الـجـمـهـوريـــة الجـزائـــريـــة الـديمقراطـيـة الـشعبيـة
People's Democratic Republic of Algeria

وزارة التـــعليـم العـالـــي والبـحـث العلمـــي
Ministry of Higher Education and Scientific Research

University of Amar Telidji, Laghouat
Faculty of Sciences
Department of Biology

جـامعة عـمـار ثـلـيـجي -الأغـواط –
كلية العلوم
قسم البيولوجيا

Master's Thesis for the Diploma of Applied Microbiology

Biological Sciences

Nature and life Sciences

Theme

Techniques for the isolation and identification of halotolerant *Penicillium* species from saline terrestrial environments

Presented by:

Souhila ROUANE

Zohra ZAABANE

Defended on October 11th, 2020 before Jury Members:

PRESIDENT: Mr. Youcef BOUBRIMA, Assistant Professor A, Department of Biology

EXAMINER: Ms. Djamila AMEUR, Assistant Professor A, Department of Agronomy

SUPERVISOR: Mrs. Djalila TAKHI, Assistant Professor A, Department of Biology

Academic Year 2019-2020

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Acknowledgments

In the name of Allah, Most Gracious, Most Merciful, Praise be to Allah, and blessing and peace be upon our prophet Mohammed, his family and his companions.

The first thanks go to he who created us and made us in the best creation, to he who inspired us the way to reach this point, to the one and the only one, Allah.

When we first started our journey in school, this day seemed so far away. Nonetheless, through the encouragement and the guidance of many special teachers, this day has come. We would like to thank all the teachers that helped us to reach this day.

A special acknowledgment to our supervisor Mrs. Takhi. It is such a pleasure and a great experience to work under the supervision of such a dedicated and inspiring person. The things you taught us are irreplaceable. You have our heartfelt gratitude.

We also would like to express our wholehearted thanks to our families for the generous support they provided us throughout our entire lives, particularly through the pursuit of the master's degree. Because of their unconditional love and prayers, we have the chance to complete this thesis.

Dedication

I dedicate the fruit of my work and my effort to my mother who brought me to this world. I always find a way to thank others, except you. There is no word good enough to thank you mom. Now finally Allah accepted your prayers to see me graduate.

To my dad, I don't believe in superheroes, but you are my superhero. Thank you for raising me the way you did.

I want to dedicate this work to my brothers, sisters and my other family members, especially my sister from another mother Nadjwa.

To my partner Souhila, for her courage and determination in the face of adversity. Despite it all, she did her best in the completion of this work.

Last but not least, to my best friends who supported me through all the past years: Messaouda, Meriem, Loubna, and Marwa. My lovely friends, our beautiful memories together will never fade. You made my graduation years extra special.

I apologize to those I did not mention in these dedications despite their great impression on my life, you are so many. I will just say thank you to all the people whose path crossed mine. I hope I have positively enriched your lives the way you did to mine.

Zohra

Dedication

It is my genuine delight to dedicate this work to:

First and foremost, my beloved parents for their unconditional love, care, and support throughout the years of my life, who's considerate words have taught me to work hard for the things I aspire to achieve. I hope that I have made you proud.

My sisters and brothers, who have never left my side, who have cheered me up in my saddest times. Thank you, for having always been there for me whenever I needed someone to laugh with or a considerate shoulder to lean on. I know I do not say it enough, but I love you all so dearly.

All my family members, aunties, uncles and cousins. You deserve my wholehearted thanks as well, for your support throughout the process.

My best friend and partner Zohra, all my gratitude and appreciation. Thank you for always having my back, for being there through thick and thin during this journey, for being a constant source of support and encouragement and for all your inspiring and hopeful words, I wouldn't have made it without you.

My dearest friends, Messaouda, Meriem, Loubna, for their kind and thoughtful words, for their understanding and encouragement in many, many dark moments. Our friendship makes my life wonderful.

All the people who touched my heart.

Souhila

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Chapter 2 - A preview on halotolerant *Penicillium* species diversity in some saline terrestrial habitats

Techniques for the isolation and identification of halotolerant *Penicillium* species from saline terrestrial environments

Souhila ROUANE and Zohra ZAABANE

Abstract

Penicillium is a well-characterized fungus found in a diverse range of habitats especially in soil. This genus is one of the dominant mycobiota in hypersaline terrestrial environment. Traditionally, morphological identification methods were used to identify fungi such as *Penicillium*. However, with the emergence of more accurate and rapid molecular identification tools, scientists embraced the polyphasic approach to address fungal diversity study challenges. The aim of our study is presenting a summary of isolation and identification techniques of halotolerant species of *Penicillium* as well as a preview on their diversity in some saline terrestrial habitats. From all the studies analyzed, 67 isolates were distributed on 47 different species that have been isolated from 11 different regions of saline soils, in which these regions have different physicochemical properties. The distribution of different *Penicillium* species varies from a region to another. The species variability is likely due to the variation in physicochemical characteristics of each studied habitat, such as pH, salinity, temperature… etc. The isolated *Penicillium* species exhibited different tolerance levels to these parameters. Most of the isolated species were alkalotolerant (67.19%), whereas some were acidotolerant (32.81%). All of the isolates were halotolerant tolerating up to 20% NaCl, and a few could tolerate up to 22% of NaCl on solid medium. However, these isolates were unable to grow in salinity beyond 15% NaCl in liquid cultures. In all of the cited studies, *Penicillium* was one of the most dominant genera, with *P. chrysogenum* as the most abundant specie (7.46%). Four studies about non-saline soils were used as an element of comparison. These soils shared 22 similar species (46.81%) with those found in the explored saline soils. *Penicillium* species can enhance sporulation and produce various secondary metabolites to adapt to extreme environmental conditions. Furthermore, the diversity of micromycetes community in hypersaline habitats is more dependent on the physicochemical parameters of habitat than on the geographical location of their habitat. The species inhabiting extreme environments can be used as unique sources of enzymes or secondary metabolites, tolerant to extreme conditions, with a biotechnological utility.

Key words: *Penicillium*, identification techniques, isolation methods, halotolerance, saline soils, fungal diversity, physicochemical parameters.

Techniques pour l'isolement et l'identification des espèces halotolérantes de *Penicillium* d'environnements terrestres salins

Souhila ROUANE and Zohra ZAABANE

Résumé

Penicillium est un champignon bien connu, qui se trouve dans une gamme diversifiée d'habitats, en particulier dans le sol, et l'une des espèces de mycobiota qui domine les environnements terrestres hypersalins. Initialement des méthodes d'identification morphologique ont été utilisées ; mais avec l'émergence de méthodes plus précises et outils d'identification moléculaire rapide, les scientifiques ont adopté l'approche polyphasique pour relever les défis de la diversité. Le but de notre étude est de présenter un résumé des techniques d'isolement et d'identification des espèces halotolérantes de *Penicillium* ainsi qu'un aperçu de leur diversité dans certains habitats terrestres salins. De toutes les études analysées, 67 isolats ont été distribués sur 47 espèces différentes qui ont été isolées de 11 régions différentes de sols salins, dans lesquelles ces régions ont des propriétés physico-chimiques différentes. Compte tenu de la répartition des espèces de *Pénicillium* sur ces régions elles diffèrent d'une région à l'autre en raison des variations de pH, de salinité, de température, etc., chaque espèce peut tolérer une certaine valeur de ces paramètres. Par conséquent, sur la base de ces paramètres, la plupart des espèces sont alcalotolérantes (67.19%) tandis que certaines d'entre elles sont acidotolerantes (32.81%), également tous les isolats sont halotolérants qui pourraient tolérer 20% de NaCl, et peu pouvaient tolérer 22% de sel en milieu solide, mais ils ne pouvaient pas croître au-delà de 15% de NaCl dans des cultures liquides. Dans toutes les études, *Pénicillium* était l'un des genres les plus dominants, dans lequel *P. chrysogenum* était l'espèce la plus abondante (7,46%). Nous avons investiguer quatre études sur des régions de sols non salins ; nous avons constaté qu'elles partageaient 22 espèces similaires avec un pourcentage de 46.81% avec celles trouvées dans les sols salins. Les espèces de *Pénicillium* peuvent améliorer la sporulation et produire divers métabolites secondaires pour s'adapter à des environnements extrêmes. De plus, la diversité de la communauté des micromycètes en habitats hypersalins est plus dépendante des paramètres physico-chimiques de l'habitat que de la position géographique. En outre, les espèces vivant dans des environnements extrêmes peuvent être utilisées comme sources uniques d'enzymes ou de métabolites secondaires à potentiel biotechnologique ou pharmaceutique.

Mots-clés : *Penicillium*, halotolérance, milieux terrestres salins, diversité fongique, paramètres physico-chimiques, isolement, identification.

تقنيات عزل و تحديد أنواع فطر *Penicillium* المتحملة للملوحة من بيئات أرضية مالحة

سهيلة روان و زهرة زعبان

البنسليوم (*Penicillium*) هو نوع من الفطريات الشائعة، والتي توجد في مجموعة متنوعة من البيئات وخاصة في التربة، وواحد من أنواع الفطريات التي تهيمن على البيئات الأرضية شديدة الملوحة. في البداية تم استخدام التحديد المورفولوجي للتمييز بين السلالات، لكن مع ظهور أدوات التحديد الجزيئي أكثر دقة، تبنى العلماء النهج متعدد الأطوار لمواجهة تحديات التنوع. الهدف من دراستنا هو تقديم ملخص لتقنيات العزل والتعرف على أنواع البنسليوم المتحملة للملوحة بالإضافة إلى معاينة تنوعها في بعض البيئات الأرضية المالحة. من جميع الدراسات التي تم تحليلها، تم توزيع 67 عزلة على 47 نوعًا مختلفًا تم عزلهم من 11 منطقة مختلفة من التربة المالحة، حيث تتميز هذه المناطق بخصائص فيزيائية كيميائية مختلفة. بالنظر إلى توزيع أنواع البنسليوم في هذه المناطق، فإنه يختلف من منطقة إلى أخرى بسبب الاختلافات في درجة الحموضة والملوحة ودرجة الحرارة وما إلى ذلك، حيث يمكن لكل نوع تحمل قيمة معينة لهذه المؤشرات. لذلك، بناءً على هذه المؤشرات، فإن معظم الأنواع تتحمل درجة الحموضة قاعدية (67.19) في حين أن بعضها مقاوم للأحماض (32.81)؛ كما أن جميع العزلات لديها قدرة تحمل للملوحة يمكنها من تحمل 20٪ من NaCl. وقليل منهم فقط يمكن أن يتحمل 22٪ من NaCl في الوسط الصلب، ومع ذلك لم يتمكنوا من النمو فوق 15٪ من NaCl في الوسط السائل. في جميع الدراسات، كان البنسيليوم من أكثر الأنواع انتشارًا، حيث كان *P.chrysogenum* هو المهيمن (7.46٪). قمنا بالتحقيق في أربع دراسات حول مناطق التربة غير المالحة؛ ووجدنا أنها تشترك في 22 نوعًا مشابهًا بنسبة 46.81% لتلك الموجودة في التربة المالحة .يمكن أن تعزز أنواع البنسليوم التبوغ وتنتج أيضات ثانوية مختلفة للتكيف في البيئات القاسية. علاوة على ذلك، فإن تنوع مجتمع الفطريات الدقيقة في المناطق شديدة الملوحة يعتمد بشكل أكبر على المؤشرات الفيزيائية والكيميائية للبيئة منه على الموقع الجغرافي.يمكن استخدام الأنواع التي تعيش في البيئات القاسية كمصادر فريدة للإنزيمات أو للأيضات الثانوية للتكنولوجيا الحيوية أو الإمكانيات الصيدلانية.

