UNIVERSIDADE FEDERAL DO PARANÁ

GISELA MANUELA DE FRANÇA BETTENCOURT

INDOLE-3-ACETIC ACID PRODUCING ENDOPHYTIC BACTERIA WITH POTENTIAL TO BE APPLIED AS BIOINOCULANT

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Co-orientador: Dr(a). Juliana Degenhardt

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Success is going from failure to failure without losing your enthusiasm.

(Winston Churchill, ano, p.)

RESUMO

Bactérias endofíticas têm a característica única de entrar e colonizar os tecidos das plantas e manter uma interação benéfica com o hospedeiro. São responsáveis direta e indiretamente por diversas características relacionadas à nutrição e defesa do crescimento das plantas. Bactérias endofíticas produtoras de auxina podem ser potencialmente usadas como bioinoculantes para promoção de crescimento de plantas. Neste trabalho, foi realizada a análise da diversidade das bactérias endofíticas de folhas de *Eucalyptus urophylla* AEC224 in vitro a partir da metagenômica e posteriormente isolados por método dependente de cultura, para obtenção de isolados com habilidades de promoção de crescimento de plantas. Das estirpes isoladas, as duas maiores produtoras de auxina foram identificadas como *Enterobacter* sp. e *Paenibacillus polymyxa* por 16S rDNA. As cepas isoladas e em conjunto com outra bactéria endofítica *Bacillus megaterium* DEBB B-353, foram avaliadas para a produção de IAA, avaliando os componentes dos meios de cultura e otimização do processo, escalonamento e aplicação no crescimento de plantas. Entre os parâmetros avaliados, o pH à 5±0.2, a água de maceração de milho (milhocina) e o glicerol foram escolhidos como fontes de nitrogênio e carbono. A melhor concentração de triptofano variou entre 1030 a 1250 $\mu g.mL^{-1}$, porcentagem de inóculo de 3,5 à 4%. A maior produção de IAA do isolado *Enterobacter* sp. DEBB B-355 foi de 824.09 $\mu g.mL^{-1}$, obtida em frascos após 96 h de cultura, 750.05 $\mu g.mL^{-1}$ em biorreator de 2 L e aumentou para 910.27 $\mu g.mL^{-1}$ quando escalonado para biorreator de 10 L, após 128 h. O rendimento de produção de IAA com o *B. megaterium* foi de 672.15 $\mu g.mL^{-1}$ em frascos, 750.61 $\mu g.mL^{-1}$ e 639.45 $\mu g.mL^{-1}$ em biorreatores de 2 L e 10 L, respectivamente. As produções otimizadas do isolado *P. polymyxa* promoveram um rendimento de IAA de 725.01 $\mu g.mL^{-1}$ em frascos, 567.18 $\mu g.mL^{-1}$ em biorreator de 2 L e teve um incremento para 812.49 $\mu g.mL^{-1}$ usando o biorreator de 10 L. Após o escalonamento, a produção de IAA reduziu de 72 para 32 h para *B. megaterium e P. polymyxa*. A síntese do IAA pelos isolados foi confirmada por HPLC e FTIR. A biomassa bacteriana e o sobrenadante com as auxinas produzidas demonstraram promover a germinação de sementes e o desenvolvimento de plantas e raízes. Além disso, foi desenvolvido um meio de baixo custo usando a milhocina e o glicerol como os principais componentes do meio de cultivo. A produção de AIA obtida para as estirpes de *B. megaterium* e *P. polymyxa*, foi superior em relação a toda produção de IAA já reportada em outros estudos. Por fim, nanopartículas mono e bimetálicas de Fe e Mn obtidas por um método simples e rápido, utilizando-se o sobrenadante de cultivo bacteriano contendo o complexo de auxinas (AIA) foram avaliadas como nano-fertilizantes de plantas. A biossíntese das nano-partículas de FeOx-NPs, MnOx-NPs e bimetálicas MnOx/ FeOx-NPs foi bem sucedida, conforme observado pelas técnicas de espectroscopia UV/Vis, MET e FTIR. As NPs aumentaram as taxas de germinação, o crescimento das raízes, assim como o peso fresco de mudas de milho e mostraram-se adequadas para serem utilizadas como nano-fertilizantes de micronutrientes.

Palavras-chave: Bioinoculantes. Biofertilizante. Bioestimulante. IAA. Nanopartículas de ferro. Nanopartículas de manganês

ABSTRACT

Endophytic bacteria have the unique characteristic of entering and colonizing plant tissues and maintaining a beneficial interaction with the host. They are directly and indirectly responsible for several characteristics related to plant nutrition and defense. Auxin-producing endophytic bacteria can potentially be used as bioinoculants to promote plant growth. In this work, an analysis of the diversity of endophytic bacteria from *Eucalyptus urophylla* AEC224 leaves was performed in vitro from the metagenomics and later isolated by culture-dependent method, to obtain isolates with plant growth promotion abilities. From the isolated strains, two major auxin producers were identified as *Enterobacter* sp. and *Paenibacillus polymyxa* by 16S rDNA. Both isolated strains and another endophytic strain *Bacillus megaterium* DEBB B-353, were evaluated for IAA production, evaluating culture medium components and optimization of the process, scale-up and application in plant growth. Among the parameters evaluated, the pH at 5 ± 0.2, corn steep liquor (CSL) and glycerol were chosen as sources of nitrogen and carbon. The best tryptophan concentration varied between 1030 to 1250 $\mu g.mL^{-1}$, percentage of inoculum from 3.5 to 4%. The highest production of IAA from *Enterobacter* sp. was 824.09 $\mu g.mL^{-1}$ in flasks after 96 h of culture, 750.05 $\mu g.mL^{-1}$ in a 2 L bioreactor and increased to 910.27 $\mu g.mL^{-1}$ when scaled to a 10 L bioreactor, after 128 h. The yield of IAA production with *B. megaterium* was 672.15 $\mu g.mL^{-1}$ in flasks, 750.61 $\mu g.mL^{-1}$ and 639.45 $\mu g.mL^{-1}$ in 2 L and 10 L bioreactors. Optimization of the *P. polymyxa* isolate promoted an IAA yield of 725.01 $\mu g.mL^{-1}$ in flasks, 567.18 $\mu g.mL^{-1}$ in a 2 L bioreactor and increased to 812.49 $\mu g.mL^{-1}$ using the bioreactor 10 L. After scale-up, the peak of IAA production decreased from 72 to 32 h for *B. megaterium* and *P. polymyxa*. The synthesis of IAA by the isolates was confirmed by HPLC and FTIR. Bacterial biomass and the supernatant with the produced auxins showed positive promotion on seed germination and plants and root development. So far, a low-cost medium was developed using CSL and glycerol as the main components of the culture medium. And, the results obtained for the strains *B. megaterium* and *P. polymyxa*, were higher in relation to all IAA production previously reported in other studies. Finally, the synthesis of mono and bimetallic nanoparticles of Fe and Mn was evaluated by a simple and fast method, using the bacterial culture supernatant containing the auxin complex (IAA), and evaluated as plant nano-fertilizers. The biosynthesis of FeOx-NPs, MnOx-NPs and bimetallic MnOx/FeOx-NPs nano-particles was successful, confirmed by the techniques of UV/Vis spectroscopy, TEM and FTIR. NPs increased germination rates, root growth as well as fresh weight of corn seedlings and proved to be suitable for use as micronutrient nano-fertilizers.

