensor strain *C. violaceum* by potent bacterial endophytes of *C. sativa* L. plants. **(g-j)** Concentration of the different AHLs (ng/mL) quenched by the CFS extracts of the four endophytic bacterial strains B3, B4, B8, and B11. Control represents the biosensor strain *C. violaceum*

4.4.4. Visualization of spatial distribution of quorum sensing signals and their quenching by endophytes using MALDI-imaging-HRMS

Since the production and release of AHLs range from intercellular to intracellular or extracellular, we investigated the spatial localization and distribution of the four AHLs in *C. violaceum* colony and periphery, and their quenching by endophytes using MALDI-Q-Exactive and MALDI-LTQ Orbitrap XL instruments. The resulting signal intensity was made visible by a color coding system (Fig. 2) ranging from dark red (low intensity) to bright red (high intensity). Mass window was deliberately kept very small at $\Delta \leq 2$ ppm from the theoretical masses for all the four AHLs measured. The untreated (not challenged by endophytes) *C. violaceum* showed visible production of violacein under the microscope (Fig. 2a, d), which was further sprayed with suitable matrix and shot by controlled laser beam. C6-HSL was produced by *C. violaceum* colony and released into the agar (Fig. 2c). The other three AHLs, namely C8-HSL (Fig. 2f), C10-HSL (Fig. 2g), and 3-oxo-C10-HSL (Fig. 2h) were not distributed far from the producing cells and only accumulated in the nearest vicinity. In fact, these three AHLs accumulated directly below the colony (visualized after scraping off a part of the colony; see Fig. 2d grey dotted box). When *C. violaceum* was grown in agar containing the extracts of bacterial endophytes B4, B8, B11 and B3 at similar concentrations as that used in the violacein assay (see Fig. 1) for each AHL, selective quorum quenching was observed (Fig. 2j-m). The C6-HSL released profusely into the agar by *C. violaceum* was quenched by CFS of the endophytic bacterial isolates and only the remnants after being quenched were observed (Fig. 2j). Interestingly, C10-HSL and 3-oxo-C10-HSL were not only selectively quenched but also observed in the agar in the vicinity of the *C. violaceum* colony after quenching.

4.5. DISCUSSION

In this study, we used a combination of high performance liquid chromatography high-resolution mass spectrometry (HPLC-ESI-HRMSⁿ) and matrix assisted laser desorption ionization imaging high-resolution mass spectrometry (MALDI-imaging-HR-MS) to quantify and visualize the spatial distribution of cell-to-cell quorum sensing signals in the biosensor strain, *Chromobacterium violaceum*. We further investigated the quenching of the quorum sensing signals by potent endophytic bacterial isolates of medicinally important *C. sativa* plants. Our preliminary analysis of violacein production by *C. violaceum* and further quenching by selected bacterial isolates prompted us to study the specific modulation of the different AHLs conveying quorum sensing in *C. violaceum*. A lot of research in the recent past has focused on different aspects of targeting the AHL signaling cascades for cell-to-cell communication in various microbial systems (Galloway et al. 2011; O'Loughlin et al. 2013).

The implementation of MALDI-MS techniques has also gained importance in various aspects of research on microbial crosstalk. Recent studies have highlighted the use of such techniques in identification of microorganisms, clinical microbiology and natural product biochemistry (Shih et al. 2014).

The four endophytic isolates showing potent quenching capability in the violacein assay were further analyzed using LC-HRMS/MS using both external reference standards and an internal standard. This provided a comprehensive understanding of the correlation between the endophytic bacterial species and their species-specific and selective ability of modulating different AHLs at different concentrations leading to an overall intervention of *C. violaceum* signaling cascade. Interestingly, we observed a trend of decreasing concentration of C6-HSL with an increase in the production of C10-HSL when treated with the CFS of endophytic bacterial isolates. This exemplified the fact that in *C. violaceum*, C10-HSL-mediated violacein production and transcription of *vioA* is inhibited by C6-HSL, as suggested by Morohoshi et al. (2010). It is further conceivable that *C. violaceum* triggered an increased production of C10-HSL as a counterstrategy when all other AHLs were being disrupted. Our work, thus, demonstrated that a single bacterial species can mount a multifaceted antivirulence defense strategy by simultaneously targeting the aggregation of different AHLs and modulate them at different concentration levels with the overall goal of minimizing the signaling potential of an invading pathogen. Studies on anti-quorum activities within the scope of research on medicinal plants, using bioactive extracts from different sources against several pathogenic biosensor strains, are also gaining impetus (Kim and Park 2013; Lau et al. 2013; Samoilova et al. 2014). Such studies on different facets of microbial metabolism and crosstalk can serve as an important tool for future biotechnological purposes (Safari et al. 2014).

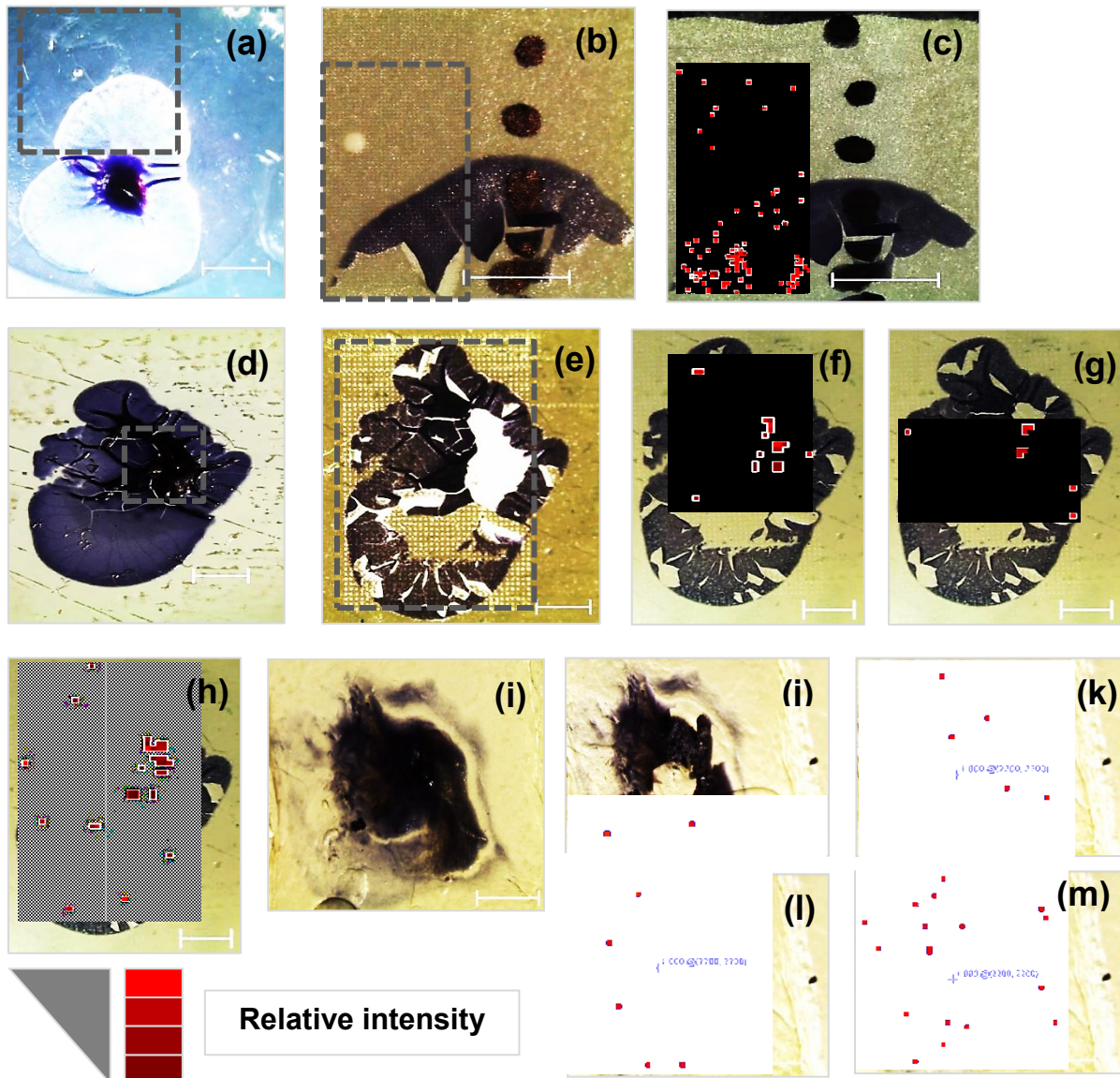


Fig. 2 Localization of the four different AHLs in the biosensor strain and their selective quenching by CFS extracts of the four endophytic bacterial strains B3, B4, B8, and B11. **(a, d)** Microscopic images of untreated (not challenged by endophyte CFS) *C. violaceum* showing visible production of violacein (violet color). Grey dotted insert shows area shot by laser beam. **(b, e)** Microscopic images of *C. violaceum* after spraying with suitable matrix showing the crystals of uniform matrix covering the colony and its periphery. Grey dotted inserts show areas shot by laser beam. **(c)** Localization of C6-HSL ($[M+H]^+$; $m/z = 200.12810$; $\Delta < 2$ ppm). **(f)** Localization of C8-HSL ($[M+H]^+$; $m/z = 228.15940$; $\Delta < 2$ ppm). **(g)** Localization of C10-HSL ($[M+H]^+$; $m/z = 256.19070$; $\Delta < 2$ ppm). **(h)** Localization of 3-oxo-C10-HSL ($[M+H]^+$; $m/z = 270.16998$; $\Delta < 2$ ppm). **(i)** Microscopic image of *C. violaceum* treated with CFS extract of bacterial endophyte strain B8. No visible production of violacein.

(j) Remnants of C6-HSL after being quenched by CFS extract of bacterial endophyte strain B8 ($[M+H]^+$; $m/z = 200.12810$; $\Delta < 2$ ppm). (k) Remnants of C8-HSL after being quenched by CFS extract of bacterial endophyte strain B8 ($[M+H]^+$; $m/z = 228.15940$; $\Delta < 2$ ppm). (i) Remnants of C10-HSL after being quenched by CFS extract of bacterial endophyte strain B8 ($[M+H]^+$; $m/z = 256.19070$; $\Delta < 2$ ppm). (m) Remnants of 3-oxo-C10-HSL after being quenched by CFS extract of bacterial endophyte strain B8 ($[M+H]^+$; $m/z = 270.16998$; $\Delta < 2$ ppm). All scale bars represent 1 mm. Insert grey box shows the color-coded relative intensities of the detected AHLs in the panels **c**, **f-h**, and **j-m**

Our investigation of the spatial localization and distribution of the four AHLs in *C. violaceum* by MALDI-imaging-HRMS revealed the release of C6-HSL on the periphery of the colony and successively diffusing into the agar. This finding corroborated the concept of CviI/CviR synthase-receptor regulated C6-HSL production followed by free passive diffusion across the cell envelope to accumulate in the local environment (McClellan et al. 1997; LaSarre and Federle 2013). This visually confirmed in high spatial resolution that in *C. violaceum*, C6-HSL is first released into the extracellular environment after production which might then be taken up by another cell in the same population to begin violacein production (McClellan et al. 1997). The other three AHLs (C8-HSL, C10-HSL and 3-oxo-C10-HSL) did not diffuse freely into the agar and were found accumulating in the immediate vicinity of *C. violaceum* that could only be visualized directly below the colony itself. These AHLs were not passively released into the agar as compared to C6-HSL, revealing that they might be actively transported across the cell membrane in a controlled manner as suggested by LaSarre and Federle (2013). The biosensor strain, when treated with the CFS of endophytic bacterial isolates, displayed selective quorum quenching of all the four AHLs. This visually confirmed the differential quorum quenching abilities of the selected bacterial endophytes. Interestingly, C10-HSL and 3-oxo-C10-HSL remnants were observed in the agar in the vicinity of the *C. violaceum* colony, lending evidence to the fact that the endophytes were capable not only of preventing the production of these AHLs by the biosensor strain but also stalled their active transportation post-production.

This study provides fundamental insights into the potential of endophytic bacteria as biocontrol agents against bacterial phytopathogens as well as antivirulence agents that might be useful in quorum-inhibiting therapies. Almost all Gram-negative bacterial pathogens maintain pathogenicity in their hosts (plants or animals, including humans) by cell-to-cell communication using quorum sensing signaling (Sifri 2008; Morohoshi et al. 2013; Amaral et al. 2014; Christiaen et al. 2014; Schafhauser et al. 2014). Attenuation of these signals will lead to suppression of pathogen virulence without introducing additional resistance-inducing

selection pressures. It is well-known that endophytes are capable of maintaining mutualistic associations with their host plants over a period of their life cycle (Kusari et al. 2012, 2013, 2014), which might lead to co-evolution of certain functional traits (such as production of bioactive secondary metabolites). However, during their co-existence with host plants, endophytes encounter invasion by a plethora of specific and generalist pathogens. Therefore, in order to survive in their ecological niches (internal plant environment), endophytes might evolve additional defense strategies that prevent the pathogens from developing resistance to their arsenal of bioactive secondary metabolites (used in chemical defense). Quorum quenching is one of such antivirulence strategies that are developed by selected endophytic bacteria. This work, thus, highlights an important biological role played by endophytes in different ecological niches, not only in host plant defense but also in maintaining colonization and their own survival inside plants. Interestingly, the bacterial interactions and antivirulence strategies might differ in environmental niches where different microbial communities interfere with the signaling systems. Recently for example, challenges of multiple signaling in bacterial communities in a particular environment with regard to their perception of different signaling molecules, has been highlighted (Cornforth et al. 2013). Admittedly, our work provides the insights into quorum quenching strategies employed by endophytic bacteria against a single biosensor strain. These quenching strategies might differ during multiple signaling events under different environmental conditions. Our study, however, provides a scientific handle to further investigate in planta quorum quenching by endophytes and elucidate the exact role of AHL-mediated gene expression and regulation within complex ecological niches of multispecies microbial communities.

4.6. REFERENCES

- Aly AH, Debbab A, Proksch P (2013) Fungal endophytes - secret producers of bioactive plant metabolites. *Pharmazie* 68:499-505
- Amara N, Krom BP, Kaufmann GF, Meijiler MM (2011) Macromolecular inhibition of quorum sensing: enzymes, antibodies, and beyond. *Chem Rev* 111:195-208
- Amaral L, Martins A, Spengler G, Molnar J (2014) Efflux pumps of Gram-negative bacteria: what they do, how they do it, with what and how to deal with them. *Front Pharmacol* 4:168 doi: 10.3389/fphar.2013.00168
- Bjarnholt N, Li B, D'Alvise J, Janfelt C (2014) Mass spectrometry imaging of plant metabolites – principles and possibilities. *Nat Prod Rep* doi: 10.1039/c3np70100j
- Burmølle M, Thomsen TR, Fazli M, Dige I, Christensen L, Homøe P, Tvede M, Nyvad B, Tolker-Nielsen T, Givskov M, Moser C, Kirketerp-Møller, K Johansen HK, Høiby N, Jensen P, Sørensen SJ, Bjarnholt T (2010) Biofilms in chronic infections –a matter

- of opportunity - monospecies biofilms in multispecies infections. *FEMS Immunol Med Microbiol* 59:324-336
- Choo JH, Rukayadi Y, Hwang J-K (2006) Inhibition of bacterial quorum sensing by vanilla extract. *Lett Appl Microbiol* 42:637–641
- Christiaen SEA, Matthijs N, Zhang X-H, Nelis HJ, Bossier P, Coenye T (2014) Bacteria that inhibit quorum sensing decrease biofilm formation and virulence in *Pseudomonas aeruginosa* PAO1. *Pathog Dis* 70:271-279
- Claessen D, Rozen DE, Kuipers OP, Søggaard-Andersen L, van Wezel GP (2014) Bacterial solutions to multicellularity: a tale of biofilms, filaments and fruiting bodies. *Nat Rev Microbiol* doi:10.1038/nrmicro3178
- Clatworthy AE, Pierson E, Hung DT (2007) Targeting virulence: a new paradigm for antimicrobial therapy. *Nat Chem Biol* 3:541-548
- Cornforth DM, Popat R, McNally L, Gurney J, Scott-Phillips TC, Ivens A, Diggle SP, Brown SP (2014) Combinatorial quorum sensing allows bacteria to resolve their social and physical environment. *Proc Natl Acad Sci USA* 111:4280-4284
- Galloway WRJD, Hodgkinson JT, Bowden SD, Welch M, Spring DR (2011) Quorum sensing in gram-negative bacteria: small-molecule modulation of AHL and AI-2 quorum sensing pathways. *Chem Rev* 111:28-67
- Grotenhermen F, Müller-Vahl K (2012) The therapeutic potential of *Cannabis* and cannabinoids. *Dtsch Arztebl Int* 109:495-501
- Hosni T, Moretti C, Devescovi G, Suarez-Moreno ZR, Fatmi MB, Guarnaccia C, Pongor S, Onofri A, Buonauro R, Venturi V (2011) Sharing of quorum-sensing signals and role of interspecies communities in a bacterial plant disease. *ISME J* 5:1857-1870
- Kharwar RN, Mishra A, Gond SK, Stierle A, Stierle D (2011) Anticancer compounds derived from fungal endophytes: their importance and future challenges. *Nat Prod Rep* 28:1208-1228
- Kim S-H, Park H-D (2013) Ginger extract inhibits biofilm formation by *Pseudomonas aeruginosa* PA14. *PLOS ONE* 8: e76106
- Kusari S, Spiteller M (2011) Are we ready for industrial production of bioactive plant secondary metabolites utilizing endophytes? *Nat Prod Rep* 28:1203-1207
- Kusari S, Zühlke S, Spiteller M (2009a) An endophytic fungus from *Camptotheca acuminata* that produces camptothecin and analogues. *J Nat Prod* 72:2-7
- Kusari S, Lamshöft M, Spiteller M (2009b) *Aspergillus fumigatus* Fresenius, an endophytic fungus from *Juniperus communis* L. Horstmann as a novel source of the anticancer pro-drug deoxypodophyllotoxin. *J Appl Microbiol* 107:1019-1030