الكلمات المفتاحية: Penicillium، الأحياء المتحملة للملوحة، البيئات الأرضية المالحة، تنوع الفطريات، المؤشرات الفيزيائية والكيميائية، العزل، التحديد.

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Abbreviations list

- **bp:** base pair.
- **°C:** Degree Celsius.
- **18S:** ribosomal RNA 18 (S: Svedberg).
- **a_w:** Water Activity.
- **CaM:** Calcium Modulated protein.
- **Cct8:** Chaperonin Containing TCP1 Subunit 8.
- **Cd:** Cadmium.
- **CFU:** Colony Forming Uni.
- **CREA:** Creatine Sucrose Agar.
- **CYA:** Czapek Yeast Extract.
- **CzA:** Czapek's medium.
- **EC:** Electrical Conductivity.
- **ETS:** External Transcribed Spacer.
- **Fig:** Figure.
- **G25N:** 25% Glycerol Nitrate Agar.
- **H_2O_2:** Hydrogen peroxide.
- **IGS:** Inter-Genic Spacer.
- **ITS:** Internal Transcribed Spacer.
- **kDa:** kilodalton.
- **LSU:** large subunit.
- **Lsu-rDNA:** Large Subunit Ribosomal Deoxyribo -Nucleic Acid.
- **MA2:** 2% malt agar.
- **MEA:** Malt Extract Agar.
- **NaCl:** Sodium chloride.
- **NCBI:** National Center for Biotechnology Information.
- **NTS:** Non-Transcribed Spacer.
- **Opt:** Optimum.
- ***P.*: *Penicillium*.**
- **PCA:** Potato-Carrot Agar.
- **PCR:** Polymerase Chain Reaction.
- **PDA:** Potato Dextrose Agar.
- **rDNA:** Ribosomal Deoxyribo-Nucleic Acid.

- **RNA:** Ribo-Nucleic Acid.
- **RPB:** RNA Polymerase II (B) subunit.
- **rRNA:** Ribosomal Ribo-Nucleic Acid.
- **SA:** Atarvation Agar.
- **SEA:** Soil Agar with rose Bengal and glucose.
- **SMs:** Secondary metabolites.
- **Spp/ sp:** Species.
- **SSU:** small subunit.
- **TEF-1α:** Translation Elongation Factor 1α.
- **Tsr1:** Ribosome Maturation Factor 1.
- **YES:** Yeast Extract Sucrose Agar.

Glossary

Acidotolerant: a microorganism capable of growing at low values of pH (such as 4), which might also grow at neutral and slightly alkaline pH (Rai and Gaur, 2001).

Alkaliphilic: The term alkaliphilic microorganisms or "alkaliphiles," generally refers to microorganisms that grow optimally at alkaline pH (9 to 10–13) (Preiss et al., 2015).

Alkalitolerant: they are organisms that grow optimally at neutral pH as well as their ability to grow at alkaline pH (Seckbach, 1999). However, they cannot normally grow above pH 9.5 (Seckbach, 2000).

Anamorph: an asexual reproductive stage of a fungus by producing asexual spores (Samson and Pitt, 1986).

Antimicrobial activity: a derived term from the Greek *anti* (against), *micro* (little) and *bios* (life). It refers to all chemical agents capable of killing or slowing the growth of other microbes (Asif, 2017).

Ascospores: sexual spores that result from meiosis, they are produced in an ascus. These spores are specific to fungi classified in *Ascomycetes* (Samson and Pitt, 1986).

Branch of *Penicillium*: an element in the *Penicillus* between metulae and stipe (Samson and Pitt, 1986).

β –tubulin (BenA): a type of tubulins, which is encoded by two genes β1 and β2**,** but most of the *Ascomycetes* have only a single β_1-tubulins. β-tubulin genes have different roles in hyphal such as growth and fungicide resistance (Zhao et al., 2014), and it is the best option for a secondary identification marker for fungi (phylogenetic classification) (Visagie et al., 2014).

Calmodulin (CaM): a ubiquitously expressed 16.7kDaCa^{2+} binding protein best known for its ability to activate CaM-dependent kinases in a variety of cells (Hill and Olson, 2012). CaM is used as a secondary marker options for identification of fungi (phylogenetic classification) (Visagie et al., 2014).

Chestnut soil: or Kastanozems (from Latin, castaneo, chestnut, and Russian, zemlja, earth), is composed of lighter-colored topsoils as well as intense accumulations of calcium carbonate or gypsum, and it mainly occur in the driest section of the steppe zone (Nachtergaele, 2017).

Cleistothecium: a nonostiolate ascocarp with a well defined peridium (Samson and Pitt, 1986)

Colony: a population of cells visible to the naked eye (Krieger, 2010). CFU is also defined as single, viable propagules that produce a single colony on an appropriate semisolid growth medium (Krieger, 2010).

Conidium (plural **conidia):** an exogenous mitotic spore produced during the asexual reproduction by microscopic fungi.

Conidiophore: the complete fruiting structure which bears conidia (Samson and Pitt, 1986).

DNA barcode: a short-standardized sequence of DNA (400-800 bp) which should be easily characterized for all species on the planet (Kress and Erickson, 2008).

Edapho-climatic factors: consist of type of soil, organo-mineral status, geomorphology, vulnerability to hydro-wind erosion, rainfall, etc. which are one of the factors that determine the dynamics of biodiversity (Butare et al., 2004).

Electrical conductivity: or specific conductance is the measure of the amount of electrical current a material can carry, it is denoted by the symbol σ and has SI units of siemens per meter (S/m) (Helmenstine, 2020).

Exudate: droplets of liquid exuded by colonies on the obverse (Samson and Pitt, 1986).

Fasciculate or Coremiform: hyphae and /or conidiophores in little groups (Flannigan et al., 2011), due to aggregation of conidiophores into upright fascicles or bundles (McGinnis, 1980).

Filamentous fungi: in the filamentous fungi, hypha is the basic unit of structure, the hyphae form a mycelium when assembled together (Peberdy, 1980). The individual hyphae are tube-like structures that display polarised growth and may form branches at sub-apical sites (Peberdy, 1980).

Floccose or lanose: a fungal colony having a wooly texture (Reiss et al., 2012) as a result of being covered with or consisting of woolly tufts (Stevenson and Waite, 2011).

Halophiles: microorganisms able to adapt and live in hypersaline habitats (Niknejad et al., 2013).

Halotolerant: are microorganisms able to survive in the presence as well as in the absence of salt (Niknejad et al., 2013).

Hypha (plural **hyphae**)**:** a long branching filamentous structure of a fungus, an *Oomycete*, or an *Actinobacterium* (Kiyasudeen et al., 2016). Hyphae normally grow away from one another to form mycelium. It is also the filamentous vegetative growth mode of most fungi (Ivarsson et al.,2020).

Immuno-competent: or immuno-competence a word used to describe the overall level of function of the immune system (Rose and Mackay, 2014).It is the ability to produce an effective immune response (Jackson et al., 2020).

Immuno-suppressants: drugs prescribed to avoid rejection of transplanted tissues and organs. They are also used in the treatment of autoimmune diseases (Fireman et al., 2004).

Loam soil: is an approximately equivalent mixture of clay, sand, and silt, in which they construct an optimal mixture of macro and micropores (Kaufmann and Cleveland, 2008).

Mesophiles: are microorganisms that grow best in moderate temperature, neither too hot nor too cold; they have growth optima around 20 to 45°C (Boundless, 2020).

Metulae: a specialized cell giving rise to a verticil of phialides (Samson and Pitt, 1986).

Monophyletic group: in phylogeny, a group of organisms descending from a single shared ancestor (Madigan et al., 2019).

Mycobiota (plural noun, no singular)**:** are a group of all the fungi present in a particular area or geographic region (Chander, 2018).

Mycotoxins: are large varieties of toxic secondary metabolites produced by fungi commonly found in the environment (Sariaslani and Gadd, 2014).

Phialides: are vase-shaped cells that develop on conidiophores, and who produce chains of spores, they are very common among *Ascomycetes* (Watkinson et al., 2016).

Phylogenetic: it is the study of evolutionary relationships among various species. According to evolutionary theory, the similarity in structure and function of molecules like DNA and proteins between organisms and groups of organisms is attributable to their descent from a common ancestor (Choudhuri, 2014).

Phyllosphere: the aerial region of the plant colonized by living organisms such as fungi, algae, protozoa, and nematodes that inhabit the leaves and stem surfaces (Jørgensen and Fath, 2008).

Polyphasic approach: it is a technique used for the identification and the classification of living organisms by combining morphological, physiological, biochemical and molecular characters (Adan and Samson, 2011).

Polyphyletic group: a group in which there are two or more separate groups, each with a separate common ancestor (Simpson, 2010).

Primary metabolites: metabolites excreted during the exponential growth phase of the microorganisms, these metabolites are essential for cells growth because they are formed as part of energy metabolisms (Madigan et al., 2012).

Psychrotolerant microorganisms: organisms able to grow at low temperatures with much lower growth rates. Their optimal growth is achieved in the range of mesophilic organisms (20°C - 40°C) (Hoover and Pikuta, 2010).

Secondary metabolites (SMs): are compounds non-essential for the growth and reproduction of the producing organism. These SMs allow to the organism to survive in their ecological niches (Gupta et al., 2014). In Microorganisms, SMs are produced in the late exponential growth phase and during the stationary phase (Madigan et al., 2012). Their production is often related to the process of sporulation in sporulating microorganisms

Solonchaks: (from Russian, sol, salt) are soils distinguished by their high concentration of soluble salts (Nachtergaele, 2017).