Key words: Bioinoculants. Biofertilizer. Biostimulant. IAA. Iron nanoparticles. Manganese nanoparticles. Nano-fertilizer. Plant growth promoting bacteria.

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LIST OF ABREVIATIONS

ACC - 1-aminocyclopropane-1-carboxylate
AS - Ammonium sulphate
BTB - Bromothymol blue
CCD - central composite design
CSL – corn steep liquor
EtOAc - ethyl acetate
FeO-NP – Iron oxide nanoparticles
FTIR - Fourier Transform Infrared spectrophotometer
HPLC – High-performance liquid chromatography
IAA - indole acetic acid
IAAld - indole-3-acetaldehyde
IAAld - indole-3-acetaldehyde
IAM - indole acetamide
IAN - indole-3-acetonitrile
IPDC - IPyA decarboxylase
IPyA - indole-3- pyruvate
IPyA - indole-3-pyruvate
LB - lysogeny broth
MnO/FeO-NP – bimetallic Manganese and Iron nanoparticle
MnO-NP – Manganese oxide nanoparticles
NPs - nanoparticles
OD – optical density
OTUs - operational taxonomic units
PGR - producing plant growth regulators
RSM - Response surface methodology
SAED - selected area electron diffraction
SEM - Scanning electron microscopic
STR – stirred tank reator
TAM - tryptamine
TEM - Transmission Electron Microscopy
Trp – Tryptophan
TSO - and tryptophan side-chain oxidase
YE - Yeast extract

SUMARY

INTRODUCTION

Agriculture plays socio-economic and environmental vital importance for humanity. Currently, agriculture practice has been facing major challenges owing to world population growth, climate change, urban area expansion and the detriments of chemical fertilizers application over the years. According to the United Nations, world population is expected to increase more than 2 billion people by 2050, and food safety is among the major concerns for future. This leads to a worldwide run for food production increase but with new agriculture methods that are in accordance with environmentally sustainable practices (Pérez-Montaño et al., 2014; Sudha et al., 2012).

Plant growth-promoting bacteria (PGPB) play a vital role in agriculture by promoting the uptake of the nutrients, improve soil fertility and crop yield, and reducing the dependency in chemical fertilizers (Pérez-Montaño et al., 2014; Swethaa and Padmavath, 2016). This group is normally established in the soil ecosystem or as plant endophytes, well adapted at different conditions, with direct and indirect benefits mechanisms to promote plant growth and defense (Bhattacharyya and Jha, 2012; Cook, 2002). To mention some direct proprieties, those related especially to vital process in the plant life cycle and nutrition as solubilization of minerals like phosphates and other nutrients, sequestration of iron for plants by siderophores, hormone production, nitrogen fixation, and indirectly by the synthesis of substances with biocontrol activity to suppress disease-causing microbes or by competition for sites on plant tissues (Lucy et al., 2004; Pérez-Montaño et al., 2014; Swethaa and Padmavath, 2016).

The endophytic association between plants and microorganisms come all along since the ancient era, developed very early in evolution. It's a reciprocal interaction, where the host plant provides a protective niche for the endophytic, and in exchange the endophytic can produce useful metabolites and signals, helping on plant development and defense (Kawaguchi and Minamisawa, 2010; Saikkonen et al., 2004; Xia et al., 2015). The endophytes have the advantage to be closer to plant internal tissue and with less competition over other microorganisms.

Similar to plants, bacteria are capable to produce phytohormones which are chemical messengers related to trigger specific biochemical, physiological, and morphological plant responses (Arshad and Frankenberger, 1991). Besides, phytohormones act as well as mediators in communications between the plant host

and its microflora, for their symbiotic associations or pathogenesis (Arshad and Frankenberger, 1991; Spaepen et al., 2007; Tsavkelova et al., 2006). Phytohormones are classified into five classes: auxins, gibberellin, cytokinins, abscisic acid, and ethylene (Arshad and Frankenberger, 1991).

Auxin is one of the most important plant hormone class, responsible in the regulation at different plant stages, from the plant cell cycle, cell division, cell elongation, differentiation, root initiation, apical dominance, tropism response, flowering, fruit ripening and senescence (Arshad and Frankenberger, 1991). The capability of auxin production is widespread among various bacteria genera, which we can divide from those which the production aims to virulence interactions toward the plant and the others which the synthesis may be beneficial to the plant, stimulating the growth and development (Arshad and Frankenberger, 1991; Tsavkelova et al., 2006).

Exploiting endophytes PGPB abilities for agriculture application has raised as an important environmental method to increase crop productivity and pathogen defense, and in order to reduce the application of chemical fertilizers and pesticides. Biofertilizer market it is an expanding market, the estimated value for 2019 was at USD 2.0 billion, and is projected to increase and reach around USD 3.8 billion by 2025. All this scenario is being driven by the increasing demand of consumers for organic products, consequently increasing the demand for organic production, globally (Intelligence, 2019a, 2019b). On the other way, the acceptance for biofertilizers by the farmers has been raising, as they observed similar results on productivity when comparing to chemical fertilizers, especially after the propelling of advanced farming with drip irrigation and sprinkles, and better awareness of the environmental problem involving chemical products.

Seeking for an auxin-producing bacterium can increase the chance to discover a potential strain to be used as bioinoculant. This was the strategy used in this work. While studying the community of endophytic bacteria from in vitro *Eucalyptus* leaves, isolated bacteria strains were used to produce auxin and develop a biotechnology process, to produce a final bioproduct as bioinoculant to be used in agriculture and increase yield and minimize the negative effects of modern.

Important factors were taken in account at the process of the bioprocess development, starting from the choice of the microorganism strain, its nutritional requirements, the correct carbon and nitrogen ratio, the concentration of the cells,

amount of precursors in the medium culture, physical parameters such as temperature, pH, aeration, and posterior application to seed and plant growth.

Eucalyptus is the most widely planted hardwood trees, highly adaptable, with rapid growth and desirable wood properties (Myburg et al., 2014). Yet, *Eucalyptus urophylla* clone BRS07-01 was one of the target species for micropropagation and genetic transformation studies (Bettencourt et al., 2018; Oliveira et al., 2016). This clone presented highly efficient in vitro organogenesis results and was added to a genetic breeding program. On the other way, while investigating genetic transformation protocol, endophytic bacteria was currently seeing during the cultivations process, which aroused the interest to study their bacteria endophytic community.

GENERAL OBJECTIVE

Prospect IAA production by endophytic bacteria and their application for plant growth promotion.