- Kusari S, Hertweck C, Spiteller M (2012) Chemical ecology of endophytic fungi: origins of secondary metabolites. *Chem Biol* 19:792-798
- Kusari P, Kusari S, Spiteller M, Kayser O (2013) Endophytic fungi harbored in *Cannabis sativa* L.: diversity and potential as biocontrol agents against host plant-specific phytopathogens. *Fungal Divers* 60:137-151
- Kusari S, Singh S, Jayabaskaran C (2014) Biotechnological potential of plant-associated endophytic fungi: hope versus hype. *Trends Biotechnol* 32:297-303
- Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) *Nucleic acid techniques in bacterial systematics*. Wiley, New York, NY, pp 115–175
- LaSarre B, Federle MJ (2013) Exploiting quorum sensing to confuse bacterial pathogens. *Microbiol Mol Biol Rev* 77:73-111
- Lau YY, Sulaiman J, Chen JW, Yin W-F, Chan K-G (2013) Quorum sensing activity of *Enterobacter asburiae* isolated from lettuce leaves. *Sensors* 13:14189-14199
- Limsuwan S, Voravuthikunchai SP (2008) *Boesenbergia pandurata* (Roxb.) Schltr., *Eleutherine americana* Merr. and *Rhodomyrtus tomentosa* (Aiton) Hassk. as antibiofilm producing and antiquorum sensing in *Streptococcus pyogenes*. *FEMS Immunol Med Microbiol* 53:429–436
- Márquez LM, Redman RS, Rodriguez RJ, Roossinck MJ (2007) A virus in a fungus in a plant: three-way symbiosis required for thermal tolerance. *Science* 315:513-515
- McClellan KH, Winson MK, Fish L, Taylor A, Chhabra SR, Camara M, Daykin M, Lamb JH, Swift S, Bycroft BW, Stewart GSAB, Williams P (1997) Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acyl homoserine lactones. *Microbiology* 143:3703-3711
- Morohoshi T, Fukamachi K, Kato M, Kato N, Ikeda T (2010) Regulation of the violacein biosynthetic gene cluster by acylhomoserine lactone-mediated quorum sensing in *Chromobacterium violaceum* ATCC 12472. *Biosci Biotechnol Biochem* 74:2116-2119
- Morohoshi T, Tokita K, Ito S, Saito Y, Maeda S, Kato N, Ikeda T (2013) Inhibition of quorum sensing in gram-negative bacteria by alkylamine-modified cyclodextrins. *J Biosci Bioeng* 116:175-179
- O'Loughlin CT, Miller LC, Siryaporn A, Drescher K, Semmelhack MF, Bassler BL (2013) A quorum-sensing inhibitor blocks *Pseudomonas aeruginosa* virulence and biofilm formation. *Proc Natl Acad Sci USA* 110:17981-17986
- Ou CC, Lu TM, Tsai JJ, Yen JH, Chen HW, Lin MY (2009) Antioxidative effect of lactic acid bacteria: intact cells vs. intracellular extracts. *J Food Drug Anal* 17:209-216
- Porrás-Alfaro A, Bayman P (2011) Hidden fungi, emergent properties: endophytes and microbiomes. *Annu Rev Phytopathol* 49:291-315

- Rasko DA, Sperandio V (2010) Anti-virulence strategies to combat bacteria-mediated disease. *Nat Rev Drug Discov* 9:117-128
- Rishi P, Preet S, Kaur P (2011) Effect of *L. plantarum* cell-free extract and co-trimoxazole against *Salmonella* typhimurium: a possible adjunct therapy. *Ann Clin Microbiol Antimicrob* 10:9
- Rompp A, Spengler B (2013) Mass spectrometry imaging with high resolution in mass and space. *Histochem Cell Biol* 139:759–783
- Safari M, Amache R, Esmailshirazifard E, Keshavarz T (2014) Microbial metabolism of quorum-sensing molecules acyl-homoserine lactones, γ -heptalactone and other lactones. *Appl Microbiol Biotechnol* 98:3401-3412
- Samoilova Z, Muzyka N, Lepekhina E, Oktyabrsky O, Smirnova G (2014) Medicinal plant extracts can variously modify biofilm formation in *Escherichia coli*. *A Van Leeuw J Microb* 105:709-722
- Schafhauser J, Lepine F, McKay G, Ahlgren HG, Khakimova M, Nguyen D (2014) The stringent response modulates 4-hydroxy-2-alkylquinoline biosynthesis and quorum-sensing hierarchy in *Pseudomonas aeruginosa*. *J Bacteriol* 196:1641-1650
- Schulz B, Guske S, Dammann U, Boyle C (1998) Endophyte-host interactions II. Defining symbiosis of the endophyte-host interaction. *Symbiosis* 25:213-227
- Shih C-J, Chen P-Y, Liaw C-C, Lai Y-M, Yang Y-L (2014) Bringing microbial interactions to light using imaging mass spectrometry. *Nat Prod Rep* doi: 10.1039/c3np70091g
- Sifri CD (2008) Quorum sensing: bacteria talk sense. *Clin Infect Dis* 47:1070-1076
- Taura F, Sirikantaramas S, Shoyama Y, Shoyama Y, Morimoto S (2007) Phytocannabinoids in *Cannabis sativa*: recent studies on biosynthetic enzymes. *Chem Biodivers* 4:1649-1663
- Teplitski M, Mathesius U, Rumbaugh KP (2011) Perception and degradation of N-acyl homoserine lactone quorum sensing signals by mammalian and plant cells. *Chem Rev* 111:100-116
- Thenmozhi R, Nithyanand P, Rathna J, Pandian SK (2009) Antibiofilm activity of coral-associated bacteria against different clinical M serotypes of *Streptococcus pyogenes*. *FEMS Immunol Med Microbiol* 57:284–294
- Zhao Q, Mutukumira A, Lee SJ, Maddox I, Shu Q (2011) Functional properties of free and encapsulated *Lactobacillus reuteri* DPC16 during and after passage through a simulated gastrointestinal tract. *World J Microbiol Biotechnol* 28:61–70

Chapter 5

BIOCONTROL POTENTIAL OF ENDOPHYTES HARBORED IN *RADULA MARGINATA* (LIVERWORT) FROM THE NEW ZEALAND ECOSYSTEM

Parijat Kusari, Souvik Kusari, Michael Spitteller, Oliver Kayser

PK, SK, MS and OK jointly conceived, planned and designed the study; PK performed all the experiments and analyzed the data; PK wrote the manuscript with inputs from all coauthors; SK supervised PK and maintained scientific surveillance; MS and OK oversaw the entire project as senior authors and supervisors of PK

Antonie van Leeuwenhoek Journal of Microbiology (2014) 106:771-788

5.1. ABSTRACT

Radula marginata and *Cannabis sativa* L. are two phylogenetically unrelated plant species containing structurally similar secondary metabolites like cannabinoids. The major objective of our work was the isolation, identification, biocontrol efficacies, biofilm forming potential and anti-biofilm ability of endophytic microbial community of the liverwort *R. marginata*, as compared to bacterial endophytic isolates harbored in *C. sativa* plants. A total of 15 endophytic fungal and 4 endophytic bacterial isolates were identified, including the presence of a bacterial endosymbiont within an endophytic fungal isolate. The endosymbiont was visible only when the fungus containing it was challenged with two phytopathogens *Botrytis cinerea* and *Trichothecium roseum*, highlighting a tripartite microbe-microbe interaction and biocontrol potency of endophytes under biotic stress. We also observed sixteen types of endophytic fungal-pathogen and twelve types of endophytic bacterial-pathogen interactions coupled to varying degree of growth inhibitions of either the pathogen or endophyte or both. This showed the magnitude of biocontrol efficacies of endophytes in aiding plant fitness benefits under different media (environmental) conditions. Additionally, it was ecologically noteworthy to find the presence of similar endophytic bacterial genera in both *Radula* and *Cannabis* plants, which exhibited similar functional traits like biofilm formation and general anti-biofilm activities. Thus far, our work underlines the biocontrol potency and defensive functional traits (in terms of antagonism and biofilm formation) of endophytes harbored in liverwort *R. marginata* as compared to the endophytic community of phylogenetically unrelated but phytochemically similar plant *C. sativa*.

Keywords: *Radula marginata*; *Cannabis sativa*; endophytic bacteria; endophytic fungi; phytopathogens; antagonism; biofilm formation

5.2. INTRODUCTION

Liverworts are small, simple and non-vascular plants existing in almost all ecosystems, though they are abundant in the tropical niches. However, these small plants are highly rich in terpenoids and aromatic compounds. Some are also known to produce specific compounds with novel carbon skeleton that serve as significant markers of different genus of liverworts (Ludwiczuk and Asakawa 2008). *Radula marginata* (Radulaceae) is a species of liverwort commonly found in the New Zealand. Species belonging to *Radula* (for example *R. perrottetti*, *R. complanata*, *R. kojana*, and others) have been reported to contain aromatic compounds and prenyl bibenzyls (Asakawa et al. 1991a, b; Toyota et al. 1994). These compounds are known to have antimicrobial, antioxidant, antifungal, cytotoxic and other important biological activities (Ludwiczuk and Asakawa 2008). Recent investigations on *Radula marginata* led to the identification of bibenzyl cannabinoids (namely perrottetinene and perrottetinenic acid), with structural similarity to tetrahydrocannabinol, the major psychoactive secondary metabolite of *Cannabis sativa* L. plants (Toyota et al. 2002; Park and Lee 2010). However, isolation of perrottetinene was also reported earlier from other species of *Radula*, viz. *R. perrottetti* and *R. laxiramea* (Toyota et al. 1994; Cullmann et al. 1999). Cannabinoids are one of the extensively studied secondary metabolites of *Cannabis* plants, which typically accumulate in glandular trichomes (Flemming et al. 2007; Happyana et al. 2013). In spite of the high content of Δ^9 -tetrahydrocannabinol, a psychoactive metabolite known as the 'drug of abuse', cannabinoids either singly or synergistically are known to have innumerable therapeutic benefits like analgesic, anti-inflammatory, anti-tremor, antioxidant, neuroprotective, immunosuppressive, appetite stimulant, antineoplastic and others (Kusari et al. 2014a). Perrottetinene and its acid have also been reported to have antimicrobial and antifungal activities (Na et al. 2005; Na and Baek 2006).

Throughout the history of evolution, plants have coevolved with a number of associated micro- and macro-organisms, including endophytic microorganisms, pathogens, parasites, herbivores, and so on. With concomitant coevolution of endophytic microorganisms (or 'endophytes') with plants, they (endophytes) have developed biosynthetic pathways leading to a plethora of bioactive secondary metabolites (Kusari et al. 2012; Kusari et al. 2014c; Brader et al. 2014). Endophytic colonization is primarily a mutualistic type of association that occurs within the internal tissues of plants without progressing towards disease. Such associations have proved beneficial for plant fitness in various ecological niches, often triggered by biotic selection pressures like invading phytopathogens (Arnold et al. 2003; Reinhold-Hurek and Hurek 2011; Hamilton and Bauerle 2012; Ansari et al. 2013; Berg et al. 2014). In our previous works, we have explored the potential of endophytic microbial

community of *Cannabis sativa* L. plants in host biocontrol as well as antivirulence agents (Kusari et al. 2013; Kusari et al. 2014b). These promising results further prompted us to explore the potency of endophytic microorganisms harbored in *R. marginata* owing to its production of structurally similar cannabinoids as those found in *C. sativa*. It is compelling that similar biosynthetic principles apply to both phylogenetically unrelated plants with regard to production of structurally and functionally similar compounds. With this background and rationale, we attempted to elucidate the biocontrol efficacies of endophytic community living in *R. marginata* against the phytopathogens of *C. sativa* plants, namely *Botrytis cinerea* and *Trichothecium roseum*. *B. cinerea* and *T. roseum* have been found to be associated with *Cannabis* plant diseases like 'gray mold', 'damping off' and 'pink rot', respectively (McPartland 1996; Kusari et al. 2013). These diseases are also known to cause epidemic and green house disasters attacking different plant tissues and ages, ranging from small seedlings to leaves, stem and flowers of mature plants (McPartland 1991; van der Werf and van Geel 1994).

Taking cues from our previous work on the endophytic community of *C. sativa*, this manuscript demonstrates the biocontrol prospects of fungal and bacterial endophytes harbored in *R. marginata*. Furthermore, this study compares and evaluates the ecological significance and antagonistic potential of bacterial endophytic community of *R. marginata* as compared to that of *C. sativa*.

Bacteria are known to perform cell-to-cell communication via quorum sensing signaling enabling microbe-microbe interaction, virulence, pathogenesis and colonization (Hartmann et al. 2014; Safari et al. 2014). Successive aggregation of bacterial communities results in mono- or multi-species biofilm formation in a particular ecological niche (Claessen et al. 2014; Cornforth et al. 2014). Given the fact that our recent findings have accentuated fundamental insights into the antivirulence strategies (by quorum quenching) of bacterial endophytic isolates of *C. sativa* (Kusari et al. 2014b), we further analyzed and compared the magnitude of biofilm formation by the bacterial isolates of the two plants at two different temperatures with reference to generalist biofilm forming pathogens, namely *Pseudomonas aeruginosa* POA1, *Staphylococcus aureus* and *Escherichia coli*. In addition, we also compared the anti-biofilm capability of the isolates against the same pathogenic biofilm formers.

The basic objective of this work was to explore the complete endophytic microbial community (both fungi and bacteria) of the liverwort *R. marginata* with respect to diversified functional traits of the endophytic isolates. This manuscript therefore deals with the isolation, identification, biocontrol potential, biofilm and anti-biofilm magnitudes of the endophytes

harbored in *R. marginata*, compared to the endophytes harbored in *C. sativa* L. plants. This underlines the similar and discrete traits of endophytic community of plants from different ecological niches with similar secondary metabolite (cannabinoids) production. To the best of our knowledge, this is the first report of isolation and evaluation of bacterial and fungal endophytic community harbored in liverwort *R. marginata*.

5.3. MATERIALS AND METHODS

5.3.1. Collection of the plant material

The liverworts were collected in August 2012 from their natural population at Waitakere Ranges Regional Park., New Zealand. The plants were identified and authenticated as *Radula marginata* by experienced botanists. Import of the plant material was allowed according to the permission of the Auckland Council, New Zealand Government. The plant material was transported to TU Dortmund, Germany in sealed plastic zip lock bags at 4 °C and processed for the isolation of endophytes within 24 h of plant collection.

5.3.2. Isolation of endophytic bacteria and fungi and establishment of axenic cultures

The isolation of the endophytes was done following previously established procedures (Kusari et al. 2013), suitably modified. The plant material (leaves) were thoroughly washed in running tap water and cut with the help of sterilized razor blade into small fragments. The fragments were then surface sterilized by sequential immersion in 70% ethanol for 1 min, 1.3 M sodium hypochlorite (3-5% available chlorine) for 3 min, and 70% ethanol for 30 s. Then the fragments were washed thoroughly in sterile double-distilled water for a couple of minutes to remove excess surface sterilants. The excess water was blotted on sterile filter paper. For the isolation of fungi, the surface sterilized fragments were placed in petri dishes containing water agar (WA; Roth) medium supplemented with streptomycin (100 mg/L) to eliminate any bacterial growth. For the isolation of bacteria, the surface-sterilized tissue fragments were placed in sterile mortar-pestle and crushed with the addition of sterile double-distilled water. The macerated tissues, thus obtained, were carefully plated on petri dishes containing Nutrient agar (NA; Roth). All the petri dishes were sealed with parafilm and incubated at $28 \pm 2^\circ\text{C}$. To ensure proper surface sterilization and isolation of endophytes, two different techniques were implemented. Firstly, the sterile double-distilled water of the final rinse were plated in NA and WA and incubated in parallel under similar conditions. Secondly, the surface-sterilized tissue fragments were imprinted simultaneously in NA and incubated under similar conditions (secondary protocol, 'imprint technique') (Sánchez Márquez et al.