Sporulation: is a complex series of events in cellular differentiation (Madigan et al., 2012), influenced by environmental and endogenous biological conditions and it leads to the formation of spores which are specialized cells that function as resting or dispersal propagules (Su et al., 2012).

Sterigma (Phialides) (plural, **sterigmata**)**:** the differentiated conidium-producing cell of *Penicillium* and related genera. The sterigma, as applied to the *Penicillia*, is a cell with a tubular body of fairly typical length and diameter that is characteristically narrowed to a conidium producing tube, or tip, from which unicellular conidia are cut off successively to form a chain of varying lengths (Refai et al., 2015).

Stipe (stalk)**:** a specialized hypha arising from a fertile hypha and supporting the *Penicillus* or conidial apparatus of *Aspergillus* and *Penicillium* (Samson and Pitt, 1986).

Synnema (plural **synnemata**)**:** in the synnematous microfungi, conspicuous stipes or fascicles can be formed by, a more or less, compacted group of long, erect and sometimes fused conidiophores which support the conidia (Heredia et al., 2018).

Teleomorph: refers to the state of a fungus which bears sexual spores such as ascospores or basidiospores (Samson and Pitt, 1986).

Tufa: is a chemical sedimentary rock developed of calcium carbonate that emerges around calcium-rich groundwater seepages or springs (Cairncross, 2010).

Ubiquitous: living organisms which are found in a diverse range of habitats and present in many environments (Visagie et al., 2014; Yadav et al., 2018).

Verticils: a cluster of more than two metulae or phialides with a common origin (Samson and Pitt, 1986).

Introduction

Extreme environments on Earth, that present unusual physicochemical features (salinity, pH, pressure, temperature… etc) (Niknejad et al., 2013), have been of great interest to microbiologist since the discovery of extremophilic microorganisms (Cimerman et al., 2005). The study of the biodiversity of organisms that thrive in these extreme environments is essential to understanding the limits of life as we know it, and with each new organism discovery, these limits are pushed further.

The term 'hypersaline environment' is defined as an extreme environment with a higher salinity than seawater; they comprise hypersaline waters and soils. In addition to salt several other factors (temperature, pH, pressure etc.) may limit the growth of organisms (Dion and Nautiyal, 2008). There are two categories of microorganisms that live in hypersaline habitats, the ones that adapt are considered halophiles and the ones that are able to survive in the presence as well as the absence of salt are halotolerant (Niknejad et al., 2013).

One of the well-characterized fungi families is the *Trichocomaceae* (Samson and Houbraken, 2011). The genus *Penicillium* is a member of this family and is considered one of its most important genera (Korejo et al., 2014), because of its worldwide spread and its important roles in breaking down organic materials (Moretti and Susca, 2017).

In general, the mycobiota that inhabit hypersaline environments are dominated by species of *Aspergillus*, *Penicillium* and some of their related teleomorphic genera (Chamekh et al., 2019).

The genus *Penicillium* is especially common in soil (Kubátová et al., 2018), covering 67% of the total fungal mass in the soil (Dhakar et al., 2013). Species of *Penicillium* are often slight to moderate halotolerant or extreme halophiles, even though some species cannot tolerate even low concentration of sodium chloride (Cimerman et al., 2005).

The members of *Penicillium* genus have numerous biotechnological applications due to its low-cost production of valuable chemicals, enzymes, vitamins, proteins and organic molecule (Ali et al., 2016). *Penicillium* species have been recognized as a prosperous source of bioactive secondary metabolites (Korejo et al., 2014), including antibacterial, antifungal substances, immuno-suppressants (Ali et al., 2011), anticancer agent, insecticidal and nematicidal activities (Korejo et al., 2014). The biological activities of these secondary metabolites have been proved and identified (Ali et al., 2011). However, it also has harmful sides because it can be a source of mycotoxins and can cause food spoilage (Ali et al., 2016).

The purposes of this study are:

- Presenting a summary of isolation and identification techniques of halotolerant specifies of *Penicillium.*
- Presenting a preview on halotolerant *Penicillium* species diversity in some saline terrestrial habitats.

Chapter 1

Bibliographic Synthesis

I. History of the discovery of *Penicillium*

It has been more than 200 years since Link (1809) introduced the generic name *Penicillium* (Visagie et al., 2014; Moretti and Susca, 2017). Since then, the genus *Penicillium* has been given more than 1000 names (Visagie et al., 2014; Singh and Khajuria, 2018). In 1981 the ICBN has reviewed the generic name of *Penicillium* (Samson and Pitt, 1986; Samson and Pitt, 1990).

The genus *Penicillium* was first discovered and described by Johann Heinrich Friedrich Link (1809) in his book, "Observations in Ordines *Plantarum Naturales*" and he described three species of *Penicillium* (Gupta, 2016). After that, between 1837 and 1839, Corda illustrated the morphology of several *P*. species (Refai et al., 2015).

The works on identification of *Penicillium* started with Dierckx in 1901 (Peberdy, 1987), then followed by Thom who was the first to publish the group concept of classification of *Penicillium* in 1915 (Refai et al., 2015).

In 1928, Scottish biologist, Sir Alexander Fleming, became the first to discover a natural powerful antimicrobial fungus by noticing a halo of inhibition around the *Staphylococcus* bacteria colonies in a plate culture. This fungus is now known as *P.notatum* (Refai et al., 2015). With help from a chemist, he named the substance extracted from the fungus "*Penicillin"* (Refai et al., 2015).

The *Penicillium* modern concept of classification, was derived from revisions of Thom, who revised all species described until 1930 (Visagie et al., 2014; Refai et al., 2015) and then accepted 300 species (Visagie et al., 2014). Later, in 1949 Raper and Thom published their famous ''Manual of the *Penicillia*'' (Refai et al., 2015) where they accepted 137 species (Visagie et al., 2014). The genus Talaromyces for the ascosporic species of *Penicillium* was created by Benjamin in 1955 (Refai et al., 2015). Later *Penicillium* species were recognized by Pitt (1979) in his book "The genus *Penicillium* and its teleomorphic states *Eupenicillium* and *Talaromyces*" who described four subgenera (Refai et al., 2015) and accepted 150 species (Visagie et al., 2014). In 1982, the "Manual and Atlas of the *Penicllia*" was published by Carlos Ramirez, in which 227 species and varieties of *Penicillium* were described (Refai et al., 2015).

II. Characteristics of the genus *Penicillium*

The genus *Penicillium* is composed of filamentous (Gupta, 2016) anamorphic fungi, phylogenetically belonging to the family *Trichocomaceae* (Samson and Houbraken, 2011). These fungi, whose name is derived from the latin *Penicillus*, for "*little brush*" (Samson and Pitt, 1986; Pitt and Hocking, 2009), produce conidiophores looking like little brushes (Samson and Houbraken, 2011).

The genus *Penicillium* is one of the most common eukaryotic life forms on earth (Shalini and Robinka, 2018). It has more than 350 species (Nigam et al., 2018); some of them play important roles in breaking down of organic materials and causing destructive rots in the food industry, where they produce a wide variety of mycotoxins (C.M. Visagie et al, 2014; Moretti and Susca, 2017). Because of the saprophytic nature of *Penicillium*, it rarely causes invasive infection in immuno-competent individuals (Zander et al., 2008).

A. Habitat

Penicillium is a well-known fungus, which is found in a diverse range of habitats (ubiquitous) (Visagie et al., 2014; Yadav et al., 2018). Since 1957, several novel species have been isolated from soil, air, phyllosphere, endophytic tissue of stems, roots, seeds, marine saline soil, mangroves, cold environments, different fruit surfaces, termite mounds, gut caterpillars, and in various food products (Yadav et al., 2018). *Penicillium* main function in nature is the decomposition of organic material (Visagieet al., 2014).

Most of *Penicillium* species are strictly aerobic; do not need much nutrition, thus able to grow in a variety of physicochemical conditions (Moretti and Susca, 2017). Some *Penicillium* species are able to grow and tolerate extreme conditions such us low/ high temperature, salinity and acidic pH (Yadav et al., 2018):

- **Temperature of growth**

Most of the *Penicillium* foods borne species are psychrotolerant, some of them are hardly able to grow at 37 °C. Generally speaking, *Penicillium* species are mesophilic (Moretti and Susca, 2017). The majority grow optimally between 20°C and 30°C (Peberdy, 1987). However, the consensus is that temperatures near 25°C are the most suitable for *Penicillium* species (Samson and Pitt, 1986; Peberdy, 1987; Moretti and Susca, 2017).

- **Water Activity a_w Demand**

A large number of *Penicillium* species can grow at water activities below 0.85 (Peberdy, 1987). Magan and Lacey (l984) found that the minimum water activity for the spore's

germination for a number of *Penicillia*, at 25°C, ranged from 0.79 to 0.83; whereas for sporulation, the range was 0.83-0.89 (Peberdy, 1987).

- **Hydrogen-Ion Concentration (pH) requirement**

The genus *Penicillium* is able to grow under acidic, neutral or even alkaline pH (Yadav et al., 2018). However, most of *Penicillium* species grow well in a pH ranging between 3.0-8.0 (Peberdy, 1987).

- **Salinity tolerance**

Penicillium strains are able to tolerate high concentrations of NaCl (Leitão and Enguita, 2015). Nevertheless, salinity tolerance of *Penicillium* species is between 0-17% of NaCl concentration (Raghukumar, 2017).

B. The identification and classification of *Penicillium* genus

1. Structure and morphology of *Penicillium*

a. Macromorphology of *Penicillium* species

The study of the macromorphology of *Penicillium* species is based on the observation of the cultural characteristics of their colonies, with the naked eye and/or using a stereomicroscope, on different media and at different temperatures (Visagieet al., 2014).