Specific objectives

- Evaluate bacteria endophytes community from in vitro *Eucalyptus* explants by metagenomic
- Screening for endophytes with ability to produce IAA and other Plant growth promotions
- Select the isolates with more capability to produce IAA and optimize the production process and scale-up
- Evaluate the ability to produce Iron and Manganese nanoparticles using bacterial auxin complex, as a green synthesis approach

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PART I - ENDOPHYTIC BACTERIA AS SOURCE FOR BIOINOCULANTS APPLICATION AND THEIR ROLE ON AUXIN PRODUCTION

ABSTRACT

Endophytic bacteria have the unique characteristic to enter and colonize plant tissues, and maintain a benefit interaction within the host. They live in all known plant species, and are responsible direct and indirectly for several traits regarding plant growth nutrition and defense, such as nitrogen fixation, phosphate solubilization, phytohormones production and antimicrobial activities. To date, most of the research for plant growth promoting bacteria and further application as bioinoculants has been done focusing on rhizobacteria species, specially diazotrophic bacteria. However, endophytes from internal plant tissues are gaining more attention, and can be exploited to be used as bioinoculants. An interesting aspect is to explore their ability for auxin production and develop a scale up process, including medium components and culture conditions for higher yields production. Auxin-producing endophytes can be potentially used as bio-products, to be applied for plant growth promotions. This review focused on studies that have isolated plant endophytic bacteria and aimed to produce IAA with emphasis on medium and culture optimization, and their applications on plant growth.

Key-words: Bioinoculant; Biofertilizer; Nitrogen fixation; Plant growth promotion.

INTRODUCTION

Plant microbiome is vital for plant growth and survival and is ecologically important. The microorganisms produce a wide range of bioactive compounds useful for plants nutrition and protection and also produce several economical compounds with relevant importance to humans activities, such as antibiotics for medicine and other useful compounds to the food industry, as well as for significant environmental impact in nutrient cycling, biodegradation and bioremediation (Bacon and White, 2016; Nair and Padmavathy, 2014). Wherefore, beneficial microbiomes can be envisaged to be used for green plant biotechnology, as biocontrol agents to improve plant resistance, and as biofertilizers for plant nutrition, emerging as a promising technology for future sustainable farming systems, to help minimize the negative effects of modern practices and still increase yield (Mercado-Blanco and Lugtenberg, 2014; Podolich et al., 2015).

The best-known plant-bacterium interaction is the association between nitrogen-fixing bacteria from legume-nodulating rhizobia, specifically belonging to the

genera *Azorhizobium, Bradyrhizobium, Ensifer, Mesorhizobium, Rhizobium,* and *Sinorhizobium* (Hardoim et al., 2015). Followed by the endophytic associations with plants by beneficial microbes, that outstand similar plant growth promotions traits, as those present in the rhizosphere (Khare et al., 2018).

Endophytes microorganisms are those that do not visibly cause harm to the host plant, varying from bacteria and fungi, and has the unique ability to enter and thrive in the plant tissues, in intercellular or intracellular spaces, colonizing host cells in the cytoplasm or become situated in periplasmic spaces, between the cell wall and the cell plasma membrane (Bacon and White, 2016; Hardoim et al., 2015, 2008) of roots, leaves, stems, flowers or seed, and confer multidimensional interactions within the host plant (Khare et al., 2018; Verma et al., 2018). While the host plant provides a protective niche for the endophytes, in exchange they produce useful metabolites and signals, helping on plant development and defense (Kawaguchi and Minamisawa, 2010; Saikkonen et al., 2004; Xia et al., 2015).

Plant endophytic bacteria are grouped into one or more of three major functional groupings, according to their traits: i) Microbes that remediate host plant defense over abiotic stress; ii) Microbes that elicit defense response of host plants against biotic stressors (pathogens and herbivores) with the production of siderophores or antibiotics (Gaieiro et al., 2013); and iii) Microbes that promote host plant growth by providing nutritionally support, through nitrogen fixation, phosphorus solubilization, iron, vitamins and phytohormones production (Bacon and White, 2016; Khare et al., 2018).

Wherein, the close interaction with the host plant, with less competition for carbon sources and existing in a more protected environment, and with the ability to change from endophytic to a free lifestyle, turns endophytic bacteria more effective and intense purposive over rhizosphere microorganisms to promote plant growth and defense (Ali et al., 2017; Singh et al., 2017; Taulé et al., 2016; Yadav and Yadav, 2017). In this context, plant endophytic bacteria appeared as a suitable solution to be used as microbial inoculants, also called biofertilizers, as an environmentally friendly, sustainable alternative to chemical fertilizers, that ensures a more efficient use of natural resources and reducing the use of conventional inorganic fertilizers (Mishra et al., 2016; Ramakrishna et al., 2019; Schütz et al., 2018).

Today, the agricultural production yields are superior, which came along with the industrialization evolution, but it has been facing challenges with more frequently environmental problems caused by the climate change, which have resulted in shifts

in vegetation, and affected large areas of land worldwide by increased water scarcity, salinization and soil toxicity with heavy metals and organics (Ali et al., 2017; Egamberdieva et al., 2017; Pérez-Montaño et al., 2014). And in addition, the excessive use of chemical fertilizers caused more deterioration of water quality, acidification, and desertification of the soil, leaching, harming of beneficial organisms and daunting use of non-renewable resource for chemical fertilizers production, such as phosphates fertilizers derived from phosphorite, which has been estimated to deplete severely within the next 50-100 years (Mishra et al., 2016).

Furthermore, with the crescent world population and demographic development, the primary concern stills to provide a substantial additional agricultural production of ~2.4 × 109 t/year, albeit in accordance with sustainable practices, that should not be based on the land area but the improvement of crop productivity (Compant et al., 2019; Pedrosa et al., 2019). All these scenarios to maintain the ecological balance is increasing the demand for microbial inoculants or biofertilizers. Still, it is notable the positive impact created by the increasing trend of consumers and farmer´s preferences for organic fruits and vegetables due to the concern of food safety (Markets and Markets, 2019).

In 2019, the biofertilizer market represented about 5% of the total chemical fertilizers market (Timmusk et al., 2017), with a value around USD 2.0 billion, and it should grow up to 11.2% by 2025 (Markets and Markets, 2019). The biofertilizer market is dominated by nitrogen-fixing organisms, as nitrogen (N) is the nutrient of higher demand for cultured plants, having *Rhizobium* spp., *Bradyrhizobium* spp., *Actinorhizobium* spp., *Azotobacter* spp., and *Azospirillum* spp. as the main microorganisms used as nitrogen-supplying biofertilizers, and phosphate solubilizing bacteria, Mycorrhiza and other microorganisms (Intelligence, 2019a, 2019b; Pedrosa et al., 2019). Moreover, studies and application of endophytic microorganisms as bioinoculants or biofertilizers and for biocontrol use are still limited when compared with the utilization and benefits of rhizosphere microorganisms (Ali et al., 2017; Singh et al., 2017). Furthermore, when surveyed, the main traits of study are for nitrogen fixation and phosphate solubilization abilities (Bhattacharyya and Jha, 2012; Compant et al., 2019; Pérez-Montaño et al., 2014). In addition, less attention is been paid to isolate endophytes aiming phytohormones production, especially the auxins, their properties, production process and optimization, and their ability as bioinoculants.