2007). The plates were monitored every day to check for the growth of endophytes. The endophytic fungi which grew out from the fragments over 4-6 weeks were subcultured onto a mycological rich medium namely, Sabouraud dextrose agar (SA) and brought into pure culture. The bacterial isolates were subcultured on NA and incubated in parallel to establish pure cultures. The endophytic isolates were routinely maintained in NA (bacteria) and SA (fungi) in active form and preserved in 15% (v/v) glycerol at -80°C (spores as well as vegetative form for fungi).

5.3.3. Genomic DNA isolation, PCR amplification and sequencing

The genomic DNA of the fungal and bacterial endophytes were isolated using peqGOLD fungal DNA mini kit and peqGOLD bacterial DNA kit (Peqlab Biotechnologie GmbH, Germany) respectively. Briefly, a set of conical flasks with 500 mL capacity each with 100 mL (SA for fungi and NA for bacteria), were used with proper autoclaving. The fungal and bacterial isolates were inoculated in the respective flasks and incubated at $28 \pm 2^\circ\text{C}$ with proper shaking (150 rpm) on a rotary shaker (INFORS HT Multitron 2, Germany). The bacterial isolates were grown till mid log phase for extraction of genomic DNA; whereas the fungal isolates were cultured over 2-3 weeks. The genomic DNA was extracted strictly following manufacturer's guidelines.

The total genomic DNA was subjected to PCR amplification using the primers ITS4 and ITS5 for fungal isolates (White et al. 1990) and 27f and 1492r for bacterial isolates (Lane 1991). The PCR amplification was performed in a 50 μL reaction mixture containing 10 μL Phusion HF buffer (5X), 1 μL dNTPs (10 mM), 0.5 μL forward primer (100 μM), 0.5 μL reverse primer (100 μM), 3 μL of template DNA, 1 μL of Phusion polymerase (2U/ μL), and 34 μL of sterile double-distilled water. The PCR cycling protocol for fungal isolates consisted of an initial denaturation at 98°C for 3 min, 30 cycles of denaturation, annealing and elongation at 98°C for 10 sec, 58°C for 30 sec and 72°C for 45 sec. This was followed by a final elongation step of 72°C for 10 min. For the bacterial isolates, the cycling conditions consisted of an initial denaturation at 98°C for 3 min, 30 cycles of denaturation, annealing and elongation at 98°C for 10 sec, 60°C for 30 sec and 72°C for 45 sec. This was followed by a final elongation step of 72°C for 10 min. As a negative control, sterile double distilled water was used instead of template DNA, in both PCR amplifications.

The PCR products spanning around 500-600 bp (for fungal isolates) and 1500 bp (for bacterial isolates), were visualized by gel electrophoresis. The PCR products were purified using peqGOLD micro spin cycle pure kit (Peqlab Biotechnologie GmbH, Germany) strictly following manufacturer's instructions. The amplified products were then sequenced from both

directions at GATC Biotech (Cologne, Germany) using the above mentioned primers.

The endosymbiont R4 (strain number) was isolated from endophytic fungal isolate F13 using previously established method (Partida-Martinez and Hertweck 2005). For further confirmation, total genomic DNA was extracted from F13 and subjected to PCR (Hoffman and Arnold 2010) using bacterial 16S rRNA specific primers (27f and 1492r) following the similar cycling conditions like other bacterial isolates. The amplified product was sequenced and compared with the pure bacterial culture for the identification and presence of endosymbiont.

5.3.4. Pathogenic strains used for antagonistic assays and biofilm formations

The two host specific phytopathogens of *Cannabis sativa* L. plants and the three biofilm forming pathogenic strains, used in this work were obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. The two phytopathogens, namely *Botrytis cinerea* (accession number DSM 5145) and *Trichothecium roseum* (accession number DSM 63066), were activated and maintained as described in our previous work (Kusari et al. 2013).

The biofilm formers, namely *Pseudomonas aeruginosa* PAO1 (accession number DSM 22644), *Staphylococcus aureus* (accession number DSM 682) and *Escherichia coli* (accession number DSM 799), were activated according to DSMZ guidelines. The medium used for activation of bacterial pathogenic strains were Nutrient agar (NA; Roth) and Luria-Bertani agar (LBA; Roth). All the activated strains were routinely maintained on NA and LBA for bacterial strains, and potato dextrose agar (PDA; Roth), Malt Extract agar (MEA; Roth), and SA for fungal phytopathogens, respectively.

5.3.5. Dual-plate antagonism assay of endophytic isolates against phytopathogens

The in vitro antagonistic assay of fungal and bacterial endophytes against the host specific phytopathogens were tested according to previously established method (Chamberlain and Crawford 1999; Kusari et al. 2013), suitably modified. The assay was carried out in five different media namely NA, SA, MEA, PDA and WA. Briefly, 5 mm plugs of fungal endophytes and pathogens were co-cultured at opposite edges of the petri dishes facing each other. In case of bacterial endophytes, the isolates were streaked at the other edge of the petri dish containing the fungal pathogens. The pathogens alone were inoculated as controls. All the antagonistic assays were carried out in triplicates in all the five different

media mentioned above. The diameter of growth of fungal pathogens were monitored daily for the different endophyte-pathogen interactions and noted at 5, 10 and 15 days interval. The antagonistic inhibitions were calculated for each of the endophyte-pathogen interactions in five different media against the control plates. The calculations were performed using our previously established equation (Kusari et al. 2013). The antagonistic assays were further characterized by various macroscopic endophyte-pathogen interactions under five different media conditions. The complete antagonisms were analyzed by the combination of inhibition percentages accompanied by different endophyte-pathogen interactions on all five solid media.

5.3.6. Biofilm and anti-biofilm assay of bacterial endophytes

The bacterial endophytic isolates of *R. marginata* and *C. sativa* were monitored for their capability to form biofilms using previously established method (Merritt et al. 2011), suitably modified. The biofilm formations of bacterial isolates were analyzed in Nutrient broth (NB) at 30°C and 37°C respectively. 100 µL of overnight cultures (OD₆₀₀ of 0.1) were inoculated in microtitre plates (containing 100 µL of fresh media) and incubated at 30°C and 37°C under static condition for 24, 48, 72 and 96 hours, respectively. The planktonic cells were discarded and biofilms were stained with 0.1% crystal violet for 10 min. The adherent bacteria were washed 2 times with sterile double distilled water and solubilized in 200 µL of 30% acetic acid for 15 min. The contents were transferred into another sterile 96-well microtitre plate and analyzed at OD₅₇₀. As a positive control, *Pseudomonas aeruginosa* (PAO1), *Staphylococcus aureus* (SA) and *Escherichia coli* (EC) were used. NB alone was used as negative control. Each experiment was performed in triplicates.

All the bacterial isolates were further analyzed for their anti-biofilm activity against generalist pathogenic biofilm formers namely *P. aeruginosa* PAO1, *S. aureus* and *E. coli*. For the preparation of bacterial extracts, the isolates were grown till mid-*log* phase in NB at 30°C with proper shaking (200 rpm). The cultures were subjected to ultrasonication (Sonifier Cell Disrupter, Branson Ultrasonics Corporation, Danbury, USA) for 10 minutes with an interval of 2 minutes in an ice bath ($\leq 4^{\circ}\text{C}$). This ultrasonic disruption was consecutively repeated thrice. The cell debris was removed by ultracentrifugation at 10,000 rpm for 30 minutes at 4°C. The extract, thus obtained, was filtered twice through sterile 0.22 µm Rotilabo[®]-Spritzenfilter (Carl Roth GmbH, Germany) for the complete removal of cells. Presence of bacterial contamination was counterchecked by plating the bacterial extracts (dilution factor of 10⁻⁶ to 10⁻⁸) on NA plates. For the anti-biofilm assay, 100 µL of each of the pathogenic cultures (OD₆₀₀ of 0.1) were inoculated in 100 µL of bacterial extracts under static conditions for 24

hours. As control pathogenic cultures were inoculated in 100 μ L of NB. The effect of extracts on biofilm formation was analyzed using the same protocol mentioned above. Each experiment was done in triplicates.

5.4. RESULTS

5.4.1. Identification and characterization of endophytic isolates

A total of 15 endophytic fungal isolates and 4 endophytic bacterial isolates were obtained from the liverwort, *Radula marginata*. The fungal and bacterial isolates were identified based on ITS and 16S rRNA analyses, respectively. The amplified products (spanning around 500-600 bp for fungal isolates and 1500 bp for bacterial isolates) were used for the identification of the endophytes. The amplified sequences were matched using the Basic Local Alignment Search Tool (BLASTn) of the US National Centre for Biotechnology Information (NCBI) against the nucleotide database. The sequences obtained were aligned using EMBOSS Pairwise Sequence Alignment of the EMBL Nucleotide Sequence Database. The fungal and bacterial sequences are deposited at EMBL-Bank with accession numbers HG971763 to HG971777 and HG971778 to HG971781 respectively. The accession numbers with sequence identity similar to most closely related species are detailed in Tables 1 and 2. All the endophytic fungal isolates belonged to phylum Ascomycota with an exception of two isolates (F12 and F14) that belonged to Zygomycota. Majority of the isolates belonged to *Daldinia* sp. Others included *Rhizopus* sp., *Xylaria* sp., *Podospora* sp., *Aspergillus* sp. and *Hansfordia* sp. Although endophytic bacteria harboring *R. marginata* was lesser (isolate numbers) than the endophytic fungal population, some of the bacterial isolates were strikingly similar with those of *C. sativa* (EMBL-Bank accession numbers reported in our earlier publication; Kusari et al. 2014b). Majority of the isolates belonged to *Bacillus* sp. in both *Radula* and *Cannabis* plants. The 1500 bp amplified product of 16S rRNA analysis of F13 revealed the endosymbiont (strain number R4) as *Paenibacillus* sp.

Table 1 Summary of EMBL accession numbers, maximum identity and most closely related species of endophytic fungal isolates of *Radula marginata*

| Strain number (Fungal Endophyte) | EMBL-Bank Accession numbers | Most closely related species (Accession numbers) | Maximum identity |
|-------------------------------------|--------------------------------|---|---------------------|
| F1 | HG971763 | <i>Podospora glutinans</i> ; (AY615208.1) | 98% |
| F2 | HG971764 | <i>Aspergillus niger</i> ; (KC119204.1) | 99% |
| F3 | HG971765 | <i>Daldinia concentrica</i> ; (AM292045.1) | 99% |
| F4 | HG971766 | <i>Xylaria</i> sp.; (HM583857.1) | 99% |
| F5 | HG971767 | <i>Xylaria</i> sp.; (HM583857.1) | 99% |
| F6 | HG971768 | <i>Daldinia concentrica</i> ; (AM292045.1) | 99% |
| F7 | HG971769 | <i>Daldinia concentrica</i> ; (AM292045.1) | 99% |
| F8 | HG971770 | <i>Daldinia concentrica</i> ; (AM292045.1) | 98% |
| F9 | HG971771 | <i>Hansfordia</i> sp.; (GQ906969.1) | 97% |
| F10 | HG971772 | <i>Daldinia concentrica</i> ; (AM292046.1) | 98% |
| F11 | HG971773 | <i>Daldinia concentrica</i> ; (AM292045.1) | 98% |
| F12 | HG971774 | <i>Rhizopus oryzae</i> ; (JX661045.1) | 98% |
| F13 | HG971775 | <i>Daldinia concentrica</i> ; (AM292045.1) | 98% |
| F14 | HG971776 | <i>Rhizopus oryzae</i> ; (JX661045.1) | 100% |
| F15 | HG971777 | <i>Daldinia concentrica</i> ; (AM292045.1) | 99% |

Table 2 Summary of EMBL accession numbers, maximum identity and most closely related species of endophytic bacterial isolates of *Radula marginata*

| Strain number (Bacterial Endophyte) | EMBL-Bank Accession numbers | Most closely related species (Accession numbers) | Maximum identity |
|--|--------------------------------|---|---------------------|
| R1 | HG971778 | <i>Bacillus subtilis</i> ; (KC182058.1) | 99% |
| R2 | HG971779 | <i>Bacillus subtilis</i> ; (KC441757.1) | 99% |
| R3 | HG971780 | <i>Bacillus subtilis</i> ; (GQ280027.1) | 99% |
| R4 | HG971781 | <i>Paenibacillus</i> sp. (KF011599.1) | 99% |

5.4.2. Biofilm formation and anti-biofilm activity of endophytic bacterial isolates

All the endophytic bacterial isolates were monitored for their capability to form biofilm under two different temperature conditions, viz. 30°C (Fig. 1a) and 37°C (Fig. 1b). Although the positive controls PAO1, SA and EC formed biofilms at both temperatures, the levels of biofilm were much higher at 37°C particularly for PAO1 (approx. 4 times) and SA (approx. 9 times). EC biofilm was similar at both temperatures. Isolate B3 was found to be a strong biofilm former at 37°C. The level of biofilm formation was similar to PAO1 and much higher than EC. However, B3 did not show significant biofilm formation at 30°C when compared to PAO1. Isolates B2, B5 and B7 also formed biofilms higher than EC and close to PAO1 at 37°C. Although B5 and B7 also did not form any significant biofilm at 30°C, isolate B2 formed strong biofilm even at 30°C. The level of B2 biofilm was similar to PAO1 and SA at 30°C. Some of the isolates like B4, B6, B8, B9 and B11 formed biofilms close to PAO1 and SA levels at 30°C. In general, the biofilm formations of the organisms under our study at 30°C are much less compared to 37°C. The endophytic isolates of *R. marginata* did not form any biofilm at both temperatures. Even the endosymbiont R4 did not have any pronounced biofilm activity.

Notwithstanding that some of the endophytic isolates of *C. sativa* were similar at the species level; they did not exhibit similar potency of biofilm formation under similar temperature conditions. For instance, isolate B2 was found to form biofilm at both 30°C and 37°C whereas B1 and B6 did not exhibit any significant biofilm activity (Fig. 1). Similarly, isolate B3 being a strong biofilm former at 37°C showed completely contrasting results compared to similar

strains like B9 and B11 that were not even close to noteworthy biofilm formation. Notably, B11 formed biofilm at 30°C where B3 was unable to show any pronounced activity. A complete dissimilar result was also found in similar strains isolated from *C. sativa*. For example, B5 and B7 formed similar level of biofilms at 37°C and did not show any activity at 30°C. It is even more interesting to note that the strikingly similar strains from two different plant species namely, *R. marginata* and *C. sativa* showed identical results. Isolates R1, R2 and R3 from *R. marginata* did not form any significant biofilm at both temperatures just like isolate B10 from *C. sativa*.

Owing to the capability of biofilm formation, the isolates were further monitored for their capability to inhibit biofilms of generalist pathogens like PAO1, SA and EC. None of the endophytic isolates of *R. marginata* and *C. sativa* showed significant anti-biofilm activity at 30°C and 37°C.

5.4.3. Antagonistic assay against phytopathogens

The *in vitro* antagonistic assay of fungal and bacterial endophytic isolates against the two selected phytopathogens revealed their efficacies as well as inadequacies as biocontrol agents. A large diversity of endophyte-pathogen interactions were observed under five different media conditions. The different interaction strategies employed by the fungal and bacterial endophytic isolates were highly diverse against the phytopathogens, with the only exception of formation of inhibition zone (a clear halo) to restrict the growth of pathogen mycelium. The overall antagonisms were deciphered based on the different degree of growth inhibitions coupled to the array of individual endophyte-pathogen interactions. Based on our previously established methodology (Kusari et al. 2013), we could assign 16 different fungal endophyte-phytopathogen and 12 different bacterial endophyte-phytopathogen interactions under five different media conditions namely NA, WA, PDA, SA and MEA, respectively (Tables 3 and 4). Furthermore, every interaction in each of the five medium led to a certain degree of growth inhibition of either the pathogen or the endophytic isolate itself. The representative illustrations of each of the endophyte-pathogen associations as compared to control plates are summarized in Figs. 2 (fungus-fungus) and 3 (bacterium-fungus), respectively (control plates are shown in Fig. 4). A comprehensive antagonistic potential, illustrating the varying extent of growth inhibition coupled to specific interactions gave a clear idea about the biocontrol efficacies of the bacterial and fungal endophytic isolates (Tables 5-8). The diversified association of endophytes with phytopathogens under different media conditions highlights the understanding of endophytes' host plant fitness potential under biotic selection pressures.