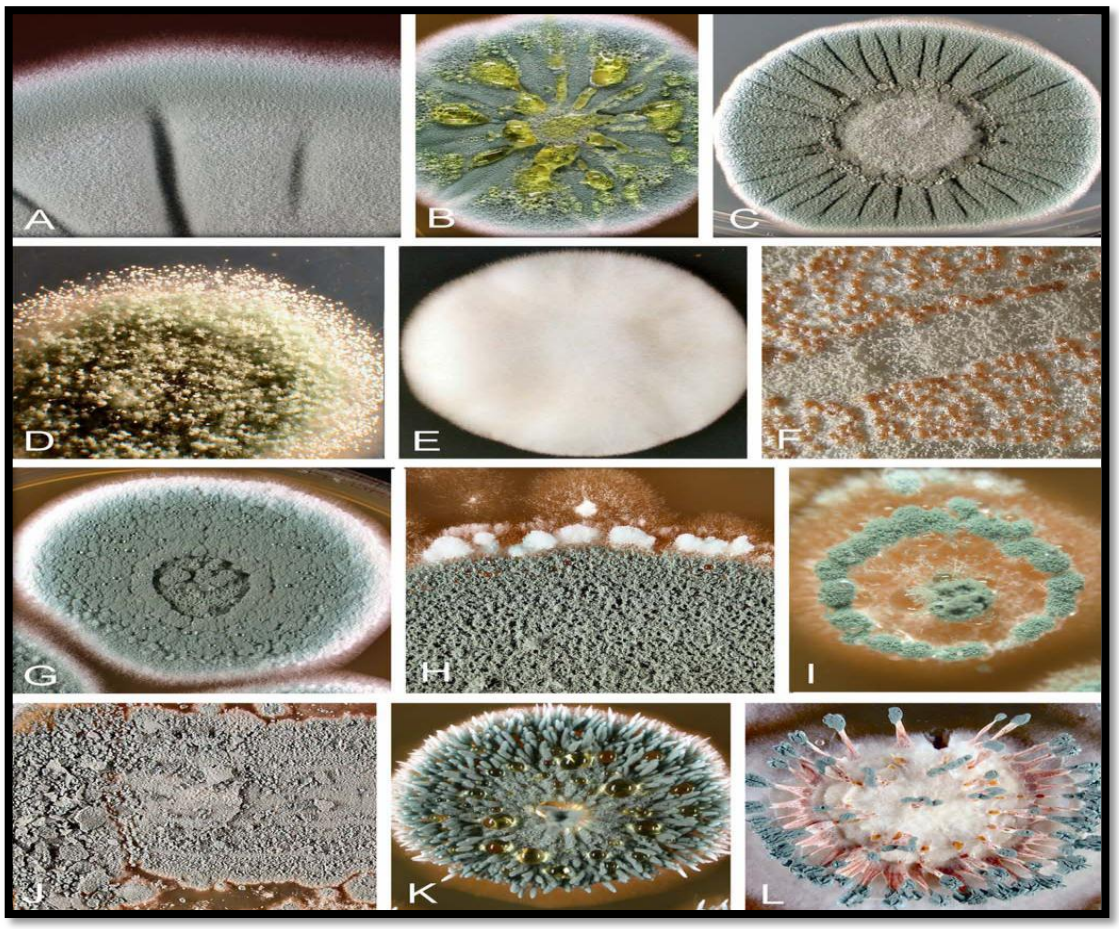
i. Appearance and texture of colonies

One of the most important macromorphological characteristic for identification is the appearance of the cultures, including colony growth, texture, colors, exudate production, the colony reverse…etc (Samson and Pitt, 1986).

The characteristic used for the description of cultures is often colony texture. Various states may be seen in one colony or during its development that lead to differences among the textures. These are not always easy to recognize (Peberdy, 1987). In the *Penicillium* genus we can recognize several textures (Fig. 1), such as Velvety colonies, Floccose or lanose colonies, Fasciculate or Coremiform colonies (Refai et al., 2015).

ii. Color of *Penicillium* colonies

The colors of *Penicillium* species colonies are quite wide. Furthermore, the same strain gives distinct colors and aspects on different media (Fig. 01) (Refai et al., 2015).



Source: Frisvad and Samson, 2004.

Fig. 01: Appearance, color and texture of *Penicillium* colonies.

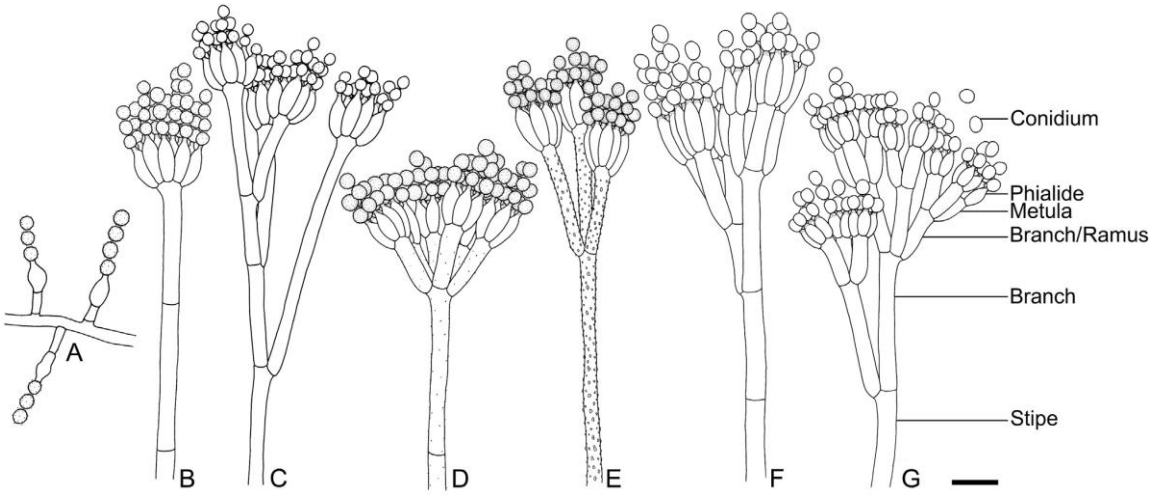
A-B. Velvety colony of *P. persicinum* and *P. chrysogenym*, **B.** typical yellow exudate in *P. chrysogenum*, **C. Velvety colony** of *P. commune* later becoming more fasciculate, **D.** large and compact conidial heads of *P, brevicompactum* **E. Floccose colony** in *P. camemberti*, **F. Sclerotia** in *P. olsonii*, **G-I. Fasciculate colonies** of *P. expansum*, **J.** crusts of conidial masses of a 10 day old colony of *P. crustosum*, **K-L.** Synnematous growth in *P. clavigerum* and *P. vulpinum*.

b. Micromorphology of *Penicillium*

The brush-like structure of the *Penicillium* genus corresponds to the reproductive asexual apparatus known as the "*penicillus*". It produces exogenous mitotic spores called conidia (Fig. 02) (Moretti and Susca, 2017).

The *Penicillus* consists of the branching of the conidiophores near the apex, in which the *Penicillia* produce conidia from conidiophores (Caballero et al., 2003). The metulae are cells that expand from the apex of the conidiophore (Caballero et al., 2003). The phialides emerge from the metulae (Caballero et al., 2003), in which metulae and phialides produce one or several verticils (Fig. 02) (Moretti and Susca, 2017). When the conidia are produced, they get pushed out in chains, and the color of the conidia's *Penicillia* that

shows up after the coloration is often in shades of gray to blue to blue-green (Caballero et al., 2003).



Source: Visagie et al., 2014.

Fig. 02: Conidiophore branching patterns observed in *Penicillium*.
A. Conidiophores with solitary phialides. **B.** Monoverticillate. **C.** Divaricate. **D, E.** Biverticillate. **F.** Terverticillate. **G.** Quaterverticillate, Scale bar 10µm.

The conidiophores of *Penicillium*, and the cleistothecia (when produced), are aspects of high taxonomic importance in this genus (Moretti and Susca, 2017). The primary taxonomic division of the genus into subgenera is resolved by the form of the *Penicillus*, in which we should count the number of branch points between phialide and stipe, down the main axis of the *Penicillus* to determine the subgenus to which a *Penicillium* isolate belongs (Fig. 02) (Pitt and Hocking, 2009).

The conidiophores range from being simple to very complex patterns with various levels of branching resulting in general symmetrical or asymmetrical patterns (Visagie et al., 2014).We can distinguish the following types of conidiophores:

- **Conidiophores with solitary phialides** (Fig. 02.A)**:** the conidiophores are comprised of solitary, swollen phialides borne on short, unbranched stipes. Conidia are associated together by connectives, short intercalary cylinders of cell wall material, to construct very long chains (Visagie et al., 2016).
- **Monoverticillate conidiophores** (simple branched) (Fig. 02.B)**:** a *Penicillus* composed of phialides with a unique branch point between stipe and conidium (Samson and Pitt, 1986). The terminal cell of the conidiophore is somewhat puffed or vesiculate (Visagie et al., 2014; Moretti and Susca, 2017).

- **Divaricate conidiophores** (irregular branched) (Fig. 02.C)**:** the conidiophores range from being simple to complex branching pattern with several subterminal branches formed, where the conidiophore parts are divergent (Visagie et al., 2014).
- **Biverticillate conidiophores** (one-stage branched)(Fig. 02.D& E)**:**a *Penicillus* which has one bearing metulae and phialides with two branching points between stipe and conidium (Samson and Pitt, 1986). The arrangement of the phialides on the stipe could be organized symmetrically or asymmetrically around the axis (Moretti and Susca, 2017).
- **Terverticillate conidiophores** (two stages branched) (Fig. 02.F)**:** a *Penicillus* bearing rami, metulae and phialides with three branching points between stipe and conidium (Samson and Pitt, 1986). The branches are usually asymmetric (Moretti and Susca, 2017).
- **Quaterverticillate conidiophores** (two stages branched) (Fig. 02 G): they have one extra level of branching beyond the terverticillate pattern, and only a few species can produce it (Visagie et al., 2014).

2. Identification Procedure

The phenotypic characters of many species of *Penicillium* are very similar which makes their identification rather complicated (Adan and Samson, 2011). Therefore, in *Penicillium* genus the concept of species has been redefined by using a polyphasic approach, combining morphological, physiological, biochemical and molecular characters (Visagie et al., 2014; Adan and Samson, 2011).

a. Determination of Morphological characteristics

Morphological descriptions are based on a combination of growth features and morphology characteristics (Peberdy, 1987). The identification of *Penicillia* is still, to a large extent, based on the examination of macro and micro-morphological characters (Samson and Pitt, 1990).

- **Macro-morphology**

This approach is based on observation with the naked eye of various characters, such as color and tint in colony surface and reverse, smell or fragrance, quantity of aerial hyphae, colony surface texture, colony margin, pattern, pigment exuded and organs formed… etc (Watanabe, 2002).

In order for the observation to be accurate, *Penicillium* isolates must be grown under standardized conditions of different media (MEA, CYA, and G25N) and temperatures, and

then incubated for a standard time of 7 days (Samson and Pitt, 2000; Pitt and hocking, 2009; Visagie et al., 2014).