Therefore, in this study we review papers that exploit IAA production by endophytic bacteria and their application for plant growth promotion.

ENDOPHYTES INTERACTION WITH THE PLANT HOST

It is believed that all plants host billions of one or more endophytes species of microorganisms (Mashiane et al., 2018; Strobel et al., 2004), wherein the opposite, a plant without endophytes would be less able to cope with pathogens and more susceptible to environmental stress conditions (Santoyo et al., 2016).

Endophytes diversity varies from host specificity, plant species, different organs, type of tissues, developmental stage and a range of biotic and abiotic factors, such as soil pH, salinity, soil type, soil structure and moisture, and soil organic matter, exudates, pathogen presence and human practices (Compant et al., 2019; Mei and Flinn, 2010; Vijayabharathi et al., 2016).

Plants actively attract and recruit microorganisms from surrounding microbial reservoirs such as the rhizosphere, phyllosphere, anthosphere, spermosphere and the carposphere by chemotaxis (Compant et al., 2019, 2011; Mishra et al., 2016). Rhizosphere which is one of the most complex ecosystems and concentrates the major community of microbial activity have been considered to be the primary source and route for endophytes colonization, where the microbes are horizontally transferred (Fig. 1) (Compant et al., 2019; Mercado-Blanco and Lugtenberg, 2014). This explains the close relationship between endophytic bacterial diversity to the rhizosphere bacteria population (Bhattacharyya and Jha, 2012; Santoyo et al., 2016). Although bacterial endophytes are different from specialized rhizosphere living bacteria, as they employ different mechanisms to enter into the plant tissues, particularly in roots and, are able to interact in a very efficient way with their plant hosts (Ali et al., 2014; Santoyo et al., 2016).

The endophytic colonization process starts with plant root exudates, such as organic acids, amino acids, fatty acids, phenolics, plant growth regulators, nucleotides, sugars, putrescine, sterols, which serves as signal molecules for the specific and complex interactions between the bacterium and the host plant, responsible for affecting and attracting surrounding microbial (Chaturvedi et al., 2016; Haichar et al., 2008; Mandal et al., 2010). And in response, the bacteria trigger the specific genes that are responsible for the endophytic lifestyle, required for attachment, penetration, and colonization of the inner plant tissues (Ali et al., 2014; Brader et al., 2014; Van der

Lelie et al., 2009) (Fig. 1). A recent study revealed that addition of flavonoids, quercetin and diadzein, compounds present in plant exudates, positively influenced endophytic colonization by *Serratia* sp. in rice tissue, and significantly increased the *Serratia* population inside rice plants (3.84 and 3.66 log c.f.u. per g) (Balachandar et al., 2006). Normally, the endophytes attach either by pili, lipopolysaccharide, or exopolysaccharide in their cell walls and enter through passive processes or through cracks or damage of lateral roots by active mechanisms, especially from cell elongation zone or root hair zone of the apical roots (Compant et al., 2019; Hardoim et al., 2008; Vijayabharathi et al., 2016).

All these factors such as type of root exudates, bacteria motility, and lipopolysaccharide composition are highly relevant traits in the establishment of endophytes colonization of plant roots (Chaturvedi et al., 2016). As shown by mutational analyses, lipopolysaccharide composition, particularly the rhamnose content had an impact on the attachment of *Herbaspirillum seropedicae* endophytic colonization on maize roots (Balsanelli et al., 2010) and, in a study conducted on rice plants, where it was found that exopolysaccharide production was essential for plant colonization by the nitrogen-fixing endophyte *Gluconacetobacter diazotrophicus* (Meneses et al., 2011).

Besides, primary and lateral root cracks, endophytic bacteria may also enter into plants from stomata, particularly on leaves and young stems (Santoyo et al., 2016). Although, due to UV light, the lack of nutrients, and desiccation, colonization via aerial parts is more difficult to occur (Hardoim et al., 2015). Seeds also represent an important source of microorganisms, which are vertically transmitted (Fig. 1), and are critical for seedling development process and survival, and then proliferate in the roots of the developing plant (Compant et al., 2019; White et al., 2014). The seed-borne endophytes are thought to be specific and more closely-adapted to plants (Verma et al., 2018).

After entering on plant tissues, endophytic bacteria are most likely to colonize in differentiation zone and intercellular spaces in the epidermis (Compant et al., 2011; Vijayabharathi et al., 2016), as these spaces are rich with minerals, sugars and non-carbohydrate metabolites, including various amino acids and organic acids, which turns the environment more appropriate for the endophyte uptake for their own growth (Mei and Flinn, 2010). Yet, the endophytes can enter from the exodermal barrier, and reside on the site of entry, deep inside the cortex, and at the intercellular space of the

cortex. Likewise, endophytes can penetrate into the endodermal barrier and invade xylem vessels, but the colonization in this tissue is less common (Vijayabharathi et al., 2016).

The endophytic lifestyle depends whether the bacteria rely strictly on the host plant for their nutrition and survival, turning them "obligate" endophytes, or if they are facultative bacteria which can live part of the time outside of the plant and part they dwell inside the plants (Hardoim et al., 2008; Vijayabharathi et al., 2016). Facultative endophytes, may leave plant tissues, especially when plant cells are differentiating and scavenge organic or inorganic nutrients in the soil, then reentering into plant tissues where they are degraded and nutrients are transferred to plants (Bacon and White, 2016b; Verma et al., 2018). Facultative endophytes were observed as seed-borne bacterial endophytes from rice plantlets, which after being inside the roots, they were able to exit root epidermal cells during root hair formation, and from hairs left at the elongating hair tip where the cell wall was thin (Verma et al., 2018). This phenomenon occurs normally when plant cells are differentiating.

Once inside and established in the plant tissues, endophytic bacteria have an advantage over bacteria inhabiting the rhizosphere, as they have the opportunity to constantly be in contact with the plants cells, with less competition and therefore, exert a direct beneficial effect (Santoyo et al., 2016), by eliciting different modes of action for phytostimulation, turning essential elements available for plant growth and development and synthesis of bioactive compounds that are used by plant´s for defense against pathogens (Nair and Padmavathy, 2014).