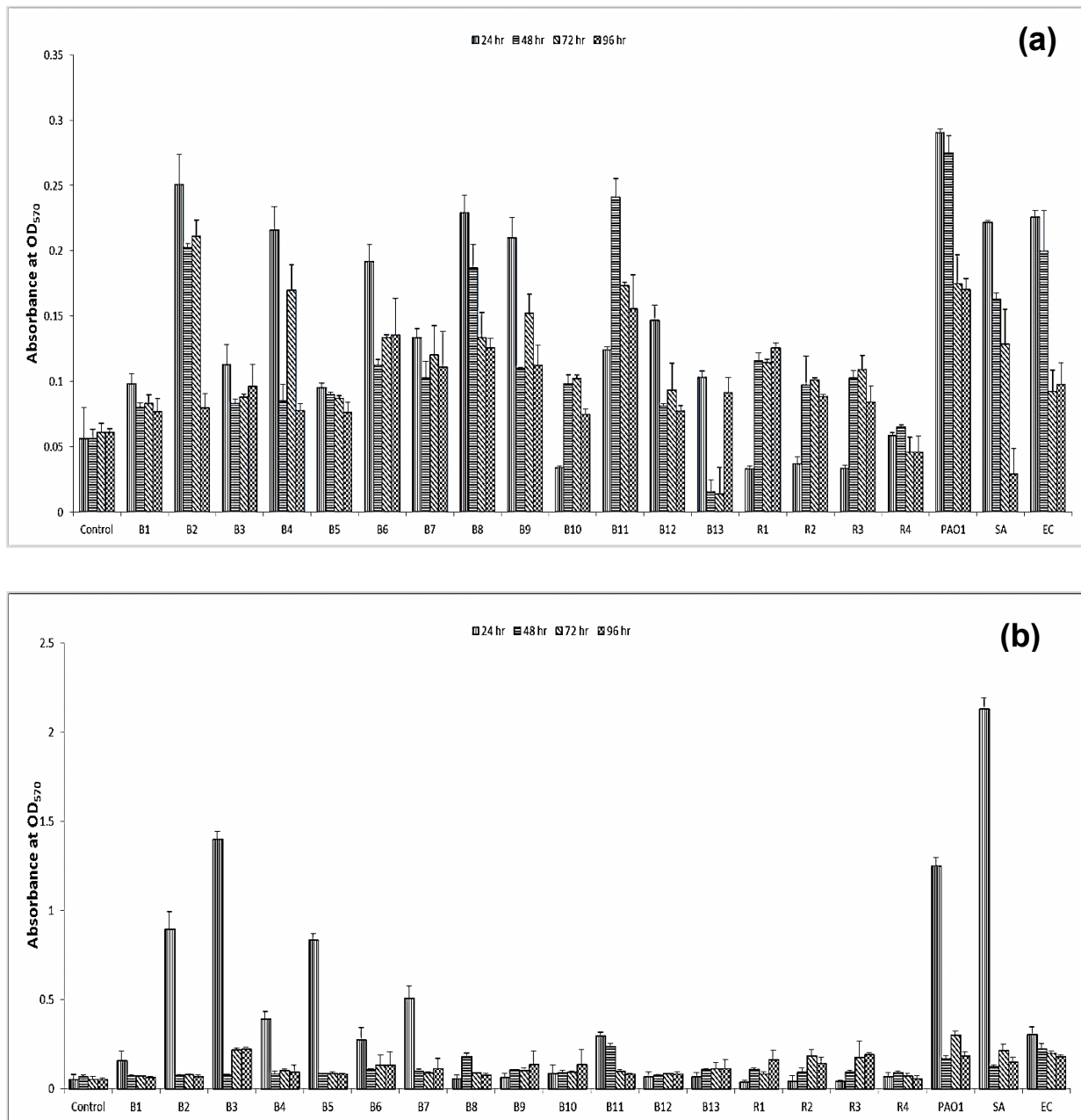


Fig. 1 Biofilm formation by endophytic bacterial isolates of *Cannabis sativa* and *Radula marginata* (strain numbers B1 to B13 and R1 to R4) in comparison to pathogenic biofilm formers *Pseudomonas aeruginosa* (PAO1), *Staphylococcus aureus* (SA) and *Escherichia coli* (EC) at 30°C **(a)** and at 37°C **(b)**; NB alone is designated as control

Table 3 Description of different endophyte-pathogen (fungus-fungus) interactions with respective interaction codes under five different media

| Interaction code | Endophyte-pathogen interaction descriptions |
|------------------|---|
| A | Both endophyte and pathogen grow towards each other, but growth stopped and mycelia of pathogen malformed as their mycelia came in physical contact; no sporulation; no color alteration of mycelia; no release of visible exudates; no inhibition zone (no halo) |
| B | Both endophyte and pathogen grow towards each other, but growth stopped and mycelia of endophyte malformed as their mycelia came in physical contact; no sporulation; no color alteration of mycelia; no release of visible exudates; no inhibition zone (no halo) |
| C | Both endophyte and pathogen grow towards each other, but growth stopped and mycelia of both pathogen and endophyte malformed as their mycelia came in physical contact; no sporulation; no color alteration of mycelia; no release of visible exudates; no inhibition zone (no halo) |
| D | Both endophyte and pathogen grow towards each other, but growth stopped as their mycelia came in physical contact; no malformation of mycelia of endophyte or pathogen; no sporulation; no color alteration of mycelia; no release of visible exudates; no inhibition zone (no halo) |
| E | Both endophyte and pathogen grow towards each other followed by substantial mycelial overlapping of endophyte and pathogen after their mycelia came in physical contact; no sporulation; no color alteration of mycelia; no release of visible exudates; no inhibition zone (no halo) |
| F | Both endophyte and pathogen grow towards each other followed by slight overgrowth of pathogen on endophyte after their mycelia came in physical contact; no sporulation; no color alteration of mycelia; no release of visible exudates; no inhibition zone (no halo) |
| G (OV/MC) | Both endophyte and pathogen grow towards each other followed by either complete overgrowth (OV) of endophyte on pathogen or mixed culture (MC) after their mycelia came in physical contact; no sporulation; no color alteration of mycelia; no release of visible exudates; no inhibition zone (no halo) |
| H | Both endophyte and pathogen grow towards each other, but growth stopped before their mycelia came in physical contact with endophytes releasing visible exudates from the entire mycelial biomass; no malformation of mycelia of endophyte or pathogen, no sporulation; no color alteration of mycelia; no inhibition zone (no halo) |
| I | Both endophyte and pathogen grow towards each other, but growth stopped before their mycelia came in physical contact with pathogen releasing visible exudates from the entire mycelial biomass; no malformation of mycelia of endophyte or pathogen, no sporulation; no color alteration of mycelia; no inhibition zone (no halo) |
| J | Both endophyte and pathogen grow towards each other, but growth stopped before their mycelia came in physical contact and endophyte forming visible dark brown to black colored band (secondary metabolites) at the point of mycelial contact; no sporulation; no color alteration of mycelia; no release of visible exudates; no inhibition zone (no halo) |

| | |
|--------|--|
| K | Both endophyte and pathogen grow towards each other, but growth stopped before their mycelia came in physical contact and inhibition zone (clear halo) produced by endophyte around its biomass; no halo by pathogen; no sporulation; no color alteration of mycelia; no release of visible exudates |
| L | Both endophyte and pathogen grow towards each other, but growth stopped and color alteration of mycelia by pathogen as their mycelia came in physical contact; no sporulation; no color alteration of mycelia; no release of visible exudates; no inhibition zone (no halo) |
| M | Both endophyte and pathogen grow towards each other, but growth stopped and color alteration of mycelia by pathogen with release of visible exudates from the entire biomass as their mycelia came in physical contact; no sporulation; no color alteration of mycelia; no inhibition zone (no halo) |
| N | Both endophyte and pathogen grow towards each other, but growth stopped before their mycelia came in physical contact; no sporulation; no release of visible exudates; no color alteration of mycelia; no inhibition zone (no halo) |
| ESY-I | Both endophyte and pathogen grow towards each other, followed by growth of endosymbiotic bacterium from the endophytic fungal mycelia towards pathogen; no release of visible exudates; no color alteration of mycelia; no inhibition zone (no halo) |
| ESY-II | Both endophyte and pathogen grow towards each other, followed by growth of endosymbiotic bacterium from the endophytic fungal mycelia towards pathogen; formation of visible dark brown to black colored band (secondary metabolites) by endophytic fungus; no release of visible exudates; no color alteration of mycelia; no inhibition zone (no halo) |

Table 4 Description of different endophyte-pathogen (bacterium-fungus) interactions with respective interaction codes under five different media

| Interaction code | Endophyte-pathogen interaction descriptions |
|------------------|---|
| 1 | Both endophyte and pathogen grow towards each other, but growth stopped before the colony and mycelia came in physical contact, followed by inhibition and malformation of pathogen mycelia |
| 2 | Both endophyte and pathogen grow towards each other, but growth stopped after the colony and mycelia came in physical contact, followed by inhibition and malformation of pathogen mycelia |
| 3 | Both endophyte and pathogen grow towards each other, but growth stopped before the colony and mycelia came in physical contact, followed by encircling of pathogen mycelia by bacterial colony from all sides causing inhibition and malformation of pathogen mycelia |

| | |
|--------|--|
| 4 | Both endophyte and pathogen grow towards each other, but growth stopped after the colony and mycelia came in physical contact; no inhibition or malformation of pathogen mycelia |
| 5 | Both endophyte and pathogen grow towards each other but growth stopped after the colony and mycelia came in physical contact, followed by slight overlapping of endophyte and pathogen; no inhibition or malformation of pathogen mycelia |
| 6 | Both endophyte and pathogen grow towards each other, but growth stopped after the colony and mycelia came in physical contact, followed by complete overgrowth of endophyte on pathogen mycelia; complete inhibition; no malformation of pathogen mycelia |
| 7(ZOI) | Both endophyte and pathogen grow towards each other, but growth stopped before the colony and mycelia came in physical contact; no further growth of endophyte or pathogen forming a zone of inhibition in between (but no halo); no malformation of pathogen mycelia |
| 8 | Only endophyte grow towards pathogen, but growth stopped before the colony and mycelia came in physical contact; growth of pathogen limited only on and around the inoculated 5mm plug followed by huge zone of inhibition in between (but no halo); no malformation of pathogen mycelia |
| 9 | Both endophyte and pathogen grow towards each other, but growth stopped before the colony and mycelia came in physical contact, followed by change of morphology of endophyte colony (for example bulging out) causing inhibition of pathogen mycelial growth; no malformation of pathogen mycelia |
| 10 | Only endophyte grow towards pathogen, but growth stopped before the colony and mycelia came in physical contact; growth of pathogen limited only on the inoculated 5mm plug and formation of inhibition zone (clear halo) by pathogen inhibiting endophyte colony; no malformation of pathogen mycelia |
| 11 | Both endophyte and pathogen grow towards each other, but growth stopped before the colony and mycelia came in physical contact and formation of inhibition zone (clear halo) by endophyte inhibiting pathogen; no malformation of pathogen mycelia |
| 12 | Both endophyte and pathogen grow towards each other, but growth stopped before the colony and mycelia came in physical contact; followed by growth of endophyte colony on pathogen plug without growing in between causing inhibition of pathogen mycelia; no malformation of pathogen mycelia |

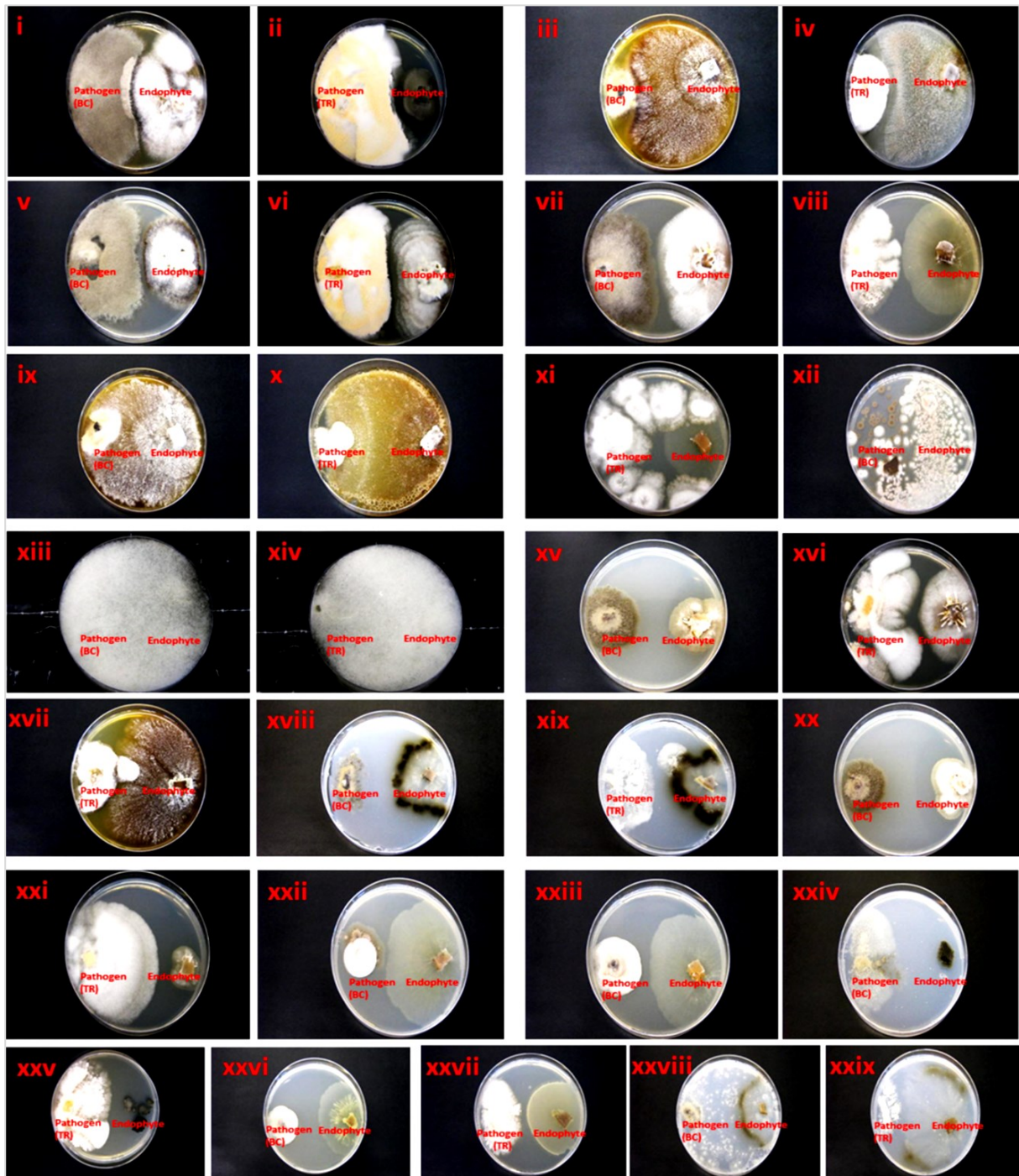


Fig. 2 Different endophyte-pathogen (fungus-fungus interactions). (i, ii) Interaction code A; (iii, iv) Interaction code B; (v, vi) Interaction code C; (vii, viii) Interaction code D; (ix, x) Interaction code E; (xi) Interaction code F; (xii) Interaction code G(MC); (xiii, xiv) Interaction code G(OV); (xv, xvi) Interaction code H; (xvii) Interaction code I; (xviii, xix) Interaction code J; (xx, xxi) Interaction code K; (xxii) Interaction code L; (xxiii) Interaction code M; (xxiv, xxv) Interaction code N; (xxvi, xxvii) Interaction code ESY-I; (xxviii, xxix) Interaction code ESY-II; (TR) Pathogen *Trichothecium roseum*; (BC) Pathogen *Botrytis cinerea*.