- **Micromorphology**

It consists in the microscopic observation of characteristics of the cells such as the number of branching points between stipe and phialides (solitary phialides to quaterverticilate), dimension, shape and texture of stipes, vesicles, metulae/branches (when present), phialides, conidia, cleistothecia, asci, and ascospores (when present) (Visagie et al., 2014).

b. Physiological characteristics

In order to establish these characteristics, the growth rates of *Penicillium* isolates on different media and at different physicochemical conditions are tested (Watanabe, 2002; Visagie et al., 2014).

c. Biochemical characteristics

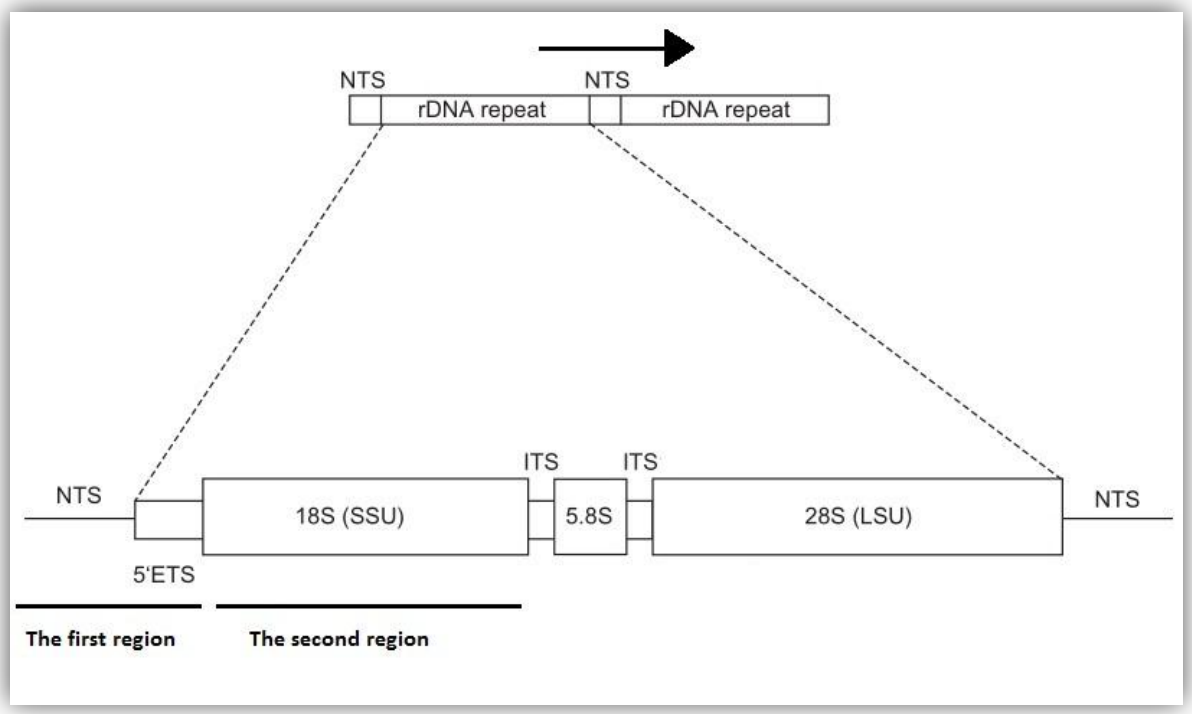
To study the characteristics of the metabolic profiles, both primary and secondary metabolisms of the fungal isolates are investigated (Samson and Pitt, 2000; Visagie et al., 2014). These include mycotoxins production and enzymatic capacities (Samson and Pitt, 2000).

d. Molecular phylogenetic characteristics

This approach is based on sequencing and analyzing conserved regions in the fungal genome such as:

- **rDNA sequences** (Fig. 03): The rDNA genes encode for the ribosomal RNAs, essential for the structure and the function of ribosomes, which are the sites of cellular protein synthesis (Gregory, 2005). The rRNA genes comprise both conserved and hyper variable regions (Ranganathan et al., 2019). This cluster encodes three subunits of ribosomal RNA (18S [small subunit (SSU)], and 5.8S and 28S [large subunit (LSU)] rRNA) and intervening regions, or internal transcribed spacers, identified as ITS1 and ITS2 (Watkinson et al., 2016).

- Using a combined analysis of four *loci* (RPB1, RPB2, Tsr1 and Cct8) for studying the phylogenetic relationships between *Penicillium* species and other members of the family *Trichocomaceae* (Samson and Houbraken, 2011).



Source: Waikagul and Thaenkham, 2014.

Fig. 03: A simplified diagram of the ribosomal DNA repeat unit of eukaryotes. The gene contains 18 S or small subunit (SSU), 5.8 S, and 28 S or large subunit (LSU) tracts. NTS is nontranscribed spacer, ITS is internal transcribed spacer having 2 segments numbered from 5' end (so-called ITS1 and ITS2, respectively). ETS is external transcribed spacer. The arrow indicates the direction of transcription.

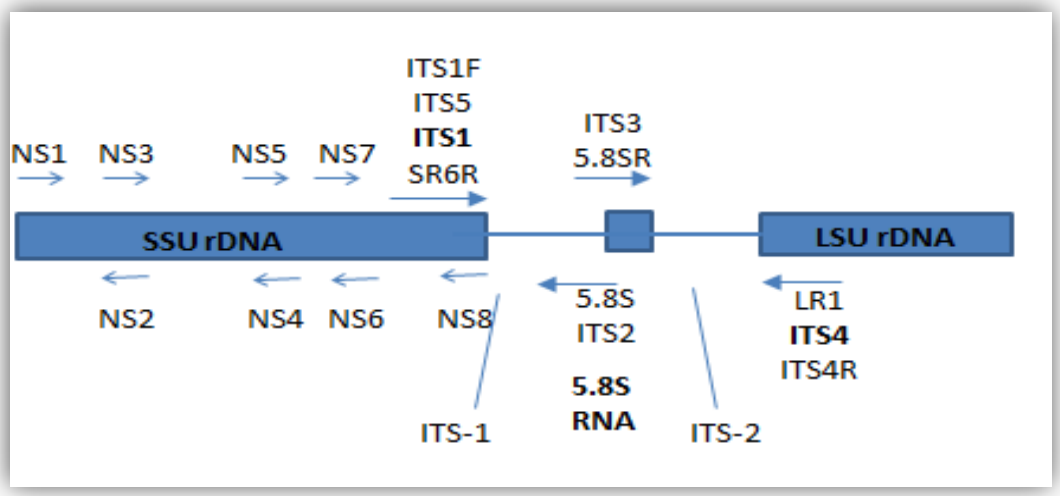
- **ITS regions of rDNA:** The new commonly applied technique for the identification and the phylogenetic analysis of *Penicillium* genus is sequencing of the ITS region along with the ‘‘house- keeping genes’’ (Pitt and Hocking, 2009). These genes are regions in a genome that tend to be highly conserved, their roles in the preservation of basic cellular functions are necessary for the existence of a cell (Ranganathan et al., 2019), such as Calmodulin, β-tubulin (BenA) genes and elongation factor 1-α genes (Pitt and Hocking, 2009).

The DNA barcode for fungal kingdom has been designated as the ITS region considering that it displays a more advanced degree of variations as to the other regions of rDNA, because it is able to separate sequences into species level. The ITS region is located in the rRNA operon with a length that ranges from 450 to 750 bp, and it exists in two parts, ITS1 and ITS2, which are divided by the 5.8S rDNA (Fajarningsih, 2016).

Procedure of DNA barcoding using ITS genes to identify fungal species

The amplification of the targeted DNA barcode regions is one of the important parts of DNA barcoding approach (Fajarningsih, 2016).

After the extraction of the genomic DNA, the ITS gene of interest is amplified by PCR, the use of specific primers (Fig 04) is strict in order to get exact amplified DNA barcode region. After that Electrophoresis is applied to analyze the PCR products, and the amplicons are used as template for DNA sequencing (Fajarningsih, 2016).



Source: (Fajarningsih, 2016).

Fig. 04: Diagram of primers location within the ribosomal cassette consisting of SSU, ITS1, 5.8S, ITS2, and LSU rDNA.

Pitt's classification techniques use the morphological and physiological characters of *Penicillium* (Visagie et al., 2014). It takes into account the branching of conidiophores and growth on different media and at different temperatures (incubation for 7 days) (Pitt and Hocking, 2009; Visagie et al., 2014):

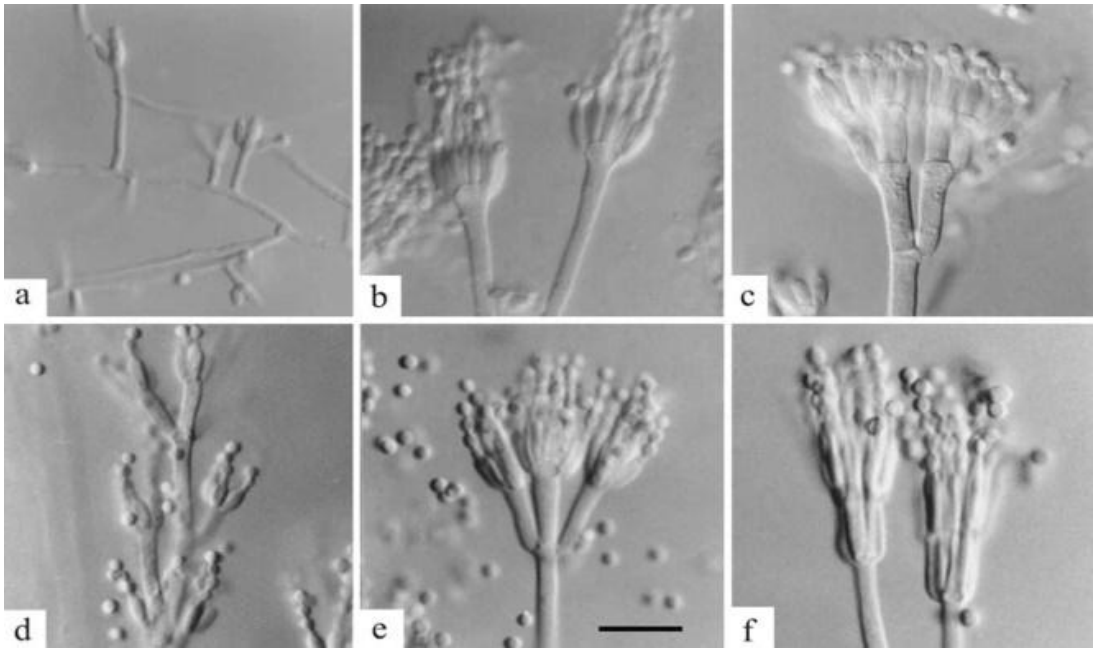
- ✓ CYA at 5°C, 30 °C & 37 °C (Pitt and Hocking, 2009).
- ✓ MEA at 25 °C (Pitt and Hocking, 2009).
- ✓ G25N 25% at 25 °C (Pitt and Hocking, 2009).

