Chapter-1

Introduction

1.1 General introduction

The plant-microbes interaction relies on intact organisms and environmental factors, affecting plant physiology and nutrition (Moutia *et al*., 2010). "Endophytes" are microorganisms found in the living plants without showing antagonistic effects to the host (Stone *et al*., 2000). The endophytes pass through the host tissue, seed, and plant propagules (Carroll, 1988). They are distinct from plant pathogenic microorganisms being not deleterious; cause no diseases to plants. On the contrary, endophytes promote plant growth and yield and demote the growth of pathogens enduring in the plant. Endophytes residing in the medicinally important plant possess the host characters with similar bioactive compounds of biological activity, which can consider endophytes a good replacer of their host plant. Formerly, endophytes comprise fungi and bacteria inhabiting the plant endosphere during their entire life cycle (Wilson, 1995). Lately, Hardoim *et al*., (2012) defined endophytes as microorganisms that include archaea, bacteria, fungi, and protists that colonize the interior of plants, nevertheless of the benefit of their association.

Bacteria are among the endophytes that colonize many different types of plants and are host-specific. The studies revealed that the richness and diversity of endophytic bacteria are enormous under field conditions (Rosenblueth and Martinez-Romero 2006). These bacteria can be isolated from different parts of plants such as roots, stems, cotyledons, or less from flowers, fruits, and seeds (Lodewyckx *et al*., 2002). They also originate from seeds, vegetative plant cuttings, rhizosphere soil, and phylloplane and can penetrate through plant tissues like stomata, lenticels, wounds, germinating radicles, or actively colonize in the apoplast, conducting vessels and in intercellular spaces (Quadt-Hallmann *et al*., 1997). Endophytic bacteria utilize

carbohydrates, amino acids, and inorganic nutrients from plants for their metabolism. In addition, endophytes influence the quantity and chemical compounds of exudates from the host root (Ferreira *et al*., 2008). The colonization of endophytes confers an ecological niche indistinguishable from phytopathogens, thereby producing biocontrol agents (Hallmann *et al*., 1997).

Research on the plant-bacteria symbiotic interaction opens ways in agricultural biotechnology to improve plant growth and yields. Also, these associations may increase the ability of plants to utilize nutrients from the soil by increasing root development and resist the entry of soil-borne pathogens into plants (Whipps, 2001). Endophytic bacteria are the prime source of biopesticide and biocontrol agents (Antoun and Prevost, 2006). In addition, they produce enormous antimicrobial compounds (Wang *et al*., 2010), bioactive secondary metabolites (Sari *et al*., 2014), and antibiotics (Strobel *et al*., 2004; Bandara *et al*., 2006; He *et al*., 2009); also induce defense mechanism of plants (Bakker, *et al*., 2007; Ardanov *et al*., 2012; Patel *et al*., 2012).

Endophytes promote plant growth and help to fix nitrogen, solubilize phosphate uptake (Richardson *et al*., 2009), and assimilate minerals (Ryan *et al*., 2008). They also improve plant nutrient acquisition and promote plant growth (Johnson *et al*., 2004). They establish a role in biofertilizer, rhizoremediator, and photostimulation (Lugtenberg and Kamilova, 2009). The endophytes synthesize phytohormone, namely indole acetic acid (Shi *et al*., 2010), gibberellins, cytokinin, siderophores and lytic enzymes (O'Sullivan & O'Gara, 1992). They repress ethylene production, aid phytoremediation, and confer plant tolerance to biotic and abiotic stress (Bastian *et al*., 1998; Gaiero *et al*., 2013; Lebeis, 2014). Studies on endophytic bacteria generate

pharmaceutical substances of biotechnological interest with industrial and medical uses (Strobel *et al*., 1996). They are highly involved in the heavy metals removal and biodegradation of organic wastes (El-Deeb *et al*., 2013).