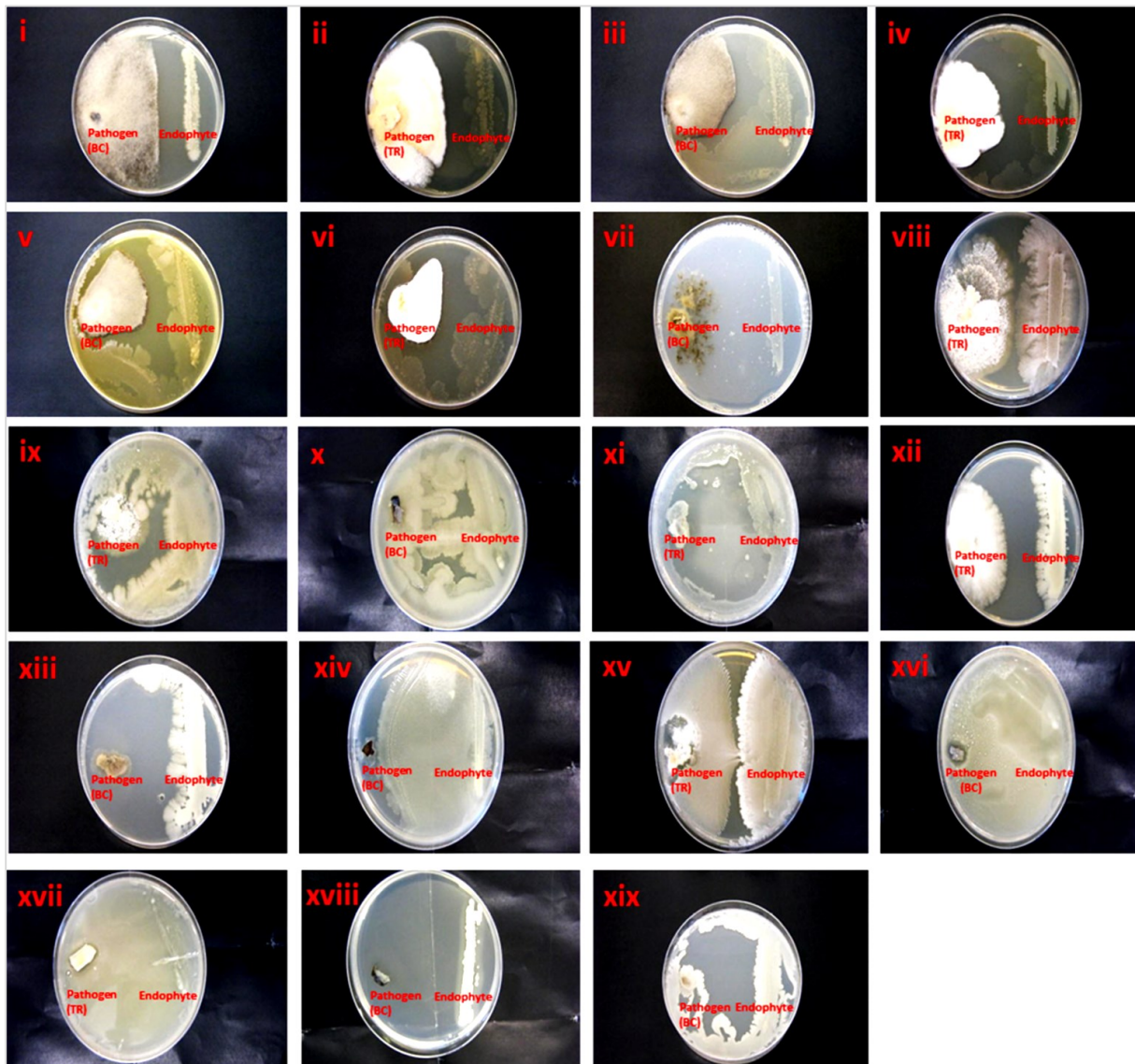


Fig. 3 Different endophyte-pathogen (bacterium-fungus) interactions. (i, ii) Interaction code 1; (iii, iv) Interaction code 2; (v, vi) Interaction code 3; (vii, viii) Interaction code 4; (ix) Interaction code 5; (x, xi) Interaction code 6; (xii) Interaction code 7; (xiii) Interaction code 8; (xiv, xv) Interaction code 9; (xvi, xvii) Interaction code 10; (xviii) Interaction code 11; (xix) Interaction code 12; (TR) Pathogen *Trichothecium roseum*; (BC) Pathogen *Botrytis cinerea*.

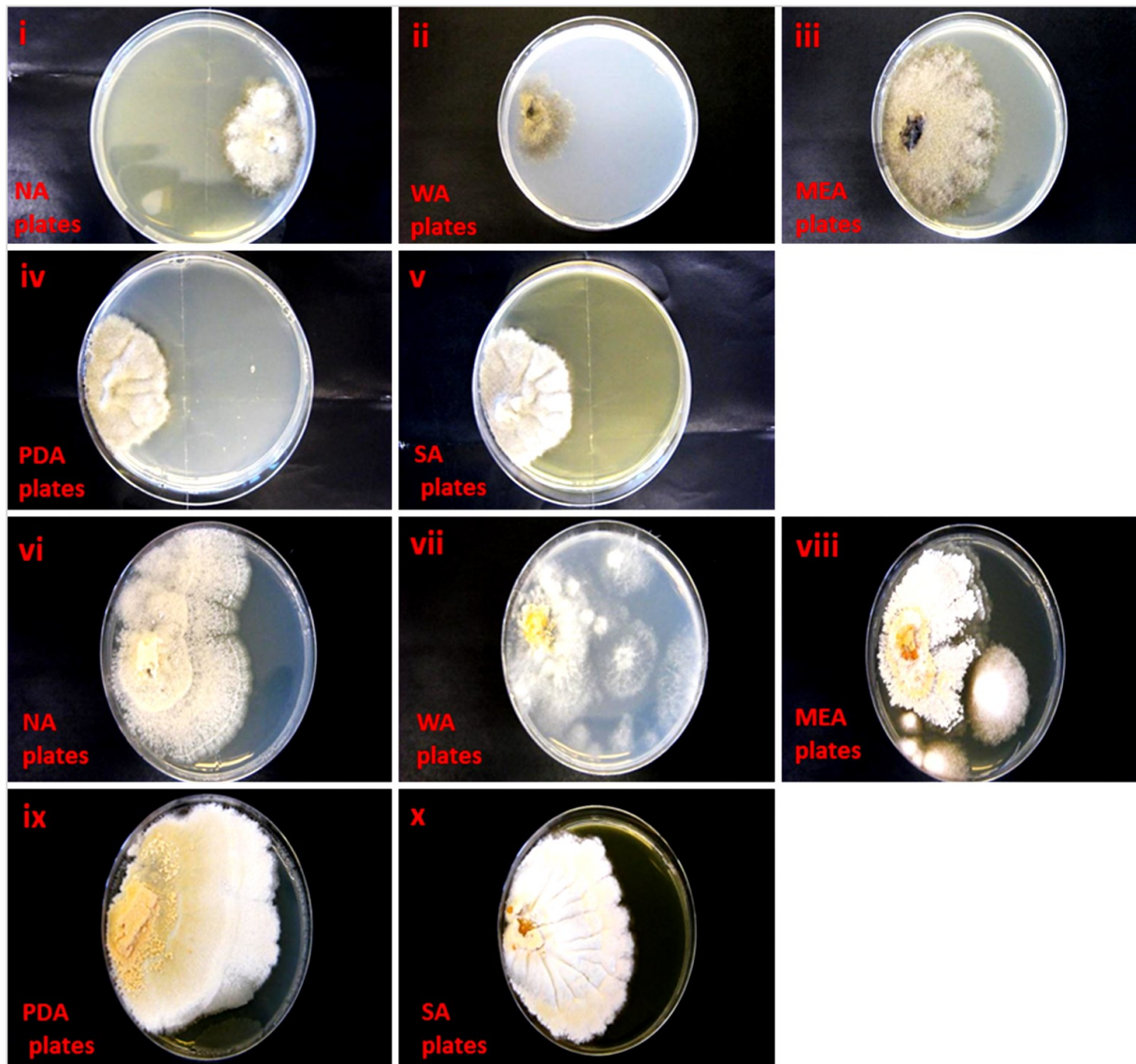


Fig. 4 Control plates of *Botrytis cinerea* (BC) and *Trichothecium roseum* (TR) in five different media. (i) BC in Nutrient agar plate; (ii) BC in Water agar plate; (iii) BC in Malt extract agar plate; (iv) BC in Potato dextrose agar plate; (v) BC in Sabouraud agar plate; (vi) TR in Nutrient agar plate; (vii) TR in Water agar plate; (viii) TR in Malt extract agar plate; (ix) TR in potato dextrose agar plate; (x) TR in Sabouraud agar plate.

Table 5 Summary of antagonistic inhibition of phytopathogen *Botrytis cinerea* by fungal endophytes of *Radula marginata* accompanied by different endophyte-pathogen interactions under five different media

| Fungal Endophyte Strain number | Antagonistic inhibition (% inhibition) against <i>Botrytis cinerea</i> in five different media | | | | | Different endophyte-pathogen interactions under five different media (interaction code, see Table 3) | | | | |
|--------------------------------|--|-----|-----|-----|-----|--|-------|-------|-------|--------|
| | WA | SA | PDA | MEA | NA | WA | SA | PDA | MEA | NA |
| F1 | -20 | -29 | 33 | 40 | MC | N | L | L | L | G(MC) |
| F2 | 0 | 0 | 22 | 40 | 64 | D | L | D | L | H |
| F3 | 60 | 57 | 67 | 70 | 55 | J | E | B | E | M |
| F4 | -20 | 0 | 11 | 10 | 45 | D | A | A | A | N |
| F5 | 0 | -14 | 44 | 30 | 64 | D | A | A | C | K |
| F6 | -80 | 57 | 67 | 70 | 9 | J | E | E | E | L |
| F7 | -60 | 57 | 67 | 70 | 73 | J | E,L | E,L | E,L | N,L |
| F8 | 60 | 57 | 67 | 80 | 73 | J | E | B | E | M |
| F9 | 40 | 43 | 44 | 70 | 82 | N | E | B,J | E | H |
| F10 | 60 | 57 | 78 | 70 | 73 | J | E | J | J | N |
| F11 | 80 | 71 | 78 | 90 | 82 | J | E,H | E | E | D |
| F12 | 100 | 100 | 100 | 100 | 100 | G(OV) | G(OV) | G(OV) | G(OV) | G(OV) |
| F13 | 60 | 57 | 67 | 70 | 82 | ESY-II | ESY-I | ESY-I | ESY-I | ESY-I* |
| F14 | 4 | 71 | 67 | 70 | 73 | B,J | E | E,L | E,L | N |
| F15 | 0 | 57 | 78 | 70 | 73 | B,J | E | E | E | L |

WA, Water agar; SA, Sabouraud agar; PDA, Potato dextrose agar; MEA, Malt extract agar; NA, Nutrient agar

MC, Mixed culture of endophyte and pathogen

ESY-I*, Only for NA plates, the interaction is accompanied by color alteration of pathogen mycelia

Table 6 Summary of antagonistic inhibition of phytopathogen *Trichothecium roseum* by fungal endophytes of *Radula marginata* accompanied by different endophyte-pathogen interactions under five different media

| Fungal Endophyte Strain number | Antagonistic inhibition (% inhibition) against <i>Trichothecium roseum</i> in five different media | | | | | Different endophyte-pathogen interactions under five different media (interaction code, see Table 3) | | | | |
|--------------------------------|--|-----|-----|-----|-----|--|-------|-------|-------|-------|
| | WA | SA | PDA | MEA | NA | WA | SA | PDA | MEA | NA |
| F1 | 56 | 9 | 29 | 36 | 9 | N | A | A | A | D |
| F2 | 63 | 73 | 50 | 36 | 18 | N | D | A | C | N |
| F3 | 75 | 55 | 64 | 79 | 36 | B,J | E | E | E | F |
| F4 | 63 | 73 | 50 | 36 | 18 | D | D | C | H | N |
| F5 | 63 | 18 | 50 | 43 | 9 | D | A | A | C | K |
| F6 | 69 | 55 | 64 | 64 | 55 | B,J | B | B | B | D |
| F7 | 81 | 64 | 79 | 79 | 27 | J | B | B | B | E |
| F8 | 69 | 67 | 79 | 71 | 45 | J | E | E | E | D |
| F9 | 75 | 73 | 79 | 79 | 36 | B | B | B | E | D |
| F10 | 56 | 64 | 79 | 71 | 18 | N | E,H | E | E,I | E |
| F11 | 88 | 73 | 86 | 71 | 36 | B | H | E | E | E |
| F12 | 100 | 100 | 100 | 100 | 100 | G(OV) | G(OV) | G(OV) | G(OV) | G(OV) |
| F13 | 44 | 64 | 71 | 71 | 27 | ESY-II | ESY-I | ESY-I | ESY-I | ESY-I |
| F14 | 75 | 73 | 42 | 79 | 36 | B,J | B | E | E | D |
| F15 | 69 | 67 | 79 | 79 | 45 | N | B,E | E | E | D |

WA, Water agar; SA, Sabouraud agar; PDA, Potato dextrose agar; MEA, Malt extract agar; NA, Nutrient agar

Table 7 Summary of antagonistic inhibition of phytopathogen *Botrytis cinerea* by bacterial endophytes of *Radula marginata* and *Cannabis sativa* accompanied by different endophyte-pathogen interactions under five different media

| Bacterial Endophyte Strain number | Antagonistic inhibition (% inhibition) against <i>Botrytis cinerea</i> in five different media | | | | | Different endophyte-pathogen interactions under five different media (interaction code, see Table 4) | | | | |
|-----------------------------------|--|-----|-----|-----|----|--|-------|-------|-------|-------|
| | WA | SA | PDA | MEA | NA | WA | SA | PDA | MEA | NA |
| R1 | 0 | 15 | 22 | -10 | 55 | 4 | 2 | 2 | 1 | 3 |
| R2 | 20 | -29 | 33 | 10 | NG | 4 | 1 | 3 | 1 | 12 |
| R3 | 20 | 0 | 11 | 20 | 82 | 4 | 3 | 1 | 1 | 4 |
| R4 | 60 | 57 | 67 | 70 | 82 | ESY-II | ESY-I | ESY-I | ESY-I | ESY-I |
| B1 | 75 | 86 | NG | 83 | NG | 8 | 8 | 10 | 8 | 6 |
| B2 | 50 | NG | NG | 83 | 75 | 8 | 8 | 10 | 8 | 9 |
| B3 | NG | NG | NG | NG | NG | 6 | 6 | 9 | 9 | 6 |
| B4 | 75 | 57 | NG | 50 | NG | 8 | 8 | 10 | 8 | 6 |
| B5 | 0 | 57 | NG | 67 | NG | 8 | 8 | 10 | 8 | 12 |
| B6 | NG | NG | NG | NG | NG | 6 | 6 | 10 | 6 | 6 |
| B7 | NG | NG | NG | NG | NG | 6 | 6 | 10 | 6 | 10 |
| B8 | NG | NG | NG | NG | NG | 6 | 6 | 10 | 6 | 10 |
| B9 | NG | NG | NG | NG | NG | 6 | 6 | 10 | 6 | 6 |
| B10 | NG | NG | NG | NG | NG | 6 | 6 | 10 | 6 | 9 |
| B11 | NG | NG | NG | NG | NG | 6 | 6 | 10,11 | 6 | 6 |
| B12 | NG | NG | NG | NG | NG | 6 | 6 | 6 | 6 | 6 |
| B13 | NG | NG | NG | NG | NG | 6 | 6 | 6 | 6 | 6 |

NG, No growth of pathogen; Every NG is accompanied by respective interaction that justifies the antagonistic inhibitions (% inhibitions) either for pathogen or endophyte respectively

ESY-I/ ESY-II, The endosymbiont interactions from Tables 5 and 6

Table 8 Summary of antagonistic inhibition of phytopathogen *Trichothecium roseum* by bacterial endophytes of *Radula marginata* and *Cannabis sativa* accompanied by different endophyte-pathogen interactions under five different media

| Bacterial Endophyte Strain number | Antagonistic inhibition (% inhibition) against <i>Trichothecium roseum</i> in five different media | | | | | Different endophyte-pathogen interactions under five different media (interaction code, see Table 4) | | | | |
|-----------------------------------|--|-----|-----|-----|----|--|----------|--------|--------|--------|
| | WA | SA | PDA | MEA | NA | WA | SA | PDA | MEA | NA |
| R1 | 69 | 27 | 43 | 43 | 4 | 4 | 2 | 2 | 2 | 4 |
| R2 | 63 | 55 | 64 | 50 | 55 | 4 | 3 | 3 | 1 | 4 |
| R3 | 75 | 45 | 43 | 50 | 36 | 4 | 3 | 2 | 1 | 4 |
| R4 | 44 | 64 | 71 | 71 | 27 | ESY-I | ESY-I | ESY-I | ESY-I | ESY-I |
| B1 | 25 | 15 | -50 | 0 | 39 | 7(ZOI) | 7(ZOI) | 5 | 5 | 4 |
| B2 | 13 | 57 | -33 | 25 | 39 | 7(ZOI) | 7(ZOI) | 7(ZOI) | 7(ZOI) | 4 |
| B3 | 88 | 36 | NG | 88 | 54 | 7(ZOI) | 4 | 6 | 7(ZOI) | 7(ZOI) |
| B4 | 13 | 43 | 33 | -17 | NG | 7(ZOI) | 7(ZOI) | 7(ZOI) | 4 | 3 |
| B5 | 38 | 43 | 0 | 13 | NG | 7(ZOI) | 7(ZOI) | 7(ZOI) | 7(ZOI) | 10 |
| B6 | 63 | 89 | 17 | 35 | NG | 7(ZOI) | 7(ZOI) | 4 | 7(ZOI) | 6 |
| B7 | 63 | 68 | -67 | -25 | NG | 7(ZOI) | 7(ZOI) | 7(ZOI) | 7(ZOI) | 6 |
| B8 | 13 | -7 | 17 | -43 | 77 | 7(ZOI) | 7(ZOI) | 7(ZOI) | 4 | 3 |
| B9 | 13 | 15 | -83 | -13 | NG | 7(ZOI) | 7(ZOI) | 4 | 7(ZOI) | 10 |
| B10 | 63 | 57 | NG | 38 | 54 | 7(ZOI) | 7(ZOI),9 | 6 | 5 | 5 |
| B11 | 25 | -6 | -23 | -38 | NG | 7(ZOI) | 5 | 4 | 4 | 6 |
| B12 | 0 | -81 | -23 | -13 | 15 | 7(ZOI) | 5 | 7(ZOI) | 5 | 4 |
| B13 | 13 | 36 | 0 | 13 | 46 | 7(ZOI) | 7(ZOI) | 7(ZOI) | 7(ZOI) | 7(ZOI) |

NG, No growth of pathogen; Every NG is accompanied by respective interaction that justifies the antagonistic inhibitions (% inhibitions) either for pathogen or endophyte respectively

ESY-I/ ESY-II, The endosymbiont interactions from Tables 5 and 6

5.5. DISCUSSION

Microbial communities contribute to the occurrence and function of diversified interactions ongoing between various macro- and micro-organisms in different ecological niches. Such interactions include constant communication between endophytes, epiphytes, pathogens and host plants (Partida et al. 2005; Newton et al. 2010; Rodriguez et al. 2012; Werner et al. 2014; Kusari et al. 2014c). Plants are natural habitats for bi-, tri- and multi-trophic microbial interactions of endophytic microbial community ensuring a certain level of positive impact on host plants against natural pathogens (Clay 2014; May and Nelson 2014). In this study, we focused on two host plants, the liverwort *Radula marginata* and the hemp *Cannabis sativa* L., with similar biosynthetic principles owing to the production of cannabinoids as bioactive secondary metabolites. To ensure a better understanding of host plant fitness benefits due to endophytic contributions against the phytopathogens, we further evaluated the biocontrol functional traits (in terms of antagonism) of endophytic microbial community of *R. marginata* when challenged against the two major phytopathogens of *C. sativa* L. plants, namely *B. cinerea* and *T. roseum*. We challenged the endophytes under five different media conditions, namely WA, SA, PDA, NA and MEA respectively, to justify and compare the potent benefits and challenges encountered by endophytic isolates against the pathogens under changing nutritional conditions. It is immensely important to understand the reaction and stability of endophytes in any microbe-microbe interactions due to biotic selection pressures, outside the host environment. Thus, monitoring the magnitude of biocontrol efficacies under different media conditions not only provide information correlating to the well-known OSMAC (One Strain MAny Compounds) approach but also evaluates the probable contributions and capabilities of endophytes in aiding host fitness against the pathogens.