There are two types of observations after seven days of cultivation (Visagie et al., 2014):

- ✓ Macroscopic observations
- ✓ Microscopic observation

Pitt differentiates four subgenera among *Penicillium* genera (Pitt and Hocking, 2009; Samson and Houbraken, 2011):

- ✓ *Aspergilloides:* Monoverticillate (Fig. 05**a, b**)
- ✓ *Furcatum*: Biverticillate (Fig. 05**d, e**)
- ✓ *Biverticillium*: Biverticillate (Fig. 05**f**)
- ✓ *Penicillium:* Terverticillate (Fig. 05**c**).



Source: Pitt and Hocking, 2009.

Fig. 05: *Penicillus* types in *Penicillium* species (**a, b**) Monoverticillate; (**c**) Terverticillate; (**d, e**) Biverticillate, subgenus *Furcatum*; (**f**) Biverticillate, subgenus *Biverticillium*; bar = 10 µm

The differences between the two subgenera *Furcatum* and *Biverticillium* are summarized in the table 01.

Traditionally a morphological concept was used for *Penicillium* identification and classification (Visagie et al., 2014). During the 1990's, DNA sequencing began to be used, and later, molecular identification became of a wider use (Visagie et al., 2014).

In 2010, the most commonly occurring *Penicillium* in a dichotomous key was keyed out by Samson and his colleagues (Adan and Samson, 2011). This key is established on a database with phenotypical and molecular (partial s-tubulin sequences) characters. This website (http://www.cbs.knaw.nl/indoor/) provides a database for identifying indoor *Penicillia* (Adan and Samson, 2011).

Table 01: Characters distinguishing subgenus *Furcatum* from subgenus *Biverticillium*

Characteristics	Subgenus *Furcatum*	Subgenus *Biverticillium*
Ratio of metulae length to phialide length	Much greater than one	Approximately one (1–1.2)
Metulae per stipe	Not exceeding five	Usually exceeding five
Colony diam on G25N	9–18 mm	Less than 10 mm
Phialide shape	Flask shaped, gradually tapering to neck (ampulliform)	Parallel sided, abruptly tapering toneck (acerose)

Source: Pitt and Hocking, 2009.

3. Classification

The first researcher who introduced a subgeneric classification system for *Penicillium* genus was Dierckx in 1901.

In 1930, Thom introduced a new system with four divisions, 12 sections and 18 subsections (Samson and Houbraken, 2011). In the same year he published the first comprehensive monograph of the genus *Penicillium* and described a useful key to identified and accepted species, which was based on morphological characteristics (Samson and Pitt, 2000).

Later in 1980, Pitt described four subgenera, *Aspergilloides*, *Furcatum*, *Penicillium*, and *Biverticillium* (Samson and Houbraken, 2011).

Recently, the genus *Penicillium* has been divided in two genera using polyphasic approach: *Penicillium* and *Talaromyces* (Moretti and Susca, 2017). The first clade comprise *Penicillium* subgenus *Biverticillium* and *Talaromyces*; which shown to form a monophyletic clade distinct from the other subgenera of *Penicillium* (Moretti and Susca, 2017), where the second clade of *Penicillium* genera comprise the species of subgenera *Eupenicillium*, *Penicillium*, *Furcatum* and *Aspergilloides* (Samson and Pitt, 2000; Samson and Houbraken, 2011).

Taxonomy of the Genus *Penicillium* (Haber et al., 2015; Singh and Khajuria, 2018).

- **Domain**: Eukaryota
- **Kingdom:** Fungi.
- **Phylum:** *Ascomycota*.
- **Class:** *Euascomycetes*.
- **Order:** *Eurotiales*.
- **Family:** *Trichomaceae*.
- **Genus:** *Penicillium.*

Teleomorphs of the genus include *Eupenicillium*, *Talaromyces*, *Hamigera*, and *Trichocoma* (Haber et al., 2015).

C. Secondary metabolism of *Penicillium*

Penicillium genus is one of the largest groups of fungi, worldwide distributed and plays a vital role in ecosystems. These fungi are one of the most important tools in biotechnology (Al-Daamy et al., 2018). Some *Penicillium* species can be used in many applications: industrial, agricultural, medicinal, and environmental (Al-Daamy et al., 2018).

Penicillium species are capable of producing various range of bioactive secondary metabolites along with confirmed biological activities; therefore, their importance has been proved. *Penicillium* secondary metabolites include antifungal and antibacterial substances (Rabha and Jha, 2018).

However, some species of *Penicillium* are deleterious to other microorganisms and can inhibit or even destroy them completely. Therefore, they are used to treat infections caused by bacteria and other organisms that can cause illness to both humans and animals by different modes of action (Al-Daamy et al., 2018).

The products of the secondary metabolism are capable to induce growth, survival and adaptation process of a fungus in a habitat. Many secondary metabolites produced by *Penicillium* species are specific and useful for identification of species as well as being widely used in industry (Kumar et al., 2018).

Chapter 2

A preview on halotolerant *Penicillium* species diversity in some saline terrestrial habitats

I. Results of some studies on the isolation of halotolerant *Penicillia*

Ten studies have been chosen in the context of isolation and identification of *Penicillium* species from various terrestrial saline habitats. In which we performed a summary for each study, to better understand the genus diversity along with the methods used for its isolation and identification.

A study was conducted in 2004, by Steiman et al, in order to access the mycoflora diversity in the hypersaline and alkaline soil (pH 7.50 to 9.75) and tufa of Mono Lake area (California). The samples sites contain lots of debris of dead organisms; in which 67 fungal species which were isolated and identified on the basis of morphological characteristics, 5 of them belonging to the genus *Penicillium* (*P. aurantiogriseum*, *P. citrinum*, *P. griseoroseum*, *P. janczewskii* and *P. waksmanii*). The isolates were cultured on MEA medium at different temperatures (22, 30°C). Depending on the medium and temperature of isolation it can be hypothesized that some species are present as dormant structures, while some others which were isolated at pH 8 on a medium enriched in Na and Ca, could be in a growing form adapted to alkaline and saline conditions. The isolation methods used were not suited for the isolation of the obligate halophilic fungi that might be present. Yet, it has been concluded that no very specific fungal flora was found in these habitat (Steiman et al., 2004).

Another study was realized during 2003–2005 by Hujslová and co., on the diversity of filamentous fungi in saline and acidic soils of the Soos National Natural Reserve, Czech Republic. They collected 28 soil samples from 4 different sampling sites that had different types of vegetation cover, conductivity, pH values, and moisture content were established in the southwestern part of Soos. The identification of isolates was based on morphological and molecular characteristics by rDNA, and β-tubulin sequences. They identified 92 taxa, belonging to 39 genera, where the dominant genus was *Penicillium* (24 species). The isolates were influenced by the effects of environmental factors, and the majority of the isolates grew optimally without salt. Subsequently, they were classified as halotolerant because of their ability to tolerate different concentration of salt. Almost all isolates could grow optimally at minimum incubation temperatures (5, and 24°C); while few species could grow with low richness at 37°C. The majority of isolates grew over a pH range from 3 to 8 (Opt. 4–8), 2 species of *Penicillium* were able to grow at extremely low pH values; they may be classified as acidotolerant. The results showed that these species are highly

adaptable to the extreme conditions; they suggested that the species composition of the fungal community is shaped by the complex effects of these factors (Hujslová et al., 2009).

In 2011, Smolyanyuk and Bilanenko, studied the halotolerant micromycetes in the Areas of Natural Salinity, in which the soil samples were collected from 3 regions: Lake Pomorie, Bulgaria (Salinity ~8%), Ein Bokek, Palestine (Salinity ~34%), and Baskunchak, Russia (salinity ~30%). They identified the isolates based on morphological and cultural characters. The first region was the richest region, in which they identified 39 species; 5 of them belong to *Penicillium*, and *P. triangularis* gave the highest values of specific abundance index during the growth. *P. chrysogenum* and *P. triangularis* were the only species to grow on medium with 20% of NaCl. In the second region they identified only 8 species of micromycetes, 3 of them belong to the genus *Penicillium*, the highest specific abundance index was represented by *P. chrysogenum* (72.5%), and *P. citrinum* (66.67%). *Penicillium* was the most identified genus with 10 species in the third region, in which *P. dierckxii* and *P. rugulosum* demonstrate the highest values of specific abundance index (100%). 3 species were able to support 20% of NaCl: *P. lanosum*, *P. miczynskii*, and *P. rugulosum*. The most frequently isolates in all 3 regions belong to *Penicillium* (*P. rugulosum*, *P. miczynskii*, *P. citrinum*, and *P. lanosum*) (Smolyanyuk and Bilanenko, 2011).

Georgieva et al, (2012), conducted a study of the diversity of mycelial Fungi in Saline Soils, in the Western Transbaikal Region, Russia, from the soda solonchaks, sulfate solonchaks and solonchakous chestnut soil. These areas present differences in the degree and type of salinization (0.01-13.94%), along with their humus content, and their pH values that ranged from 6 - 10. The vegetation on the lake's coast is scarce; and the soil's surface is covered with moss and litter (dead grass). Forty species of fungi were isolated, belonging to 24 genera. The halo-alkali-tolerant and alkalophilic micromycetes were dominant in the soda solonchak area where *P. spinulosum* was isolated only from the first sample (pH 7.75). Whereas *P. aurantiogriseum* was isolated from various samples of all soils. The two isolated *Penicillium* species developed only on the standard weakly acid media; whereas, there was no growth on the selective medium with an alkaline buffer. Based on the medium, these species may be accidental for alkaline soils; they occur to be in the dormant phase (Georgieva et al., 2012).

In 2013, Dhakar and co., studied temperature, pH and salt tolerance of 25 isolates of *Penicillium*, these isolates were identified based on polyphasic approach. All the cultures were isolated from soil samples collected from subalpine and alpine zones of Pindari Glacier region high altitudes in the Indian Himalayan region. They examined the colony characters performed on 5 different media which gave different results. The fungal isolates could tolerate pH from 2 to 14 (Opt. 5–9), and 7 isolates tolerated pH 1.5. While salt tolerance of *Penicillium* isolates was between 10 and 22 %; and 10 isolates showed tolerance above 20 %, these species can be categorized as halotolerants .All the *Penicillium* isolates were identified based on ITS region, 25 isolates showed maximum similarity between 95 and 100 % to their respective available strains in NCBI database (Dhakar et al., 2013).