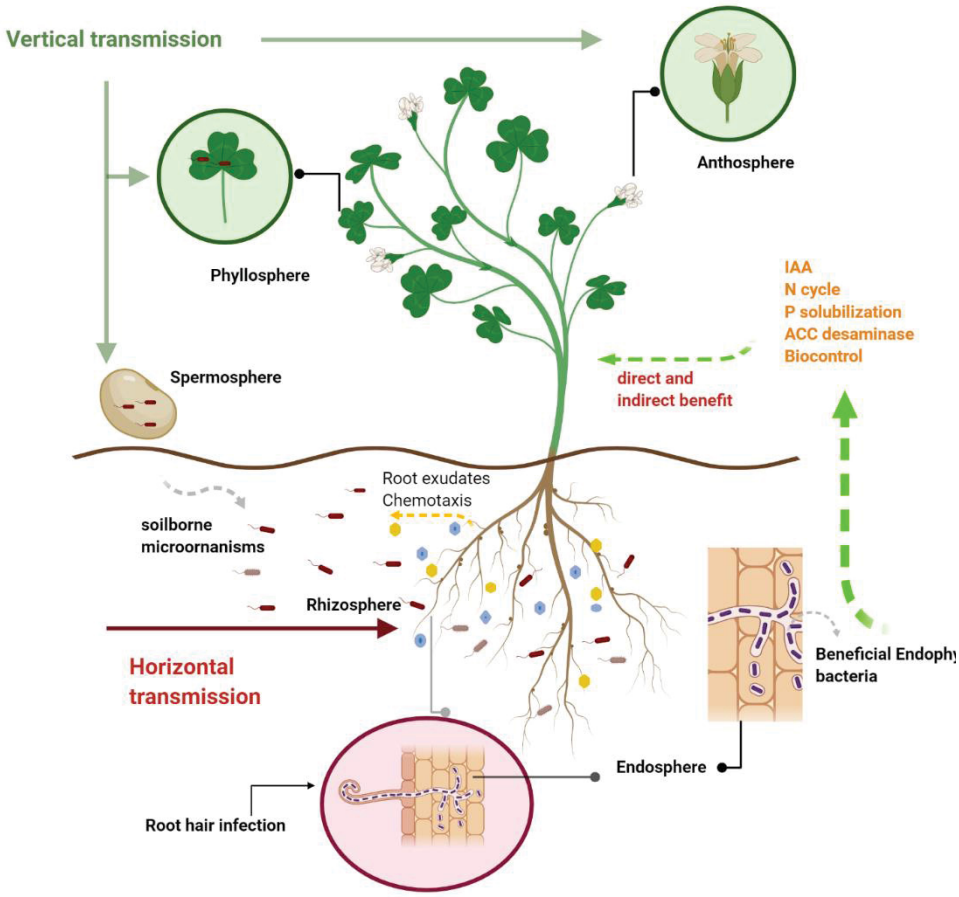

Figure 2. Sites of plant colonization by endophytic bacteria. Microorganisms from spermosphere, anthosphere, phyllosphere are transmitted via vertical. Seeds endophytes can spread to rhizosphere and integrate soilborne microorganisms' communities. Rhizosphere microorganisms are attracted by root exudates and then invade the internal plant tissues through cracks formed at the sites of lateral root emergence and root tips, and colonize internal plant tissues. Competent endophyte bacteria can promote plant growth promotion and defense by IAA production, nitrogen cycle, phosphate solubilization, ACC deaminase, and other bio compounds. Schematic diagram design using BioRender APP. Figure by the author.

Furthermore, endophyte-plant interaction and plant growth responses hinge upon their genotypes (Mei and Flinn, 2010; Park et al., 2017). Different responses were observed with different poplar cultivars and different endophytes in a preliminary experiment (Taghavi et al., 2009; Van der Lelie et al., 2009). This turns necessary to understand the interactions between endophytic bacteria and their host plant or other plant species in order to test the ability of these microorganisms to colonize diverse plant species, or to discard any disease symptoms or abnormalities, prior to the application as bio inoculants (Zinniel et al., 2002).

PRODUCTION OF PHYTOHORMONE BY ENDOPHYTES: AUXIN CLASS

Phytohormones are secondary metabolites, known as chemical messengers which regulate plant responses over different environmental conditions at extremely low concentrations. These compounds are capable to promote either biochemical, physiological, and morphological responses for plant growth and development, as well as to induce systemic tolerance to stresses (Ali et al., 2017; Arshad and Frankenberger, 1991). Although microorganisms do not need phytohormones to exist (Pirog et al., 2018), they have the ability to produce phytohormones, for their effect on plant growth promotion and symbiotic associations, and also to manipulate the plant internal hormonal balance to mediate plant-pathogenies mechanisms (Hwang et al., 2015).

Phytohormones produced by endophytic bacteria have a similar effect on plant growth stimulation, nutrient acquisition, and plant tolerance to stresses as phytohormones and exogenous phytohormone supplementation (Egamberdieva et al., 2017; Etesami et al., 2015; Patten and Glick, 1996). They function as molecular signals, to elicit transcriptional changes in hormone, defense-related and cell wall-related genes, regulating the endogenous plant hormone balance in response to environmental factors, to boost growth and stress responses (Backer et al., 2018).

Since Charles Darwin observed the movement of plants toward the sunlight and reported the existence of a growth-regulating signal responsible for phototropism and geotropism phenomenon, in 1880, scientists focused to understand how plants regulate their growth and what substances were related do the signal mechanisms (Patten and Glick, 1996). Later on, in 1946, Haagen-Smit and collaborators reported that the auxin IAA in higher plants implicated in virtually all aspects of plant growth and development (Arshad and Frankenberger, 1991; Spaepen et al., 2007). Hitherto, phytohormones are classified into five major classes: auxins, cytokinins, gibberellins, ethylene and abscisic acid (Arshad and Frankenberger, 1991), but there is evidence of other compounds related to plant growth regulation as brassinosteroids, jasmonates, salicylic acid, strigolactones, among others (Ahmed and Hasnain, 2014).

Auxins are one of the most important plant growth regulator groups that act as a master control, direct or indirectly regulating most of the plant processes, at different plant stages, from plant cell cycle to cell division, cell elongation, differentiation, root initiation, apical dominance, vascular differentiation, tropism response, floral meristem initiation, fruit development, leaf abscission, parthenocarpy, differentiation of phloem

and xylem and senescence (Ahmed and Hasnain, 2014; Arshad and Frankenberger, 1991; Tanimoto, 2005). Indole-3-acetic acid (IAA) is the major naturally occurring auxin known in plants and that can be synthetized as well by bacteria and fungi (Tsavkelova et al., 2007). Other natural well-known auxins are indole-3-butyric acid (IBA), 4-chloro-IAA, in addition to the precursor indole-3-acetonitrile and others conjugate IAA forms from the indolic group, and phenylacetic acid (PAA) from non-indolic compound. All these are also considered active auxins, but little is known about their physiological roles (Ahmed et al., 2010; Cassán et al., 2014). Yet, the synthetic auxin groups with 1-naphthaleneacetic acid (NAA), 2,4-Dichlorophenoxyacetic acid (2,4-D), picloram and dicamba are also known (Arshad and Frankenberger, 1991; Nagata et al., 2007; Normanly et al., 1995).