Traditionally, endophytes are cultured from the plant tissue through surface sterilization in a nutrient-rich medium. Most of the endophytic bacterial community have identified through culture-independent approaches using specific marker genes such as V3-V4 regions of 16S rRNA gene, the internal transcribed spacer regions, ITS1 and ITS2, or by whole genome sequencing (Taghavi *et al*., 2009; Ikeda, *et al*., 2010; Turner *et al*., 2013). Bacterial endophytes that are beneficial to plant growth are across many phyla, such as Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes (Bulgarelli *et al*., 2012). Some of the bacterial endophytes, in addition, carry necessary genes for biological nitrogen fixation (BNF), enabling them to convert the nitrogen gas into usable forms of nitrogen (Bhattacharjee *et al*., 2008).

Recently, researchers have extensively applied the new technologies in connection to plant pathology and microbial ecology research (Sylla *et al*., 2013; Unterseher *et al*., 2013; Muller and Ruppel 2014; Taylor *et al*., 2014). The perspective of metagenome and Next-generation sequencing (NGS), along with the evolution of bioinformatics tools, has made it easy to globally analyze microbial communities outside or inside any substance, including plant tissues. The usage of selected barcode genes in identifying all organisms in culture-independent method gives an advantage over the culture- dependent techniques. These barcodes includes organisms that are extremely hard to culture, representing approximately 99% of the total calculated microbial communities and the rare taxa that are not in sight during culture methods (Kellenberger, 2001).

Next-Generation Sequencing (NGS) or high-throughput sequencing is a powerful sequencing tool for microbial analysis that has led to invent the field of "metagenomics" (Oulas *et al*., 2015). With the advent of these new sequencing technologies, the genetic study of microbial communities has become more accessible and more reliable. Metagenomics, also known as environmental genomics, gives an overall idea of the genome assembly of the microbial community by direct extraction and cloning of DNA from environmental samples or natural habitats (Handelsman, 2004). The metagenomic analysis of plant microbiome provides operational data rather than genomic data. Usually, metagenomics is applied to study the soil microbes (Urich *et al*., 2008) and complex marine microbial communities (Gilbert *et al*., 2008). NGS has a variety of sequencing platforms like Illumina/Solexa and Roche 454 pyrosequencing with an approximate average read length of 150 base pairs and 500 base pairs, respectively (Balzer *et al*., 2010).

Bacterial endophytes are depot of novel secondary metabolites for immense therapeutic use. Bacteria produce a wide array of volatile organic compounds (VOCs), which develop metabolic products or by-products such as hydrocarbons, aliphatic alcohols, ketones, and indole (Tan and Zou, 2001). Competition and cooperation lead to coexist of bacterial strain in active communities producing diverse secondary metabolites as a signal to adjust to biotic and abiotic stresses (Hughes and Sperandio, 2008). Organic solvents like chloroform, ethyl acetate, and ethanol are commonly used to extract intracellular and extracellular secondary metabolites by adding solvent to the culture medium (Khanna *et al*., 2011). Structure elucidation of the secondary metabolites is determined using different techniques, namely liquid chromatography-mass spectrometry (LC–MS), gas chromatography-mass spectrometry (GC–MS), and nuclear magnetic resonance (NMR). GC-MS is the most

appropriate technique to determine the number of volatile components from a complex mixture of active compounds (Tiwari *et al*., 2015).

Extracellular metabolomics is the study of low molecular weight extracellular metabolites (exometabolome) secreted by microbial cells into the surroundings like culture supernatant (Mashego *et al*., 2007). From the liquid microbial culture, extracellular metabolites can be separated using simple techniques like filtration or centrifugation, whereas intracellular metabolites achieved by breaking the cell, which is a complex process (Villas-Boas and Bruheim, 2007). Various analytical techniques are introduced to identify and quantify exometabolome present in the spent microbial culture media. These metabolites are formed due to secretion in different growth phases of microbes during cell uptake of nutrients from the external culture medium. Consequently, the quantity and quality of extracellular metabolites can easily be changed by one or more environmental factors such as temperature, pH, the concentration of nutrients, and others (Mapelli *et al*., 2008).