With this rationale, herein we report for the first time the isolation, identification, biocontrol efficacy, biofilm forming potential, and anti-biofilm ability of endophytic microbial community of the liverwort *Radula marginata*, as compared to bacterial endophytic isolates harbored in *Cannabis sativa* plants. Liverworts are inhabited by numerous fungal species (Ptaszyńska et al. 2010). Endophytes belonging mainly to Ascomycota, Basidiomycota and Zygomycota have been isolated from different species of liverworts with the exception of *R. marginata* (Forrest et al. 2006; Davis and Shaw 2008). In our work, majority of the endophytic fungal isolates belonged to Ascomycota, followed by two isolates belonging to Zygomycota. This is in complete agreement with reports of 97% liverwort endophytes discovered so far belonging to phylum Ascomycota (Davis and Shaw 2008). Moreover, Davis et al. (2003) reported the presence of *Xylaria* sp. belonging to family Xylariaceae (phylum Ascomycota), as one of the major endophytic fungal species of liverworts.

Interestingly, we found a few *Xylaria* sp. and a lot of *Daldinia* sp. (both of which belong to family Xylariaceae) as endophytes of *R. marginata*. This not only highlights the broad host range of Xylariaceae endophytes, but also points towards close relationship between the endophytic fungal communities of liverworts. Needless to mention that this observation can be extended with further investigations of *R. marginata* endophytes prospected from different ecological niches. Although the load of bacterial endophytic microbial community in *R. marginata* was quite low, there was a striking similarity with the bacterial endophytes isolated from *C. sativa* plants.

With the exception of the endosymbiont (*Paenibacillus* sp.), all the bacterial isolates belonged to *Bacillus* sp. which is also the major genus found living in *C. sativa* as endophytes. It is of immense ecological importance to note the presence of similar endophytic bacterial genus in two different plants from different geographical areas but containing similar secondary metabolites. Although *Bacillus* sp. is quite commonly found in various ecological niches, exhibiting an endophytic lifestyle in two different host plants with similar biosynthetic principles is noteworthy. The ecological context is even more highlighted by the presence of *Bacillus subtilis* strain (isolate R1, R2, R3 and B10) in both *R. marginata* and *C. sativa* as endophytes. To gain a deeper insight into the significance of presence of similar bacterial species, the bacterial endophytic isolates were further exploited for their efficacies in retaining certain 'defensive' functional traits like biofilm formation. In addition, the isolates were evaluated for their magnitude of anti-biofilm capacity against the generalist biofilm forming pathogens. An enthralling observation aiding the ecological context of isolates R1, R2, R3 and B10 was their insignificant biofilm formation capacity at both 30°C and 37°C. Apart from these four isolates, there were another two isolates, B5 and B7 (both being identified as *Bacillus pumilus*) that exhibited similar activity of forming significant biofilm only at 37°C. But the isolates R1, R2, R3 and B10 were more intriguing owing to the fact that they were harbored in two different host plants producing cannabinoids, than compared to isolates B5 and B7 harbored only in *C. sativa* plants. Whether the presence of similar species and their functional characteristic of biofilm activity are attributed to similar biosynthetic principles of different host plants needs more plant survey from different geographical locations.

Fungal and bacterial endophytic communities of *R. marginata* were further evaluated for their biocontrol potency against phytopathogens under varying media conditions. As cannabinoids are therapeutically relevant compounds, it is important to look for elimination attempts of diseases caused by constant attacks of various phytopathogens to host plants. As endophytes form a major part of plant habitat, it is worth exploring the endophyte-plant relationship in aiding plant fitness benefits. We observed different endophyte-pathogen

interactions under different media conditions with varying degree of growth inhibition. Interestingly, F12 (*Rhizopus oryzae*) was able to completely inhibit both the phytopathogens in all the five different media by completely overgrowing the pathogen mycelia. Some isolates demonstrated physical defense strategies by causing malformation of pathogen mycelia on contact. This highlights their ability of identifying confronting pathogens under close proximity. This not only point towards the selective ability of isolates towards coexisting with host plants as endophytes, but also their perception of the presence of host plant pathogens. Some isolates were able to perceive unfavorable conditions long before physical proximity, and displayed chemical defense by either releasing visible exudates, forming inhibition zone (halo) or even producing secondary metabolites in form of dark brown to black bands. Sometimes, even the same endophytic isolate was found to display physical and chemical defense strategies only by changing the media conditions. This underlines the endophytic potency of producing cryptic metabolites under slight variation of media conditions. The results further exemplify the fact that establishment of endophytic lifestyles in a particular plant niche is always accompanied by various physical and chemical associations not only with the host plant but also epiphytes, pathogens and neighboring endophytes. Interestingly, in some cases, the pathogen could also perceive confronting endophytes and take necessary actions like malformation of endophytic mycelia, forming inhibition halo and even altering own mycelia color. Taking the bacterial endophytic community into consideration, the major highlight was the presence of an endosymbiotic bacterium with an endophytic fungal isolate. The occurrence of the endosymbiont was only visible when the biotic stress was triggered by challenging with the phytopathogens. The isolate F13 was unable to counter-attack the pathogen in any of the five media conditions. Surprisingly, in all the five media conditions against both pathogens, the endosymbiont was observed protruding out of the fungal mycelia and exhibiting antagonistic potential either by slightly overgrowing the pathogen mycelia or by forming dark black secondary metabolite patterns. This is an intriguing example of tripartite microbe-microbe interaction where the endosymbiont plays the pivotal role in exhibiting biocontrol potency in aiding host plant defense as compared to the fungal endophyte itself. It is also noteworthy that most of the bacterial endophytes of *C. sativa* inhibited *B. cinerea* by completely overgrowing the pathogen mycelia on WA, SA and MEA, except on PDA where the pathogen formed an inhibition zone (halo) to counter the endophyte attack. The bacterial isolates of *R. marginata* were also active against both the phytopathogens, with some causing malformation of pathogen mycelia.

Our overall results primarily reveal the presence of endophytic microbial community of liverwort *Radula marginata*, their functional traits and biocontrol potency against

phytopathogens. It also provides insights into the possibility of harboring similar endophytic bacterial genus in different host plants with structurally similar secondary metabolite production (such as cannabinoids). It also underlines and compares the biocontrol potency of endophytes against phytopathogens of *C. sativa* plants. Admittedly, this is only the first report of *R. marginata* endophytic community; our results, however, can provide a hypothesis that host plants containing similar phytochemicals might harbor same and/or similar endophytic microflora. It would be interesting to evaluate and compare our results with more endophytic communities of this liverwort from different ecological niches to get a better concept of ecological significance of different plants harboring similar endophytes.

6. REFERENCES

- Ansari MW, Trivedi DK, Sahoo RK, Gill SS, Tuteja N (2013) A critical review on fungi mediated plant responses with special emphasis to *Piriformospora indica* on improved production and protection of crops. *Plant Physiol Bioch* 70:403-410
- Arnold AE, Mejia LC, Kyllö D, Rojas EI, Maynard Z, Robbins N (2003) Fungal endophytes limit pathogen damage in a tropical tree. *Proc Natl Acad Sci U S A* 100:15649–15654
- Asakawa Y, Hashimoto T, Takikawa K, Tori M, Ogawa S (1991a) Prenyl bibenzyls from the liverworts *Radula perrottetti* and *Radula complanata*. *Phytochemistry* 30:235-251
- Asakawa Y, Hashimoto T, Takikawa K, Tori M, Ogawa S (1991b) Prenyl bibenzyls from the liverwort *Radula kojana*. *Phytochemistry* 30:219-234
- Berg G, Grube M, Schloter M, Smalla K (2014) Unraveling the plant microbiome: looking back and future perspectives. *Front Microbiol* 5:148
- Brader G, Compant S, Mitter B, Trognitz F, Sessitsch A (2014) Metabolic potential of endophytic bacteria. *Curr Opin Biotechnol* 27:30–37
- Claessen D, Rozen DE, Kuipers OP, Søgaaard-Andersen L, vanWezel GP (2014) Bacterial solutions to multicellularity: a tale of biofilms, filaments and fruiting bodies. *Nat Rev Microbiol* 12:115-124
- Clay K (2014) Defensive symbiosis: a microbial perspective. *Funct Ecol* 28:293-298
- Cornforth DM, Popat R, McNally L, Gurney J, Scott-Phillips TC, Ivens A, Diggle SP, Brown SP (2014) Combinatorial quorum sensing allows bacteria to resolve their social and physical environment. *Proc Natl Acad Sci U S A* 111:4280–4284
- Cullmann F, Becker H (1999) Prenylated bibenzyls from the liverwort *Radula laxiramea*. *Z Naturforsch J Biosci* 54:147–150
- Davis EC and Shaw AJ (2008) Biogeographic and phylogenetic patterns in diversity of liverwort-associated endophytes. *Am J Bot* 95:914-924

- Davis EC, Franklin JB, Shaw AJ, Vilgalys R (2003) Endophytic *Xylaria* (Xylariaceae) among liverworts and angiosperms: phylogenetics, distribution, and symbiosis. *Am J Bot* 90:1661-1667
- Flemming T, Muntendam R, Steup C, Kayser O (2007) Chemistry and biological activity of tetrahydrocannabinol and its derivatives. In: Khan MTH (ed) *Topics in heterocyclic chemistry*. Springer, Berlin, pp 1-42
- Forrest LL, Davis EC, Long DG, Stotler BJC, Clark A, Hollingsworth ML (2006) Unraveling the evolutionary history of the liverworts (Marchantiophyta): multiple taxa, genomes and analyses. *The Bryologist* 109:303-334
- Hamilton CE, Bauerle TL (2012) A new currency for mutualism? Fungal endophytes alter antioxidant activity in hosts responding to drought. *Fungal Divers* 54:39–49
- Happyana N, Agnolet S, Muntendam R, Van Dam A, Schneider B, Kayser O (2013) Cannabinoid analysis of laser-microdissected trichomes of *Cannabis sativa* L. by LC-MS and cryogenic NMR. *Phytochemistry* 87:51-59
- Hartmann A, Rothballer M, Hense BA, Schröder P (2014) Bacterial quorum sensing compounds are important modulators of microbe-plant interactions. *Front Plant Sci* 5:131
- Hoffman MT and Arnold AE (2010) Diverse bacteria inhabit living hyphae of phylogenetically diverse fungal endophytes. *Appl Environ Microbiol* 76:4063-4075
- Kusari P, Kusari S, Lamshöft M, Sezgin S, Spiteller M, Kayser O (2014b) Quorum quenching is an antivirulence strategy employed by endophytic bacteria. *Appl Microbiol Biotechnol* 98:7173-7183
- Kusari P, Kusari S, Spiteller M, Kayser O (2013) Endophytic fungi harbored in *Cannabis sativa* L.: diversity and potential as biocontrol agents against host plant-specific phytopathogens. *Fungal Divers* 60:137-151
- Kusari P, Spiteller M, Kayser O, Kusari S (2014a) Recent advances in research on *Cannabis sativa* L. endophytes and their prospect for the pharmaceutical industry. In: Kharwar, R. N. et al. (eds.) *Microbial Diversity and Biotechnology in Food Security*. Springer, India, pp. 3-15
- Kusari S, Hertweck C, Spiteller M (2012) Chemical ecology of endophytic fungi: origins of secondary metabolites. *Chem Biol* 19:792–798
- Kusari S, Singh S, Jayabaskaran C (2014c) Biotechnological production of plant-associated endophytic fungi: hope versus hype. *Trends Biotechnol* 32:297-303
- Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) *Nucleic acid techniques in bacterial systematics*. Wiley, New York, pp. 115–175

- Ludwiczuk A and Asakawa Y (2008) Distribution of terpenoids and aromatic compounds in selected southern hemispheric liverworts. In: Fieldiana Botany Number 47. Field Museum of Natural History, Chicago, pp. 37-58
- Márquez LM, Redman RS, Rodriguez RJ, Roossinck MJ (2007) A virus in a fungus in a plant: three-way symbiosis required for thermal tolerance. *Science* 315:513-515
- May G and Nelson P (2014) Defensive mutualisms: do microbial interactions within hosts drive the evolution of defensive traits? *Funct Ecol* 28:356-363
- McPartland JM (1991) Common names for diseases of *Cannabis sativa* L. *Plant Dis* 75:226–227
- McPartland JM (1996) A review of Cannabis diseases. *J Int Hemp Assoc* 3:19–23
- Merritt JH, Kadouri DE, O'Toole GA (2011) Growing and analyzing static biofilms. *Curr Protoc Microbiol* 1B: 1.1-1.8
- Na YS and Baek SH (2006) Antimicrobial activity of chlorinated bibenzyl compound. *Korean J Orient Physiol Pathol* 20:719-723
- Na YS, Kim H, Oh HJ, Kim MJ, Baek SH (2005) Antifungal activity of chlorinated bibenzyl compound on the dermatophytic fungus *Trichophyton mantagrophytes*. *Korean J Orient Physiol Pathol* 19:1068-1072
- Newton AC, Fitt BDL, Atkins SD, Walters DR, Daniell TJ (2010) Pathogenesis, parasitism and mutualism in the trophic space of microbe-plant interactions. *Trends Microbiol* 18:365-373
- Park BH and Lee YR (2010) Concise synthesis of (±)-perrottetinene with bibenzyl cannabinoid. *Bull Korean Chem Soc* 31:2712-2714
- Partida-Martinez LP and Hertweck C (2005) Pathogenic fungus harbours endosymbiotic bacteria for toxin production. *Nature* 437:884-888
- Ptaszyńska A, Mułenko W, Żarnowiec J (2010) Bryophytes microniches inhabited by microfungi. *Annales UMCS, Biologia* 64:35-43
- Rheinhold-Hurek B and Hurek T (2011) Living inside plants: bacterial endophytes. *Curr Opin Plant Biol* 14:435-443
- Rodriguez Estrada AE, Jonkers W, Kistler HC, May G (2012) Interactions between *Fusarium verticillioides*, *Ustilago maydis*, and *Zea mays*: an endophyte, a pathogen, and their shared plant host. *Fungal Genet Biol* 49:578-587
- Safari M, Amache R, Esmailshirazifard E, Keshavarz T (2014) Microbial metabolism of quorum-sensing molecules acyl-homoserine lactones, γ -heptalactone and other lactones. *Appl Microbiol Biotechnol* 98:3401–3412