The ability to synthesize IAA is widely distributed among plant-associated bacteria (Lacava, 2013), such as *Azospirillum spp., Alcaligenes faecalis, Klebsiella spp., Enterobacter sp., Acetobacter diazotrophicus, Herbaspirillum seropedicae, Azotobacter spp., Rhizobium spp., Rhodospirillum rubrum, Pseudomonas fluorescens, Bacillus subtilis, Paenibacillus polymyxa* and most species of the genus Rhizobium (Lacava, 2013; Pirog et al., 2018). Evidence showed that IAA is related to microbe-plant interactions signaling for their colonization strategy and can also activate plant defense systems against phytopathogenic microorganisms, as they can interfere with gene expression regulation (Spaepen et al., 2007; Spaepen and Vanderleyden, 2011).

Auxin biosynthesis in bacteria can occur via multiple pathways, similar to how it occurs in plants (Spaepen and Vanderleyden, 2011). It can be produced via tryptophan (Trp) or not, by several possible enzymatic pathways (Arshad and Frankenberger, 1991; Normanly et al., 1995). Tryptophan is one of the main compounds present in several plant exudates and the main precursor for IAA production by bacteria. The addition of tryptophan to culture media results in most of the cases in higher IAA production (Idris et al., 2007). Mutants strains with inactivation of genes involved in tryptophan biosynthesis of *Bacillus amyloliquefaciens* FZB42, demonstrated reduction of both IAA concentration and plant-growth-promoting activity, showing that production of IAA was dependent on tryptophan (Idris et al., 2007).

At least five different pathways for the synthesis of IAA by bacteria using tryptophan have been described (Patten and Glick, 1996; Spaepen and Vanderleyden, 2011), namely according to their intermediates, as indole-3- pyruvate (IPyA), indole

acetamide (IAM), tryptamine (TAM), indole-3-acetonitrile (IAN) and tryptophan side-chain oxidase (TSO), this last was named according to the key enzyme (Cassán et al., 2014; Patten and Glick, 1996). In addition, a tryptophan-independent pathway has been suggested (Spaepen and Vanderleyden, 2011). Moreover, more than one pathway could be present in the same bacterium (Li et al., 2018; Patten and Glick, 1996), as demonstrated in the plant growth-promoting rhizobacterium *Azospirillum brasilense* (Prinsen et al., 1991; Vande Broek et al., 1999) and *P. fluorescens* (Oberhänsli et al., 1991), but it seems that in most of the bacteria IAA biosynthesis follows mainly the IAM and the IPyA routes (Fig. 2) (Ali et al., 2017).

It is important to note that not all IAA-producing bacteria are beneficial to plants. Auxin biosynthesis is also wide-spread among plant pathogens (Patten and Glick, 1996; Tsavkelova et al., 2007). Normally, phytopathogens produce auxins in ultrahigh quantities, which leads to a disruption of the plant's hormonal concentrations, leading to some diseases (Pirog et al., 2018), as in the classic example of *Agrobacterium tumefaciens,* the causal agent of crown gall disease, which is used for plant genetic transformation (Bettencourt et al., 2018; Koga et al., 1991). Yet, the resulting plant-microbe interactions can be determined by the pathway of IAA production (Ali et al., 2017). The IAM pathway has been described mainly in phytopathogenic bacteria and is involved in down-regulation of genes related to plant defense responses (Kochar et al., 2013; Robinette and Matthysse, 1990; Yamada, 1993). *Pseudomonas savastanoi,* associated to plant diseases, was described to produce IAA using Trp via IAM pathway (Ouzari et al., 2008), where tryptophan is converted to indole- 3-acetamide (IAM) by tryptophan-2-monooxigenase and IAM is metabolized to IAA by IAM-hydrolase, determined by *iaaM* and *iaaH* genes (Fig. 2) (Kochar et al., 2011; Mohite, 2013).

IPyA pathway is considered the "main" auxin biosynthetic pathway in plant and bacteria, although, the downstream steps reactions for IAA biosynthesis are quite different in bacteria and plants (Enders and Strader, 2015; Korasick et al., 2013). The bioconversion of Trp to IAA by IPyA is a simple three-step reaction, where the enzyme aromatic amino acid transferase starts converting Trp to IPyA, followed by decarboxylation of IPyA by an IPyA decarboxylase (IPDC) to indole-3-acetaldehyde (IAAld), and then oxidize to IAA by indole-3-acetaldehyde dehydrogenase (Fig. 2) (Cassán et al., 2014; Jasim et al., 2014). Molecular analysis reported that *ipdc* gene is related to IPyA pathway in IAA biosynthesis in bacterial isolates (Jasim et al., 2014). The IPyA pathway was identified in mutualistic endophytes *Piriformospora indica* that

colonizes roots in an asymptomatic manner (Swethaa and Padmavath, 2016) and in the nitrogen-fixing bacteria *Azospirillum brasilense* (Aguilar et al., 2008) and *Enterobacter cloacae* (Koga et al., 1991).

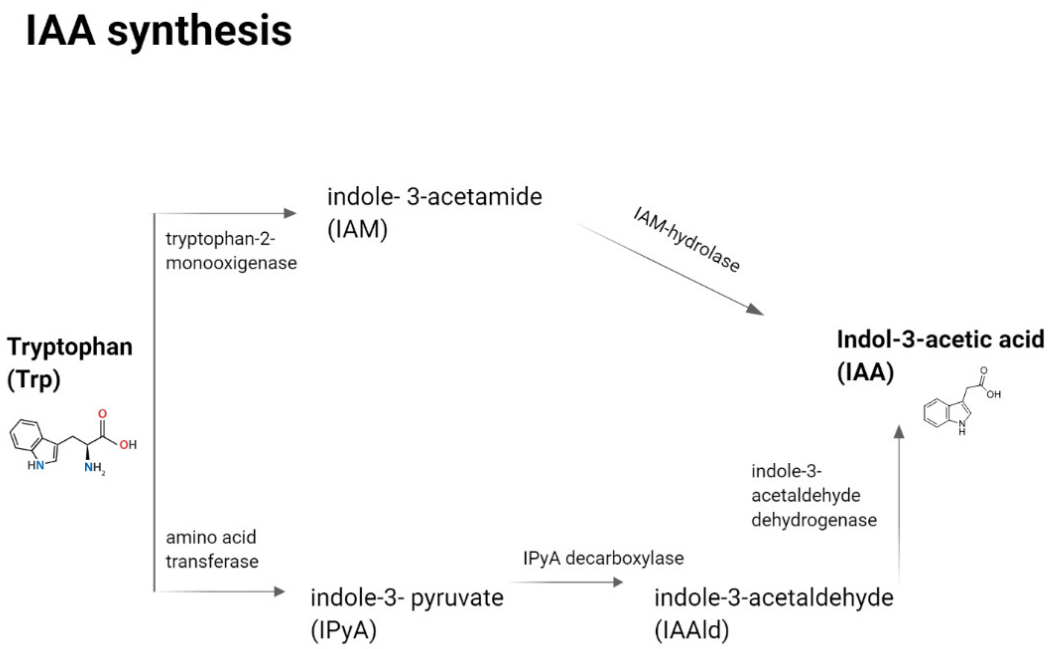

Figure 1. Overview of two indol-3-acetic acid (IAA) biosynthetic pathways in bacteria: indole-3-acetamide (IAM) and indole-3- pyruvate (IPyA) pathway and their intermediates, and Tryptophan (Trp). Adapted from Spaepen and Vanderleyden (2011).