A study of the effect of salinity on the fungal occurrence that was conducted in Al Shega Area, at Al Qasim (Saudi Arabia), by Al Tamie (2014), in which 17 halotolerant fungi have been isolated and identified. Among which, *P. canescens* was the only *Penicillium* specie identified. *P. canescens* have shown high growth rate at 5, 10, and 15% of NaCl, on the other hand the growth has stopped at 20% of NaCl. The isolated fungus was able to grow moderately at pH ranging from 6.5 to 8. The results indicated that *P.canescens* could only grow at temperature of 30°C with concentrations of 0%, 5% NaCl; while at temperatures (30, 40, 50°C), and under salinities of (10, 15%) there was no growth. The study concluded that *P. canescents* is an halotolerant specie (Al Tamie, 2014).

Jaouani and co. (2014), isolated 21 halotolerant fungi from Sebkha El Melah, a saline habitat located in the Saharan salt flat (Tunisia). The sebkha sediments are composed of several saliferous layers of rock salt and gypsum (calcium sulfate) and/or polyhalite (sulfate of potassium, calcium, and magnesium). Based on morphology, ITS and 28S rRNA genes sequencing, along with other specific genes, 5 isolates were identified as belonging to the genus *Penicillium*. The study of their tolerance of salt, on solid media, showed that they were able to grow optimally on 5 % of NaCl, however they also could grow normally beyond 10% of NaCl. Two isolated strains of *P. chrysogenum* could weakly grow at 20% NaCl. However, in liquid cultures, the strains could not grow beyond 15% of NaCl. The authors explained this result by the alteration of osmotic gradient, making the fungi expend more energy in the osmoregulatory processes, resulting in a slow growth. All the *Penicillium* isolates were considered as moderately haloalkaliphilic since they were able to grow at pH 10 and 10% of NaCl. The screening of capacity of *Penicillium* strains to

produce extracellular enzymes in the presence of 10% of NaCl, protease and amylase were the most abundant enzymes produced, then laccase and cellulose, and none of the strains could produce lipase. They concluded that these microorganisms are capable of producing extracellular enzymatic activities in extreme conditions (Jaouani et al., 2014).

Kubátová and co., in a published study in 2018, studied the taxonomic revision of the biotechnologically important species as well as the description of two new species isolated from acidic and saline soils. In order to study morphological and molecular characters, 20 strains were used, along with 12 new isolates from the Soos Nature Reserve acidic (pH 1.7–2.7) and saline soils located near Františkovy Lázně, Czech Republic. They isolated *P. soosanum*, from the saline acidic (pH 2.7) soil. The second specie was *P. diatomitis*, isolated from saline acidic (pH 1.7) soil. This isolate displayed its ability to tolerate high salinity (0.5 M Na_2SO_4) as well as low pH (pH 2) with an optimal pH of 5, which certifies its halo- and acidotolerance(Kubátová et al., 2018).

In the study of the diversity of halotolerant and halophilic mycoflora in the soil of the Great Sebkha of Oran located in northwestern of Algeria, Chamekh and her colleagues (2019), collected samples from 2 different regions. The first site was neutral, where grew cereal crops. The second was an alkaline soil without any kind of vegetation. Both sites were extremely saline. The isolates were identified based on morphology and molecular analysis (TEF-1α, β-tubulin, and ITS genes sequancing). One of the most dominant filamentous fungi genera found in the sebkha was *Penicillium*, with a frequency of 26.50% in the first site (34 species, 9 among them belong to *Penicillium*); and a less representation in the second, in which 13 species were identified; 4 from them belong to *Penicillium* and the dominant species was *P. egyptiacum*. All *Penicillium* isolates could grow in the absence of NaCl, and all of them could tolerate a salinity of 12.5%. However, only *P. vinaceum* could grow at 17.5% of NaCl with an optimum of 5%. Thus, these isolates are considered halotolerant (Chamekh et al., 2019).

A recent study of microbial diversity has been conducted in Sabkha and Desert Sites in Saudi Arabia by Alotaibi and co. (2020), in which the samples were taken from 9 different locations (Al-Aushazia, AlQasab, AlKasar, Tabuk, Al-Kharj, El- Al-Madina, Jubail, Taif and Abqaiq). On the basis of 18S rDNA sequencing, they identified 203 fungal species. *Penicillium* species were isolated from two out of nine different locations. Four *P. chrysogenum* and three *P. spp.* were isolated from AlQasab salt flats area with a percentage

of (5.7%). This location has a sandy loam soil with the highest EC (electrical conductivity) (78.6) among all studied sites, with a neutral pH (7.19). Three *P. spp.* and two *P. camemberti* were isolated from Jubail area with a percentage of (17.2%) which consists of sandy soil with a pH of 7.2 and EC 4.4. The *Penicillium* species present in Jubail are halotolerant (Alotaibi et al., 2020).

II. Analysis of the results of the presented studies

In order to catch a glimpse of the diversity of *Penicillium* species in different terrestrial saline habitats, as well as the effect of the extreme conditions of these habitats on their variability, 10 studies on the subject, from 11 different geographic regions, were analyzed, as previously presented.

Soil samples were taken from different saline habitats, in each region, and at different depths. After sampling the soils, different methods were applied for the analysis of the soil and the isolation of halophilic and halotolerant fungi. In 4 studies (Hujslová et al., 2009; Smolyanyuk and Bilanenko, 2011; Al Tamie, 2014; Chamekh et al., 2019) used two methods; the first is a direct method named soil plate method in which, few milligrams of soil were directly cultured on the selective nutrient media and each medium contained different NaCl concentrations. The second is an indirect technique, the dilution plate method, where few grams of soil were diluted, then cultured on the selective nutrient media, wherein each medium contained different NaCl concentrations. Moreover 4 studies (Steiman et al., 2004; Georgieva et al., 2012; Jaouani et al., 2014; Alotaibi et al., 2020) used only the soil plate method. The following media were used in the studies for the isolation and identification of halophilic and halotolerant fungi:

- PDA was used in 05 studies.
- Sabouraud Dextrose Maltose agar was used in 2 studies.
- Mycological agar, Vegetable-8 Juice, Dox's agar, sterile malt extract (1.5%) agar, MA2, SEA, CYA, MEA, YES, CREA, PCA, CzA, and SA, these media were used only once, each in a study.

The incubation temperature used varied from 22 to 45°C; however 25°C was the most utilized. As for the pH used it ranged from acid, neutral to alkaline, (pH 1-14), and the incubation period took several days (one to two weeks).

There are more other recent isolation techniques (that weren't applied in the analyzed studies) used to study soil microbial communities and their functional genes, specifically

Metagenomics which is, the science that analyzes directly without cultivation combined DNA or RNA from an environmental sample consisting of organisms that have not been isolated and identified. This technique is more applied for identification of microorganisms present in extreme environments, furthermore, analyses based on RNA or proteins may be used to investigate the patterns of gene expression in natural microbial communities (Madigan et al., 2019).

In all studies, the fungal isolates were identified based on morphological and cultural characters. Furthermore, 6 studies used molecular identification techniques for the phylogenetic identification of the isolates. These techniques were based on the sequencing of the rDNA genes cluster (ITS, SSU, 5.8S, LSU). Other genes were also sequenced, such as TEF-1α, and/or β-tubulin genes.

The study of physicochemical parameters of these regions shows that all of them have high salinity, depending of the studied area. A zone in the great Sebkha of Oran (Algeria) presents the highest salinity (46% of NaCl $g.L^{-1}$) among the presented studies. This region is followed by Ein Bokek (Palestine), with 34% of NaCl (g.L) due to its proximity to the Dead Sea.

Wide ranges of pH were recorded in these soils. Most of these terrestrial habitats presented slightly alkaline to alkaline pH (6 regions). The soil near to the water edge of Baskunchak lake (Russia) had the highest pH value (10.6). Three habitats had neutral pH (6-7). However, two regions had acidic pH, in which Soos National Natural Reserve (Czech Republic) had an acidic soil with the lowest pH value pH, ranging from 1.6 to 2.7.

The temperatures of these areas were not mentioned in most of the analyzed studies. However, some stated that temperatures changed seasonally. Nonetheless, some studied areas are considered as cold environments where the temperature drops to 5°C; which is the casein the high-altitude soils of the Indian Himalayan Region. This site experience heavy rainfall and snowfall. Whereas some regions are extremely warm. This is the case in Al Shega Area, at Al Qasim (Saudi Arabia), where the temperatures can reach 50°C.

The genus *Penicillium* has been isolated from all the studied saline soils. A total of 47 isolates were identified to the species level in the genus *Penicillium*. However, 22 isolates were identified as *Penicillium*, without reaching the species level, that remains unknown, because they were unable to identify the exact species.

The richest region in *Penicillium* species, among the analyzed studies, was the soil of the Soos National Natural Reserve, Czech Republic; where 24 species were isolated by Hujslová et al (2009).The second highest region in the diversity of *Penicillium* species is the high-altitude Indian Himalayan soil, where Dhakar and co., in 2013, identified 22 species. These two regions have different *Penicillium* species. This is likely due to the differences in the physicochemical properties of each soil, even though their temperatures and salinities were somehow similar.

The area with the lowest *Penicillium* diversity, among the analyzed studies, is Al Shega Area, at Al Qasim (Saudi Arabia), wherein only one *Penicillium* species has been isolated, *P. canescens*, by Al Tamie (2014). The reason for this result could be the high temperatures of this region that can reach 50°C. In all other studies *Penicillium* strains could grow optimally in a range between 20 to 30°C and even some strains could grow at low Temperature 4°C (*P. chrysogenum* and *P. canescens)*. However, none of them could grow beyond 37°C. Moreover, according to Samson and Pitt (1986), along with other authors, the so far, studied and well-characterized *Penicillium* species are more suited to temperatures around 25°C.

The circle chart down below (Fig. 06) demonstrates the percentage of the genera present in 10 studied areas. A total of 510 fungal isolates were identified as belonging to 78 different genera. In which the genus *Penicillium* is the most present in those saline terrestrial habitats with a percentage of (6.8%); it was abundant in 3 regions, and in other 4 regions it was considered as one of the abundant genera, whereas it wasn't abundant in 3 regions. Followed by *Aspergillus* (5.44%) which was present in 8 regions, *Alternaria* and *Fusarium* are present in 7 regions (4.76%), and 36.08% represents the percentage of 53 different genera; each genus found only in one region.