- Toyota M, Kinugawa T, Asakawa Y (1994) Bibenzyl cannabinoid and bisbibenzyl derivative from the liverwort *Radula perrottetti*. *Phytochemistry* 37:859-862
- Toyota M, Shimamura T, Ishii H, Renner M, Braggins J, Asakawa Y (2002) New bibenzyl cannabinoid from the New Zealand liverwort *Radula marginata*. *Chem Pharm Bull* 50:1390-1392
- van der Werf HMG, van Geel WCA (1994) Vezelhenneep als papiergrondstof, teeltonderzoek 1987–1993 Fiber hemp as a raw material for paper, crop research 1987–1993. Report nr. 177, PAGV, Lelystad, the Netherlands, p 62
- Werner GDA, Strassmann JE, Ivens ABF, Engelmoer DJP, Verbruggen E, Queller DC, Noe R, Johnson NC, Hammerstein P, Kiers ET (2014) Evolution of microbial markets. *Proc Natl Acad Sci U S A* 111:1237-1244
- White TJ, Bruns TD, Lee S, Taylor JW (1990) Amplification and direct sequencing of fungal rRNA genes for phylogenetics In *Protocols: A guide to methods and applications*. PCR Academic press, San Diego pp. 315-322

Chapter 6

DISCUSSION AND OUTLOOK

6.1. Endophytic biodiversity of *Cannabis sativa* L. and their antagonistic prospects against phytopathogens

Our work on the investigation of endophytic microbial community harbored in *C. sativa* L. was based on the recent advancements made in devising various strategies of discovering endophytes based on the rationale of their cost-benefit relationship with their hosts in order to exploit their potential beneficial efficacies. Since this plant is protected by national and international legislations and regulations, we sampled and imported the *C. sativa* L. plants from the legal farmer Bedrocan BV Medicinal Cannabis (the Netherlands) with the permission of the Federal Institute for Drugs and Medical Devices (Bundesinstitut für Arzneimittel und Medizinprodukte, BfArM), Germany under the license number 458 49 89. We then isolated a plethora of fungal and bacterial endophytes and subjected them to various culture conditions and parameters and even challenged them (dual-culture antagonistic assays of the fungal isolates) with two major phytopathogens of the *Cannabis* plant, namely *Botrytis cinerea* and *Trichothecium roseum*, which are potent greenhouse threat for the cultivars and known to cause disasters at epidemic scales (Bush Doctor 1985; Barloy and Pelhate 1962). Majority of fungal endophytes belonged to Ascomycota. *Bacillus* sp. constituted the majority of bacterial endophytes. Any plant-fungal interaction is always preceded by a physical encounter between a plant and a fungus, followed by several physical and chemical barriers that must be overcome to successfully establish a plant-endophyte association. Therefore, it is only a matter of chance that a particular fungus establishes as an endophyte for a particular ecological niche, or plant population, or plant tissue, either in a localized and/or systemic manner.

Our target was to evaluate the endophytes within the ecological and biochemical contexts, especially focusing on their biocontrol potential to thwart the host-specific phytopathogens. These led us towards the identification of potent endophytes that not only proved to be promising biocontrol agents against the specific phytopathogens, but also demonstrated qualities of being a natural reservoir of bioactive secondary metabolites. Eleven (for fungal endophytes) and twelve (for bacterial endophytes) different kinds of antagonistic interactions are observed when challenged with the phytopathogens in five different media, namely Sabouraud agar (SA), Nutrient agar (NA), Potato dextrose agar (PDA), Malt extract agar (MEA) and water agar (WA), respectively. This highlights the fact that endophytes are capable of producing different compounds under varying conditions which are otherwise 'cryptic' metabolites. All the fungal endophytic isolates showed antagonistic potency to some extent against either one or both of the phytopathogens in varying the media, but three of the isolates proved to exhibit prominent complete inhibition. Many endophytes started sporulating

in NA, as expected, revealing their response to the unfavorable condition while countering the confronting pathogen. Interestingly, the same endophyte isolates showed various other interesting inhibition patterns like formation of a clear halo (inhibition zone), release of exudates without even physical contact of mycelia, and change of mycelia color among others, which accompanied the inhibitions. Most of the bacterial endophytes inhibited *B. cinerea* by completely overgrowing the pathogen mycelia on WA, SA and MEA, except on PDA where the pathogen formed an inhibition zone (halo) to counter the endophyte attack. Interestingly, in some cases, the pathogen could also perceive confronting endophytes and take necessary actions like malformation of endophytic colony, forming inhibition halo and even altering own mycelia color.

We know that slight variations in the *in vitro* cultivation conditions can impact the range and type of secondary metabolites produced by bacteria and fungi. The varying assortment of antagonisms demonstrated by the endophytes against the host phytopathogens indicates that their efficacies are either due to production of secondary metabolites or the immediate intermediates in the biosynthetic pathway of those metabolites, triggered upon pathogen-challenge. Furthermore, random screening of endophytes in axenic cultures can often lead to rediscovery of known natural products, with a very high possibility of the “cryptic” bioactive molecules (complete agreement with the well-known ‘OSMAC’ approach), not produced under normal lab conditions. Thus, our work was devised and implemented with the scientific rational (which has been proven in a plethora of plant-endophyte systems) of function-based interpretation, i.e., qualitative evaluation of the interactions between the endophytic microbial community and the two host-specific phytopathogens. Our work not only reports endophytes as potent biocontrol agents under suitable conditions but also provides a platform to compare the endophytes of the same plant from different wild populations and collection centers (if accessible) for global scale diversity analysis.

6.2. Comparison and evaluation of endophytic efficacies and functional traits of liverwort *Radula marginata* to that of *Cannabis sativa*

In this study, we focused on two host plants, the liverwort *Radula marginata* and the hemp *Cannabis sativa* L., with similar biosynthetic principles owing to the production of cannabinoids as bioactive secondary metabolites. To ensure a better understanding of host plant fitness benefits due to endophytic contributions against the phytopathogens, we further evaluated the biocontrol functional traits (in terms of antagonism) of endophytic microbial community of *R. marginata* when challenged against the two major phytopathogens of *C. sativa* L. plants, namely *B. cinerea* and *T. roseum*. It is immensely important to understand

the reaction and stability of endophytes in any microbe-microbe interactions due to biotic selection pressures, outside the host environment. Further, we evaluated the bacterial endophytes with respect to their retaining functional traits like biofilm formation and antibiofilm activity against generalist pathogenic biofilm formers. A plethora of endophytic fungi and bacteria were isolated from the liverwort. Although the load of bacterial endophytic microbial community in *R. marginata* was quite low, an enthralling observation aiding to the ecological significance was the similarity of bacterial endophytic isolates of *R. marginata* with *C. sativa* at the species level. Although *Bacillus* sp. is quite commonly found in various ecological niches, exhibiting an endophytic lifestyle in two different host plants with similar biosynthetic principles is noteworthy. Another important observation in context to functional traits was their (similar bacterial endophytic isolates) insignificant biofilm forming capacity at both 30°C and 37°C. Whether the presence of similar species and their functional characteristic of biofilm activity are attributed to similar biosynthetic principles of different host plants needs more plant survey from different geographical locations.

Fungal and bacterial endophytic communities of *R. marginata* were further evaluated for their biocontrol potency against phytopathogens under varying media conditions. With concomitant coevolution of endophytic microorganisms (or 'endophytes') with plants, they (endophytes) have developed biosynthetic pathways leading to a plethora of bioactive secondary metabolites. Such associations have proved beneficial for plant fitness in various ecological niches, often triggered by biotic selection pressures like invading phytopathogens (Arnold et al. 2003; Reinhold-Hurek and Hurek 2011; Hamilton and Bauerle 2012; Ansari et al. 2013; Berg et al. 2014). The different interaction strategies employed by the fungal and bacterial endophytic isolates were highly diverse against the phytopathogens. Furthermore, every interaction in each of the five medium led to a certain degree of growth inhibition of either the pathogen or the endophytic isolate itself. Some isolates demonstrated physical defense strategies with their ability of identifying confronting pathogens under close proximity. Some isolates were able to perceive unfavorable conditions long before physical proximity, and displayed chemical defense by either releasing visible exudates, forming inhibition zone (halo) or even producing secondary metabolites in form of dark brown to black bands. The diversified association of endophytes with phytopathogens under different media conditions highlights the understanding of endophytes' host plant fitness potential under biotic selection pressures like invading pathogens. Taking the bacterial endophytic community into consideration, the major highlight was the presence of an endosymbiotic bacterium with an endophytic fungal isolate. The occurrence of the endosymbiont was only visible when the biotic stress was triggered by challenging with the phytopathogens. This is an intriguing example of tri-partite microbe-microbe interaction where the endosymbiont plays

the pivotal role in exhibiting biocontrol potency in aiding host plant defense as compared to the fungal endophyte itself.

Admittedly, this is only the first report of *R. marginata* endophytic community; our results, however, can provide a hypothesis that host plants containing similar phytochemicals might harbor same and/or similar endophytic microflora. However, it would be interesting to evaluate and compare our results with more endophytic communities of this liverwort from different ecological niches to get a better concept of ecological significance of different plants harboring similar endophytes.

6.3. Attenuation of quorum sensing signaling by endophytes as quantified and visualized by HPLC-ESI-HRMSⁿ and MALDI-imaging-HRMS

Our work exemplifies the association of *Cannabis sativa* L. plants with endophytes under various abiotic and biotic selection pressures leading to the development of different functional traits – an important one being the “quorum quenching” ability of endophytes to thwart invading pathogens without introducing resistance mediating selection pressures. This study provides fundamental insights into the potential of endophytic bacteria as biocontrol- as well as antivirulence agents that might be useful in quorum-inhibiting therapies. In this study, we have used a combination of high performance liquid chromatography high-resolution mass spectrometry (HPLC-ESI-HRMSⁿ) and matrix assisted laser desorption ionization imaging high-resolution mass spectrometry (MALDI-imaging-HRMS) to quantify and visualize the spatial distribution of cell-to-cell quorum sensing signals in the biosensor strain, *Chromobacterium violaceum*. We further showed that potent endophytic bacteria harbored in *Cannabis sativa* L. plants can selectively and differentially quench the quorum sensing molecules of *C. violaceum*. Therefore, the major concept and focus of our research is not inhibition of growth, but antivirulence strategies within an ecological niche. The suppression of virulence factors does not necessarily have to inhibit the growth of the pathogen; rather this should prevent the pathogens from developing resistance to their arsenal of bioactive secondary metabolites (used in chemical defense). This study is the first report of ‘visualizing’ quorum sensing using imaging mass spectrometry in high spatial resolution.

Four endophytic bacterial isolates showed potent quenching capability in the overall violacein production of biosensor strain. Violacein production in *C. violaceum* is due to quorum sensing signaling in an environmental niche via the aid of AHLs (*N*-acylated L-homoserine lactones) Further, the overall violacein being quenched were further analyzed using LC-HRMS/MS using both external reference standards an internal standard. This provided a comprehensive understanding of the correlation between the endophytic bacterial species and their

species-specific and selective ability of modulating different AHLs at different concentrations leading to an overall intervention of *C. violaceum* signaling cascade. Our work, thus, demonstrated that a single bacterial species can mount a multifaceted antivirulence defense strategy by simultaneously targeting the aggregation of different AHLs and modulate them at different concentration levels with the overall goal of minimizing the signaling potential of an invading pathogen. Our investigation of the spatial localization and distribution of the four AHLs in *C. violaceum* by MALDI-imaging-HRMS revealed the release of C6-HSL on the periphery of the colony and successively diffusing into the agar corroborated the concept of CviI/CviR synthase receptor-regulated C6-HSL production followed by free passive diffusion across the cell envelope to accumulate in the local environment (McClellan et al. 1997; LaSarre and Federle 2013). The other three AHLs (C8-HSL, C10-HSL, and 3-oxo-C10-HSL) did not diffuse freely into the agar and were found accumulating in the immediate vicinity of *C. violaceum* that could only be visualized directly below the colony itself. These AHLs were not passively released into the agar as compared to C6-HSL, revealing that they might be actively transported across the cell membrane in a controlled manner as suggested by LaSarre and Federle (2013). Interestingly, C10-HSL and 3-oxo-C10-HSL remnants were observed in the agar in the vicinity of the *C. violaceum* colony, lending evidence to the fact that the endophytes were capable not only of preventing the production of these AHLs by the biosensor strain but also possibly stalled their active transportation post-production.

Almost all Gram-negative bacterial pathogens maintain pathogenicity in their hosts (plants or animals, including humans) by cell-to-cell communication using quorum sensing signaling. Attenuation of these signals will lead to suppression of pathogen virulence without introducing additional resistance-inducing selection pressures. Quorum quenching is one of such antivirulence strategies that are developed by selected endophytic bacteria. The overall strategy is to inhibit specific mechanisms that promote infection and are essential to persistence in a pathogenic cascade (for example, binding, invasion, subversion of host defenses and chemical signaling), and/or cause disease symptoms. It is well-known that endophytes are capable of maintaining mutualistic associations with their host plants, which might lead to co-evolution of certain functional traits (production of bioactive secondary metabolites). However, during their co-existence with host plants, endophytes encounter invasion by a plethora of specific and generalist pathogens. Therefore, in order to survive in their ecological niches, endophytes might evolve additional defense strategies that prevent the pathogens from developing resistance to their arsenal of bioactive secondary metabolites (used in chemical defense). Quorum quenching is one of such antivirulence strategies that are developed by selected endophytic bacteria. This work, thus, highlights an important

biological role played by endophytes in different ecological niches, not only in host plant defense but also in maintaining colonization and their own survival inside plants

6.4. Outlook

The potential of inimitable fungal endophytes adept in biosynthesizing bioactive metabolites, occasionally those imitative to their host plants, has irrefutably been recognized. Endophytes can be accepted as new sources for gene- and drug discovery in medical sciences and will provide, by distinct genomic blueprints, new insights in gene assembly and expression control. Nonetheless, there is still no known breakthrough in the biotechnological production of these bioactive natural products using endophytes. It is imperious to expound the metabolome in endophytes correlating to their host plants on a case-by-case basis to comprehend how the biogenetic gene clusters are regulated and their expression is affected *in planta* and *ex planta* (i.e., by environmental changes and axenic culture conditions). Only a deeper understanding of the host-endophyte relationship at the molecular level might help to induce and optimize secondary metabolite production under laboratory conditions to yield desired metabolites in a sustained manner using endophytes. This can be achieved by challenging the endophytes by specific and non-specific pathogens, especially those attacking their host plants, by devising suitable co-culture and dual-culture setups (qualitative, followed by suitable quantitative experiments). The pathogens encountered can serve as an inducer that might trigger the production of defense secondary metabolites with pro-drug-like properties. Further, it would be interesting to compare our results (which were performed using *C. sativa* L. plants from Bedrocan BV) to those of *Cannabis* plants sampled from different wild and/or agricultural populations from different parts of the world. It would also be interesting to evaluate and compare the endophytic microbial communities of *R. marginata* from different ecological niches to get a better concept of underlying similar and discrete trends of endophytic efficacies and functional traits. Additionally, investigation of *in planta* quorum quenching by endophytes and further elucidating the exact role of AHL-mediated gene expression and regulation within complex ecological niches of multispecies microbial communities would aid in better understanding of the virulence suppression of pathogens and co-evolution of bioactive natural products.

Once the production of a target or non-target natural product with a desired biological activity has been achieved, techniques like genome mining, metabolic engineering and metagenomics could be utilized to influence the manipulation of secondary metabolite production by endophytic fungi or the plant itself by directed infection with beneficial endophytes. Such directed investigation with the scientific rationale of mimicking the natural

plant-endophyte-pathogen interactions should be pursued to warrant a virtually incessant discovery and sustained supply of bioactive pro-drugs against the current and emerging diseases.