Moreover, screening for phytohormones producing-bacteria among the endophytes isolates is a strategy to select those with potential abilities to be used as biofertilizers for agriculture practices. This strategy increases the chances to acquire a potential plant growth promoting bacteria (PGPB), with more than one plant growth-promotion trait, as usually auxin producing endophytic bacteria are found to have other mechanisms abilities, such as nitrogen fixation and phosphate solubilization (Verma et al., 2018).

IAA-PRODUCING ENDOPHYTES

The fact that auxins are responsible for so many morphological and structural changes in plants, turns them the most studied phytohormones, followed by cytokinins and gibberellins (Dodd et al., 2010; Mutturi et al., 2016).

Screening for auxin producing endophytic bacteria has the potential to obtain an efficient microbial inoculant for sustainable agricultural application (Ramanuj and Shelat, 2018; Verma et al., 2018; Yadav and Yadav, 2017). Endophytic bacteria isolated with the ability to produce IAA, especially using Trp as a precursor, were reported in some studies (Table 1). Most of the focused reports were on the pre-screening for IAA production and concomitantly for other plant promotion characteristics and their ability to promote plant growth.

A screening study for rice cut grass (*Leersia oryzoides L*.) seeds bacterial endophytes, reported that from ten isolates evaluated, six were able to produce IAA in a range of 13.26 $\mu g.ml^{-1}$ to 47.06 μgml^{-1}, and promoted increases in root and shoot lengths in rice seedlings. Afterward, the authors identified them as *Pantoea agglomerans*, *Pseudomonas sp.*, *Pantoea hericii*, *Chryseobacterium sp., Pseudomonas oryzihabitans* and *Pantoea vagans* (Verma et al., 2018). Indeed, IAA production by bacteria can vary among different species and strains, and it is also influenced by culture condition, growth stage, and substrate availability (Ambawade and Pathade, 2013). The ability to produce IAA does not seem to depend on the Gram-factor, since either Gram-positive or Gram-negative strains were reported to produce IAA, including *Alcaligenes spp., Acetobacter spp., Azospirillum spp., Azotobacter spp., Bacillus spp., Burkholderia spp., Enterobacter spp., Herbaspirillum spp., Klebsiella spp., Paenibacillus spp., Pantoea spp., Pseudomonas spp., Rhizobium spp., Rhodococcus spp.,* and *Streptomyces spp.* (Acuña et al., 2011; Belimov et al., 2015; de Souza et al., 2013; Fretes et al., 2019; Pereira and Castro, 2014; Pérez et al., 2016; Tabassum et al., 2017). *Azospirillum*, *Pseudomonas* and *Bacillus* genera are the most studied IAA producers (Ali et al., 2017; Cassán et al., 2015; Pindi et al., 2013).

Bacterial IAA biosynthesis is highly influenced by environmental stress, including acidic pH, osmotic and matrix stress, carbon limitation, and plant exudates (Spaepen et al., 2007). Likewise, for in vitro production, factors such as bacteria growth rate, type of carbon and nitrogen sources, tryptophan concentration, temperature, agitation, and days of incubation are related to affect directly to the concentration of IAA.

Acidity is a chemical property of soils that plays a central role in plant development and soil microbiome. Processes such as organic matter decomposition, mineralization, immobilization, ammonification, nitrification, volatilization, biological

nitrogen fixation, and insoluble inorganic phosphate solubilization, have a direct effect on pH and consequently influences the rhizosphere environment (Marra et al., 2015; Spaepen et al., 2007). Consequently, the pH of the culture medium directly influences the growth of microorganisms and the biochemical processes they perform (Marra et al., 2015). The appropriate pH for an isolate growth in vitro culture may be linked to the source from where the strains were isolated. This was supposed by Sachdev et al. (2009) when they observed no growth of *Klebsiella* strains at pH 4.0 and 5.0, and detected the optimum IAA production at pH 8, as these strains were isolated from the wheat rhizosphere (clay soil) which apparently have alkaline pH (pH 7.8-8.0). Alkaline conditions were also optimum for *Serratia marcescens* subsp. *marcescens* IAA production, showing the highest production of IAA (3.56 $\mu g.mL^{-1}$) at pH 9 (Hasuty et al., 2018). Differently, acidic pH promoted higher IAA production for *Bacillus sp*. MQH−19 and *Paenibacillus sp.* SPT−03 endophytes, around pH 6.0 and 5.0 respectively (Acuña et al., 2011). Similarly, in recent research two *Eucalyptus* endophytes isolated from micro-propagated explants, which were cultivated on pH 5.0 to 8.0, showed higher IAA production when cultured in pH 5.0 (Part II).

In the environment, bacterial production of phytohormones are determined by plant exudates or specific compounds and/or the presence of plant extracts and surfaces. Flavonoids, which are produced by the host plant and accumulate in the rhizosphere stimulates IAA production of the symbiotic bacterium *Rhizobium sp*. strain NGR234 (Prinsen et al., 1991; Theunis et al., 2004). Plant leaf extracts from *Citrus cinensis* showed to increase *Xanthomonas axonopodis* IAA production (Costacurta et al., 1998). A remarkable report was the improvement of 20-fold of IAA concentrations (642 ± 77.07 to 869 ± 78.82 $\mu g.mL^{-1}$) with the addition of pepper extract on the medium, by endophytic bacterial isolated from *Piper nigrum* (Jasim et al., 2014). The authors also investigated several parameters for IAA production optimization by comparative and quantitative analysis. The experimental approach was designed based on the assumption that once living in the microenvironment of pepper plants, the endophytic bacteria bio-molecules synthesis, as IAA, might be regulated by the plant extracts. Although it remains unclear which is the exact compound responsible for this induction, owing that plant extracts have multi bioactive compounds that can induce phytohormones production (Spaepen et al., 2007).

Tryptophan is the major precursor and indispensable component for IAA biosynthesis in the culture broth. Despite that some isolates show the ability to produce

IAA without L-Trp supplement in medium, the majority of the isolates produce IAA especially when Trp is added to the medium, and exhibits higher amounts of IAA (Ambawade and Pathade, 2013). *Klebsiella* strains enhanced up to 2.2 times of IAA amount when tryptophan was added to the medium (Sachdev et al., 2009). And, from endophytes isolated from *Cocos nucifera L*., two *Serratia marcescens subsp. marcescens* strains, showed the highest IAA when Trp concentration was increased to 25 mM, producing 10.36 μg./mL^{-1} and 11.18 μg.mL^{-1} IAA (Hasuty et al., 2018).