Microbial extracellular components are used to synthesis different metal and non-metal nanoparticles. Microbial nanoparticles synthesis is an eco-friendly method that includes both intracellular and extracellular synthesis (Mukherjee *et al*., 2001). Most of the bacteria live in ambient temperature, pH, and pressure that support the biogenic synthesis of nanoparticles. The bacterial carrier matrix improves the contact between the enzyme and metal salt that generates microbial nanoparticles, which in turn increase the surface area with more catalytic activity. Thus the biogenic nanoparticles is developed when the microbes take the target metal ions from their environment and convert into elemental ion using the enzymes generated during the cell activities (Bootharaju and Pradeep, 2012).

Bio-active compounds from endophytes are the secondary metabolites having antimicrobial, insecticides, cytotoxic, and anticancer potentialities. These bioactive compounds are secondary metabolites classified into alkaloids, phenols, terpenoids, steroids, quinones, lignans, and lactones (Zhang *et al*., 2006). A new potent drug sources are required for the replacement of existing antimicrobial drugs as various pathogenic bacteria shows resistant on these drugs (Kayalvizhi and Gunasekaran, 2010). Actinobacteria groups on fermentation produce different enzymes (Oldfield *et al*., 1998), antibiotics (Cragg and Newman, 2013; Buchanan *et al*., 2005), antitumor compounds (Kingston, 2009), and immunosuppressant agents (Mann, 2001). Many of metabolites produced by these bacteria have antibacterial activities such as dimethyl novobiocins, celastramycins A-B (Igarashi *et al*., 2007), munumbicins A-D (Hasegawa *et al*., 2006; Kang *et al*., 2012) and kakadumycins (Hasegava *et al*., 1983).

Oxidative damage of human cells or tissues pathologically leads to cancer, arthritis, atherosclerosis, cirrhosis, and emphysema (Halliwell and Gutteridge, 1984). Among the microorganisms, bacteria stand as one of the copious sources of natural antioxidants products. Antioxidants play a significant role in human nutrition due to more lipid-free radicals in diet and after food ingestion (Evans, 1997). Free radical scavenging (in vitro and in vivo) of endophytic bacteria in the cells, and cell-free extracts are found to be significant in antioxidant activity (Shen *et al*., 2011).

Microbial-based cancer therapy is one of the innovations in treating cancer diseases. Advancement in the study of bacterial by-products like enzymes, proteins, immunotoxins, fatty acids, and secondary metabolites have made to target cancer cells or to regress tumor via cell growth inhibition, inducing apoptosis or arresting cell

cycle stages (Frankel *et al*., 2000; Wei *et al*., 2008; Bernardes *et al*., 2010). Moreover, microbial drug discovery is paying an inevitable influence on cancer chemotherapy. For more than a century, bacteria reduce the growth rate or size of certain forms of cancer (Chakrabarty, 2003). Several anaerobic bacterial species and facultative anaerobic bacteria show the ability to regress or target the tumors in experimental animals (Gill and Holden, 1996).

1.2 Objectives of the study

Endophytes are among the richest sources of natural compounds, having beneficial properties like antimicrobial, antioxidants, and anticancer. So, the work aims to study such beneficial aspects of endophytic bacteria from the medicinal plants belonging to *Morinda* L. species. The objectives are comprehended below and the schematic representation with an overview of study design is shown in fig. 1.1.

- Isolation and identification of endophytic bacteria from *Morinda citrifolia* L. and *Morinda pubescens* J.E Smith.
- Metagenomic study of endophytic bacteria in *Morinda pubescens*.
- Extracellular components extraction and qualitative and quantitative chemical analysis.
- Biogenic iron oxide nanoparticle production efficacy.
- In vitro study
    - Antimicrobial activity
    - Antioxidant and
    - Cytotoxicity study.
- In vivo study
    - Toxicity study
    - Anti-inflammatory and
    - Antitumor study in mice.

Fig. 1.1 Schematic representation with an overview of the study design.