Appendix

- I. LIST OF ABBREVIATIONS
- II. LIST OF TABLES
- III. LIST OF FIGURES
- IV. LIST OF ORIGINAL CONTRIBUTIONS
- V. CURRICULUM VITAE

LIST OF ABBREVIATIONS

| | |
|----------------------------|--|
| 1/D _{mn} | Camargo's index |
| 1-D | Simpson's diversity index |
| 3-oxo-C10-HSL | <i>N</i> -(3-oxo-decanoyl)-L-homoserine lactone |
| AHL | <i>N</i> -acylated L-homoserine lactone |
| BC | <i>Botrytis cinerea</i> |
| BLAST | Basic Local Alignment Search Tool |
| bp | Base pair |
| C10-HSL | <i>N</i> -decanoyl-L-homoserine lactone |
| C6-HSL | <i>N</i> -hexanoyl-L-homoserine lactone |
| C8-HSL | <i>N</i> -octanoyl-L-homoserine lactone |
| CDA | Czapek Dox Agar |
| CFS | Cell free supernatant |
| CFU/mL | Colony forming units per milliliter |
| <i>D</i> | Simpson's index |
| d ₃ -C6-HSL | Deuterated <i>N</i> -hexanoyl-L-homoserine lactone |
| DHB | 2,5-dihydroxybenzoic acid |
| DI | Deionized water |
| <i>D</i> _{mg} | Margalef's richness |
| <i>D</i> _{mn} | Menhinick's index |
| DMSO | Dimethylsulfoxide |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxyribonucleotide triphosphate |
| DSMZ | German Collection of Microorganisms and Cell Cultures (<i>Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH</i>), Braunschweig, Germany |
| EMBL | European Molecular Biology Laboratory |
| EMBOSS | European Molecular Biology Open Software Suite |
| eV | Electron volt |
| gDNA | Genomic deoxyribonucleic acid |
| h | Hour |
| <i>H'</i> | Shannon diversity index |
| HCCA | α -cyano-4-hydroxycinnamic acid |
| HCOOH | Formic acid |
| HPLC | High performance liquid chromatography |
| HPLC-ESI-HRMS ⁿ | High performance liquid chromatography electrospray ionization high-resolution mass spectrometry |
| Hz | Hertz |
| ITS | Internal Transcript Spacer |
| kV | Kilovolts |
| L | Liter |
| L/min | Liter per minute |
| LBA | Luria-Bertani Agar |
| LC | Liquid chromatography |
| LC-FTMS ⁿ | Liquid chromatography fourier transform mass spectrometry |
| M | Molar |
| <i>m/z</i> | Mass-to-charge ratio |
| MALDI-imaging-HRMS | Matrix assisted laser desorption ionization imaging high-resolution mass spectrometry |
| MEA | Malt Extract Agar |
| mg | Milligram |
| mg/L | Milligram per liter |
| MIC | Minimal inhibitory concentration |

| | |
|-------------------|--|
| min | Minute |
| min ⁻¹ | Per minute |
| mL | Per milliliter |
| mm | Millimeter |
| NA | Nutrient Agar |
| NB | Nutrient Broth |
| NCBI | US National Centre for Biotechnology Information |
| ng | Nanogram |
| NG | No growth |
| ng/mL | Nanogram per milliliter |
| NI | Not inhibited |
| nm | Nanometer |
| OD | Optical density |
| OSMAC | One Strain Many Compounds |
| PCR | Polymerase chain reaction |
| PDA | Potato Dextrose Agar |
| P_i | Total number of species |
| ppm | Parts per million |
| rDNA | Ribosomal deoxyribonucleic acid |
| RG | Radial growth of pathogen in presence of endophyte |
| rpm | Revolutions per minute |
| rRNA | Ribosomal ribonucleic acid |
| s | Second |
| s ⁻¹ | Per second |
| SA | Sabouraud Agar |
| SD | Standard deviation |
| TR | <i>Trichothecium roseum</i> |
| V | Volt |
| v/v | Volume to volume |
| WA | Water Agar |
| ZOI | Zone of inhibition |
| α | Fisher's log series index |
| Δ 9-THC | Delta 9-Tetrahydrocannabinol |
| μ J | Microjoule |
| μ L | Microliter |
| μ L/min | Microliter per minute |
| μ M | Micromolar |
| μ m | Micrometer |

LIST OF TABLES

| Table | Table legend | Page |
|------------------|--|-------------|
| Chapter 2 | | |
| Table 1 | Important natural cannabinoids and metabolic precursors found in the <i>Cannabis</i> plants | 9 |
| Table 2 | New cannabinoids found in liverwort <i>Radula marginata</i> | 13 |
| Chapter 3 | | |
| Table 1 | Summary of the fungal endophytes isolated from various tissues of <i>C. sativa</i> with their respective strain codes, EMBL-Bank accession numbers, and closest affiliations of the representative isolates in the GenBank according to rDNA ITS analysis. | 32 |
| Table 2 | Different types of dual culture interactions between isolated endophytic fungi and the two host pathogens (<i>B. cinerea</i> and <i>T. roseum</i>) on five different solid media. | 39 |
| Table 3 | Growth inhibition (% antagonism) of the phytopathogen <i>Botrytis cinerea</i> by isolated fungal endophytes of <i>C. sativa</i> on five different media after 15 days, and the respective endophyte-pathogen interaction types | 42 |
| Table 4 | Growth inhibition (% antagonism) of the phytopathogen <i>Trichothecium roseum</i> by isolated fungal endophytes of <i>C. sativa</i> on five different media after 15 days, and the respective endophyte-pathogen interaction types | 43 |
| Chapter 4 | | |
| Table 1 | 16S rRNA based identification of bacterial endophytes isolated from <i>Cannabis sativa</i> L. plants with their respective strain codes and the EMBL-Bank accession numbers | 60 |
| Chapter 5 | | |
| Table 1 | Summary of EMBL accession numbers, maximum identity and | 87 |

most closely related species of endophytic fungal isolates of *Radula marginata*

| | | |
|----------------|---|------------|
| Table 2 | Summary of EMBL accession numbers, maximum identity and most closely related species of endophytic bacterial isolates of <i>Radula marginata</i> | 88 |
| Table 3 | Description of different endophyte-pathogen (fungus-fungus) interactions with respective interaction codes under five different media | 91 |
| Table 4 | Description of different endophyte-pathogen (bacterium-fungus) interactions with respective interaction codes under five different media | 92 |
| Table 5 | Summary of antagonistic inhibition of phytopathogen <i>Botrytis cinerea</i> by fungal endophytes of <i>Radula marginata</i> accompanied by different endophyte-pathogen interactions under five different media | 97 |
| Table 6 | Summary of antagonistic inhibition of phytopathogen <i>Trichothecium roseum</i> by fungal endophytes of <i>Radula marginata</i> accompanied by different endophyte-pathogen interactions under five different media | 98 |
| Table 7 | Summary of antagonistic inhibition of phytopathogen <i>Botrytis cinerea</i> by bacterial endophytes of <i>Radula marginata</i> and <i>Cannabis sativa</i> accompanied by different endophyte-pathogen interactions under five different media | 99 |
| Table 8 | Summary of antagonistic inhibition of phytopathogen <i>Trichothecium roseum</i> by bacterial endophytes of <i>Radula marginata</i> and <i>Cannabis sativa</i> accompanied by different endophyte-pathogen interactions under five different media | 100 |

LIST OF FIGURES

| Figure | Figure legend | Page |
|------------------|--|-------------|
| Chapter 2 | | |
| Fig. 1 | <i>Cannabis sativa</i> L. plants sampled from the Bedrocan BV Medicinal Cannabis (the Netherlands). | 6 |
| Fig. 2 | Representative plates showing emergence of endophytic fungal mycelia from surface-sterilized <i>Cannabis sativa</i> L. plant tissues on water agar media amended with antibiotic (streptomycin, 100 mg/L) | 16 |
| Chapter 3 | | |
| Fig. 1 | Phylogenetic tree based on neighbor-joining analysis of the rDNA ITS sequences of the endophytic fungal isolates obtained from various tissues of <i>C. sativa</i> . The endophytic fungal codes are shown in blue. For the closely related species, the taxonomic names are followed by their respective accession numbers in brackets. Significant bootstrap values (>50%) are indicated at the branching points. The tree has been drawn to scale. | 37 |
| Fig. 2 | Types of endophyte-host pathogen interactions observed in dual culture antagonistic assay. (a-k) Interaction types I-XI, where endophytes are shown on the left and challenging pathogen on the right of the representative Petri plates. | 38 |
| Chapter 4 | | |
| Fig. 1 | Quorum quenching of AHLs produced by biosensor strain <i>C. violaceum</i> by potent bacterial endophytes of <i>C. sativa</i> L. plants. (a) Representative LC-FTMS extracted ion chromatograms of detected AHLs (of control) quenched by CFS of endophytic bacterial strains (shown for strain B4). The ions monitored are displayed in each trace and correspond to the most abundant protonated molecules $[M+H]^+$ using a maximum deviation of 2 ppm. (b) Representative MS/MS spectra showing comparison of the main detected component, C10- HSL (m/z 256.1) in | 67 |

authenticated standard (top) and in the control biosensor strain (bottom).

- Fig. 1** Quorum quenching of AHLs produced by biosensor strain *C. violaceum* by potent bacterial endophytes of *C. sativa* L. plants. (c-f) Quorum quenching responses of the CFS of four endophytic bacterial strains (B3, B4, B8, and B11) against the biosensor strain *C. violaceum* (control). (k) Comparison of bacterial cell count of *C. violaceum* when treated with CFS extracts and when untreated (control). All the data represent the logarithmic value of CFU/mL (\pm SD). **68**
- Fig. 1** Quorum quenching of AHLs produced by biosensor strain *C. violaceum* by potent bacterial endophytes of *C. sativa* L. plants. (g-j) Concentration of the different AHLs (ng mL⁻¹) quenched by the CFS extracts of the four endophytic bacterial strains B3, B4, B8, and B11. Control represents the biosensor strain *C. violaceum*. **69**
- Fig. 2** Localization of the four different AHLs in the biosensor strain and their selective quenching by CFS extracts of the four endophytic bacterial strains B3, B4, B8, and B11. (a,d) Microscopic images of untreated (not challenged by endophyte CFS) *C. violaceum* showing visible production of violacein (violet color). Grey dotted insert shows area shot by laser beam. (b,e) Microscopic images of *C. violaceum* after spraying with suitable matrix showing the crystals of uniform matrix covering the colony and its periphery. Grey dotted inserts show areas shot by laser beam. (c) Localization of C6-HSL ([M+H]⁺; $m/z = 200.12810$; $\Delta < 2$ ppm). (f) Localization of C8-HSL ([M+H]⁺; $m/z = 228.15940$; $\Delta < 2$ ppm). (g) Localization of C10-HSL ([M+H]⁺; $m/z = 256.19070$; $\Delta < 2$ ppm). (h) Localization of 3-oxo-C10-HSL ([M+H]⁺; $m/z = 270.16998$; $\Delta < 2$ ppm). (i) Microscopic image of *C. violaceum* treated with CFS extract of bacterial endophyte strain B8. No visible production of violacein. (j) Remnants of C6-HSL after being quenched by CFS extract of bacterial endophyte strain B8 ([M+H]⁺; $m/z = 200.12810$; $\Delta < 2$ ppm). (k) Remnants of C8-HSL after being quenched by

CFS extract of bacterial endophyte strain B8 ($[M+H]^+$; $m/z = 228.15940$; $\Delta < 2$ ppm). (i) Remnants of C10-HSL after being quenched by CFS extract of bacterial endophyte strain B8 ($[M+H]^+$; $m/z = 256.19070$; $\Delta < 2$ ppm). (m) Remnants of 3-oxo-C10-HSL after being quenched by CFS extract of bacterial endophyte strain B8 ($[M+H]^+$; $m/z = 270.16998$; $\Delta < 2$ ppm). All scale bars represent 1 mm. Insert grey box shows the color-coded relative intensities of the detected AHLs in the panels c, f-h, and j-m.

Chapter 5

- Fig. 1** Biofilm formation by endophytic bacterial isolates of *Cannabis* (a, b) *sativa* and *Radula marginata* (strain numbers B1 to B13 and R1 to R4) in comparison to pathogenic biofilm formers *Pseudomonas aeruginosa* (PAO1), *Staphylococcus aureus* (SA) and *Escherichia coli* (EC) at 30°C (a) and at 37°C (b); NB alone is designated as control. **90**
- Fig. 2** Different endophyte-pathogen (fungus-fungus interactions). (i, ii) Interaction code A; (iii, iv) Interaction code B; (v, vi) Interaction code C; (vii, viii) Interaction code D; (ix, x) Interaction code E; (xi) Interaction code F; (xii) Interaction code G(MC); (xiii, xiv) Interaction code G(OV); (xv, xvi) Interaction code H; (xvii) Interaction code I; (xviii, xix) Interaction code J; (xx, xxi) Interaction code K; (xxii) Interaction code L; (xxiii) Interaction code M; (xxiv, xxv) Interaction code N; (xxvi, xxvii) Interaction code ESY-I; (xxviii, xxix) Interaction code ESY-II; (TR) Pathogen *Trichothecium roseum*; (BC) Pathogen *Botrytis cinerea*. **94**
- Fig. 3** Different endophyte-pathogen (bacterium-fungus) interactions. (i, ii) Interaction code 1; (iii, iv) Interaction code 2; (v, vi) Interaction code 3; (vii, viii) Interaction code 4; (ix) Interaction code 5; (x, xi) Interaction code 6; (xii) Interaction code 7; (xiii) Interaction code 8; (xiv, xv) Interaction code 9; (xvi, xvii) Interaction code 10; (xviii) Interaction code 11; (xix) Interaction code 12; (TR) Pathogen *Trichothecium roseum*; (BC) Pathogen *Botrytis cinerea*. **95**

- Fig. 4** Control plates of *Botrytis cinerea* (BC) and *Trichothecium roseum* (i-x) (TR) in five different media. (i) BC in Nutrient agar plate; (ii) BC in Water agar plate; (iii) BC in Malt extract agar plate; (iv) BC in Potato dextrose agar plate; (v) BC in Sabouraud agar plate; (vi) TR in Nutrient agar plate; (vii) TR in Water agar plate; (viii) TR in Malt extract agar plate; (ix) TR in potato dextrose agar plate; (x) TR in Sabouraud agar plate. **96**

LIST OF ORIGINAL CONTRIBUTIONS

Peer reviewed articles

1. **Kusari P.**, Kusari S., Spiteller M., Kayser O. (2013) Endophytic fungi harbored in *Cannabis sativa* L.: Diversity and potential as biocontrol agents against host plant-specific phytopathogens. *Fungal Divers* 60:137-151
2. **Kusari, P.**, Kusari, S., Lamshöft, M., Sezgin, S., Spiteller, M. Kayser, O. (2014) Quorum quenching is an antivirulence strategy employed by endophytic bacteria. *Appl Microbiol Biotechnol* 98:7173-7183
3. **Kusari P.**, Kusari S., Spiteller M., Kayser O. (2014) Biocontrol potential of endophytes harbored in *Radula marginata* (liverwort) from the New Zealand ecosystem. *Antonie van Leeuwenhoek* 106:771-788

Book Chapter

1. **Kusari P.**, Spiteller M., Kayser O., Kusari S. (2014) Recent advances in research on *Cannabis sativa* L. endophytes and their prospect for the pharmaceutical industry. In Kharwar, R. N. et al. (eds.) Microbial Diversity and Biotechnology in Food Security, *Springer* India, Part I, pp. 3-15

PRESENTATIONS

Oral Presentations

1. Biodiversity of endophytes harbored in *Cannabis sativa* L. and their biocontrol strategies employed against host-specific phytopathogens. At the Fourth Annual CLIB Retreat, Lünen, Germany (14th February 2013)
2. Profiling of quorum sensing response and biocontrol potential of endophytic bacteria harbored in *Cannabis sativa* L. At the Annual Conference of the Association for General and Applied Microbiology (VAAM), Bremen, Germany (13th March 2013). *BIOspektrum* 2013, pp 239: PPAV004

3. Biochemical fingerprinting of endophytes harbored in *Radula marginata* that confer plant fitness benefits. At the 61st International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (GA), Münster, Germany (4th September 2013). ***Planta Med*** 2013, 79: SL78

Poster Presentations

1. Phylogenetic study of endophytes harbored in *Cannabis sativa*. At the Third Annual CLIB Retreat, Bergisch Gladbach, Germany (23rd February 2012)
2. Phylogenetic study of endophytes harbored in *Cannabis sativa*. At the Current Aspects of European Endophyte Research, Reims, France (28th to 30th March 2012)
3. Biodiversity of endophytes harbored in *Cannabis sativa* L. and their biocontrol strategies employed against host-specific phytopathogens. At the Fourth Annual CLIB Retreat, Lünen, Germany (13th February 2013)
4. Elucidating the interspecies and multispecies cost-benefit crosstalk of endophytes harbored in *Cannabis sativa* L. At the Fifth Annual CLIB Retreat, Lünen, Germany (12th February 2014)

Contributed Presentations

1. Endophytic diversity of pharmaceutically important *Cannabis sativa*. At the International Congress on Natural Products Research (ICNPR), 8th Joint Meeting of AFERP, ASP, GA, PSE and SIF, New York, USA. ***Planta Med*** 2012, 78: PI43

CURRICULUM VITAE**Personal information**

| | |
|---------------|--------------------------------|
| Name | Parijat Kusari |
| Nationality | Indian |
| Date of birth | 15 th November 1986 |
| Gender | Female |

Educational qualification

| | |
|---------------|---|
| May 2011-2014 | PhD thesis at the Chair of Technical Biochemistry, Department of Biochemical and Chemical Engineering, TU Dortmund, Germany |
| 2008-2010 | Master of Science (M.Sc.) in Applied Microbiology at KIIT University, India |
| 2005-2008 | Bachelor of Science (B.Sc.) in Botany at Calcutta University, India |

Awards and Fellowship

| | |
|---------------|---|
| May 2011-2014 | PhD Scholarship from CLIB Graduate Cluster Industrial Biotechnology |
| December 2010 | Vice Chancellor's Medal for securing highest CGPA in M.Sc. (Applied Microbiology) |