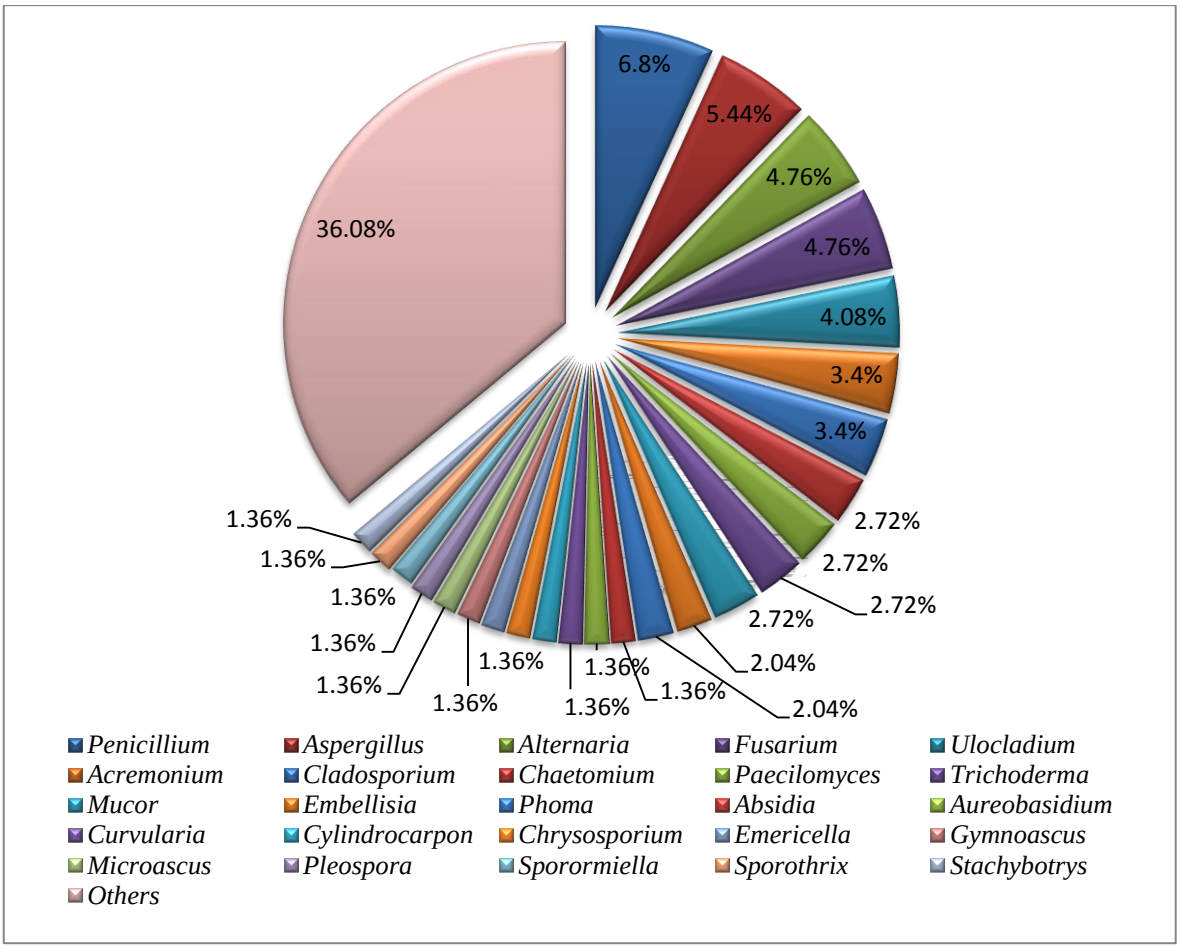


Fig. 06: Percentages of Fugal genera present in 10 different saline soil habitats.

The distribution of *Penicillium* species on these regions differs from a region to another, which could be due to the variations in pH, salinity, temperature, and other edaphoclimatic conditions. The studies show that each species has a distinct growth optimum and can tolerate certain values of these parameters:

Concerning the pH optima, most of the isolated *Penicillium* species were alkalotolerant (67.19%), some were acidotolerant (32.81%).

Regarding the response of the *Penicillium* isolates to the salinity, it has been recorded that all of the isolates were halotolerant. Most of them could tolerate up to 20% of NaCl (g/l) (example: *P. chrysogenum* and *P. triangularis*). Only a few isolates could tolerate 22% of NaCl (example:*P. glabrum*, *P. thomii*, and *P. simile*).

The effect of salt concentration on fungal growth in solid and liquid media was studied by Jaouani and co. (2014). The results indicated that all *Penicillium* isolates could grow above 10% of NaCl (g/l) in solid media, 2 strains of *P. chrysogenum* could tolerate 20% NaCl. However these strains could not grow beyond 15% of NaCl in liquid cultures. This

result was justified by the alteration of osmotic gradient, which leads the fungi to consume more energy in the osmoregulatory processes, the consequence of which is a slow growth.

According to the circle chart (Fig. 10) indicating the presence of *the first stud* species in all studied regions, *P. chrysogenum* is the most abundant specie (7.46%). It was isolated from 5 regions because of, most likely, its ability to tolerate high salinities (20% NaCl), as well as alkaline conditions (Opt. 8). It could also possess to ability to develop, both, at low (4°C) and high moderate temperatures (37°C).

The second most isolated specie is *P. citrinum* (5.98%), it has been found in four regions. Four species were found in 3 regions with a percentage of 4.48% each. Four other species were present in 2 regions with a percentage of 2.99% each; the other 56.68% is distributed on 37 different species each one found in one region (Fig. 07).

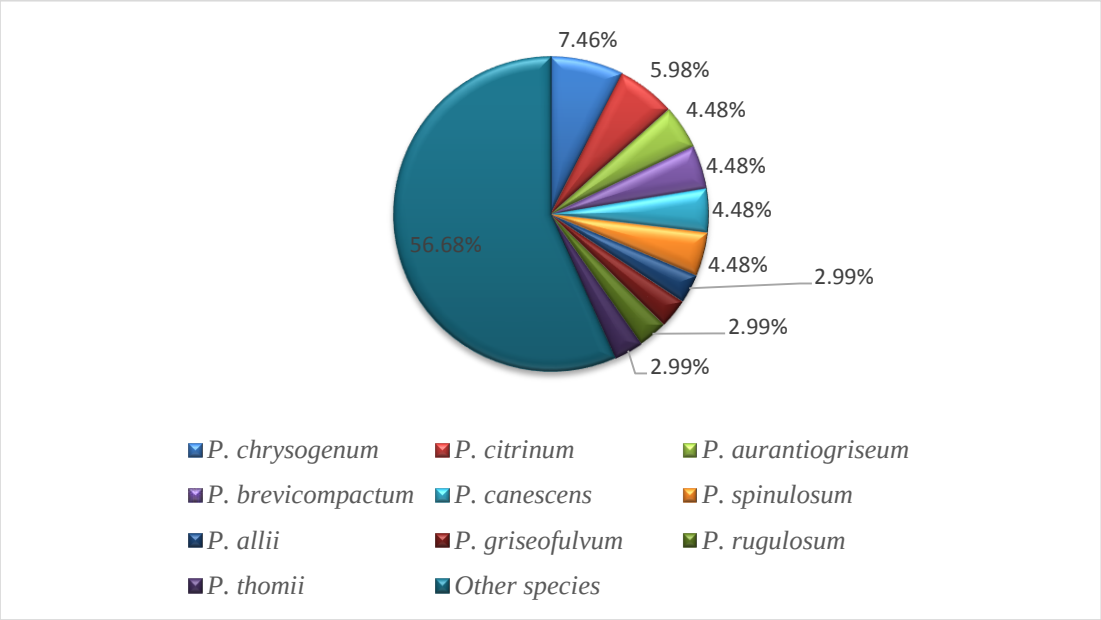


Fig. 07: Percentage of *Penicillium* species present in 11 different saline soils

In order to compare between *Penicillium* species present in the studied saline soils with those found in non-saline soil, four studies about the mycobiota of non-saline soils were analyzed.

The first study was realized by Puangsombat et al., 2010, who assessed and compared the composition, and diversity of soil fungi in five land use types in the Tha Kum-Huai Raeng Forest Reserve, Trat province, eastern Thailand; wherein 8 *Penicillium* species and 19 *P.sp* were isolated. The second study was conducted in four new reclaimed areas at Assiut Governorate, Egypt, by Zohri et al., 2014, in order to study the diversity of soil fungi, in which 7 *Penicillium* species were isolated. In the third study, Barbosa and co.

(2015) studied the diversity of *Aspergillus* and *Penicillium* in soils of the Catimbau National Park in Pernambuco, Brazil, in which they isolated 20 *Penicillium* species. The last research was conducted by Kolanlarli and co. (2019), and aimed at the isolation and the identification of *Penicillium* species from Edirne Söğütlük Forest soil (Turkey), in which 14 different species were isolated.

In the four studies 43 species has been identified, in which *P. citrinum* was found in three of these studies, along with *P. janczewskii* which was found in two studies. However these 4 regions share 22 similar species with those found in saline soil with a percentage of 46.81%; which is due to the ubiquity of the genus *Penicillium* as well as the halotolerance aspect of those species (they are not obligate halophilic species).

Penicillium species possess many characteristics such as tolerance for a wide range of pH, high salinities, and enhancement of sporulation and production of various secondary metabolites such as pigments and watery exudates at low temperature. These extraordinary capacities can be attributed to the ecological resilience possessed by these fungi (Dhakar et al., 2013); as well as their rich and particular extracellular enzymatic arsenal that allows them to adapt and survive in extreme environments (Jaouani et al., 2014).

The studied species by Hujslová and co. (2009) are decidedly adaptable to the extreme conditions. These authors suggested that the complex effects of biotic and abiotic factors shape the species composition of the fungal community. The study of Smolyanyuk and Bilanenkothe (2011) showed that the community of micromycetes of hypersaline habitats is more dependent on the physicochemical parameters of habitat that on geographical position.

The species inhabiting extreme environments may be used as unique sources of enzymes or secondary metabolites of biotechnological or pharmaceutical potential, as well as their ability to produce novel chemical compounds (Hujslová et al., 2009).

Conclusion and Perspectives

By analyzing multiple studies concerning the isolation and identification of halotolerant *Penicillium* species from different terrestrial saline soils, we can conclude that the considerably different *Penicillium* species isolated from the soils of 11 regions were influenced by the complex of physicochemical parameters.

This research clearly illustrates the diversity of the genus *Penicillium* in saline soils, in which the isolated species are not obligate halophilic fungi. Overall, the isolated species were halotolerant, and some of them are also found in non-saline soils. Their variability is probably affected by changes in the habitat characteristics. The species found in the analyzed studies about non-saline soils were similar by 47.81% with those isolated from saline soil. The isolated species, likely, managed to survive and adapt in these extreme environments by producing a diversity of enzymes and secondary metabolites.

Further studies are needed to explore and exploit the promising potential of *Penicillium* species for the production of produce new bioactive compounds. It will be interesting to investigate their biogeochemical roles in such extreme environments and explore the possible factors involved in influencing their occurrence and distribution patterns.

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