As a secondary metabolite, IAA peak production is associated with the stationary phase, indicating that the bacteria growth phase plays a critical role in determining the amount of IAA produced (Ahmed and Hasnain, 2014; Spaepen and Vanderleyden, 2011). A *Bacillus* sp. OPR 7 isolate from *Ophioglossum reticulatum L.* showed increasing production of IAA beginning at exponential phase of growth and reached its maximum after 96 h of culture, and produced nearly 40 μg.ml^{-1} of IAA under batch culture (Mukherjee et al., 2017). This confirms that IAA is produced during all stages of culture growth but increases significantly in the stationary phase (Malhotra and Srivastava, 2009).

Interestingly, carbon limitation and reduction in growth rate are both linked to the stationary phase. It clarifies the importance of microbial growth status and behavior in the IAA biosynthesis and, vice versa (Spaepen et al., 2007). Evaluating growth and IAA biosynthesis of *Azospirillum brasilense* Sp245, the authors observed that IAA biosynthesis was absent during exponential growth in micro-aerobic batch cultures, and no *ipdC* gene expression was observed (Ona et al., 2005). And when evaluating growth in fed-batch culture they clearly observed that carbon limitation and reduction in growth rate were necessary for IAA biosynthesis in *A. brasilense*. This observation confirmed that bacteria growth arrest caused by depletion of the carbon source and N limitation in the growth medium are required for IAA production for some strains (Ona et al., 2005). Similar features are also observed for other secondary metabolites production by bacteria.

The time course of IAA production varies from bacterial species, starting from the first day of culture until 7 to 12 days (Goudjal et al., 2013; Sachdev et al., 2009). Seeking to optimize the culture conditions for IAA production by *Streptomyces* sp. PT2 endophytic, the maximum concentration was after 7 days, and the production decreased after this time (Goudjal et al., 2013). This might be explained by the released enzymes, such as oxidases and peroxidase which degraded IAA, as has

been shown in *Rhizobium* sp. from *Cajanus cajan* (Datta and Basu, 2000; Goudjal et al., 2013). The same *Streptomyces sp*. PT2 produced more IAA when cultured at 25 °C. Normally, the temperature for IAA production varies in the range of 25-30 °C, and peak production occurs between 2 and 5 days of culture (Table 1).

However, most reports with endophytes capable to produce auxins are limited only to the pre-screening step (Hasuty et al., 2018), using mostly enriched medium and one culture condition, without further studies to optimize the IAA production to its maximum possible yield (Jasim et al., 2014), which is observed more regularly with rhizobacteria isolates. Once knowing the ability to produce IAA for a specific strain, the research could further investigate how to optimize the fermentation process by testing different conditions such as carbon and nitrogen source and ratio, temperature, pH, and a range of Trp concentrations to find the optimized conditions. By optimizing the medium components for the production of IAA, isolate *Pantoea agglomerans* strain PVM reached the maximum IAA production (2.191 $g.L^{-1}$) using a rapid and cost-effective medium with 8 $g.L^{-1}$ of meat extract and 1 $g.L^{-1}$ of L-Trp, at pH= 7, 30°C and for 48-h of incubation (Apine and Jadhav, 2011).

Cultural and nutritional conditions for IAA production optimization was also estimated for different varieties of sugarcane isolates of *Acetobacter diazotrophicus* endophytes (Patil et al., 2011). Effect of different concentrations of tryptophan, incubation period, pH, sucrose concentration and different nitrogen sources on IAA production was investigated separately. The results showed that LGI medium supplemented with 1.2 $g.L^{-1}$ tryptophan, after 6 days resulted in the maximum IAA yield with an agitation of 200 rpm. Further, pH at 6 produced maximum IAA, while high acidic and alkaline pH were not suitable. In reference of carbon and nitrogen source concentrations, 12% (w/v) of sucrose and 0.1% (w/v) NH_4Cl were more efficient to produce the higher IAA concentrations. Sucrose is the main carbon source present in sugarcane, therefore the most suitable carbon source for *A. diazotrophicus* growth. Thus, all these medium conditions were combined with 30°C on a shaker at 200 rpm, and reached a maximum IAA production at the stationary phase (Patil et al., 2011). Agitation is related to aeration and circulation of nutrients in the fermentation process, and might favor the growth of microbial mass and enzymatic transformations, in this case of tryptophan to auxins (Jasim et al., 2014).

So far, most of the studies used the classical single factor method, but it is time-consuming and laborious. To contour this, statistical designs as response surface

methodology (RSM), have been used in bioengineering to investigate the interaction among the influencing factors and in order to optimize bio-molecules production, which allows the evaluation of multiple variables with a minimum number of experiments (Patel and Patel, 2014; Swethaa and Padmavath, 2016). These methodologies were applied for *Enterobacter sp*. DMKU-RP206 (Nutaratat et al., 2017), *Pseudomonas putida* Rs-198 (Peng et al., 2014), and endophytic bacteria isolated from saline desert plants (Patel and Patel, 2014) for IAA production optimization.

As mentioned by Patel and Patel (2014), optimization was especially important to scale up the bulk production of IAA at an industrial scale. The study applied first the Plackett-Burman experimental design for initial screening of the factors, to see the potential factors that influence the response, and then subjected to RSM to obtain the optimum concentrations of individual variables. Using this strategy, they observed that six factors of media components including yeast extract, mannitol, $MgSO_4.7H_2O$, K_2HPO_4, NaCl, and L-Tryptophan reflected more positively to attain higher IAA yields. In sequence, they applied a 2^n factorial central composite design with 52 experiments and observed an increase of IAA production in a range of 1.22 (141.125 $\mu g.mL^{-1}$), 4.41 (148.875 $\mu g.mL^{-1}$) and 4.45 (161.875 $\mu g.mL^{-1}$) fold when compared to the standard medium (36.25 $\mu g.mL^{-1}$) (Patel and Patel, 2014).

Up to now, *Enterobacter sp*. DMKU-RP206 isolated from rice leaves, was reported to produce the highest amount of IAA regarding a plant-associated bacteria species. After optimizing the process, the optimum conditions were 0.85% of lactose as a carbon source, 1.3% of yeast extract as a nitrogen source, 1.1% of L-tryptophan as a precursor, 0.4% of NaCl, an initial pH of 5.8, with incubation temperature at 30 °C, and a shaking speed of 200 rpm, for 7 days of incubation. By using these conditions, the authors could reach up to 3158.8 ± 72.2 $mg.L^{-1}$ of IAA production. Not withstanding, performing scale up the obtained IAA amount increased to 5561.7 $mg.L^{-1}$, a 13.4-fold improvement in IAA production by the isolate (Nutaratat et al., 2017). The same research group kept improving the IAA production by *Enterobacter* sp. DMKU-RP206 and succeeded to replace lactose to an alternative low-cost carbon source, using in the medium 1.48% sweet whey, 1.42% yeast extract and 0.88% L-tryptophan, pH adjusted to 6 and with shaking at 200 rpm, at 30°C for 3 days, and produced 3,963.0 $mg.L^{-1}$ of IAA after 3 days (Srisuk et al., 2018). Thus, this result indicates that IAA in culture filtrates of *Enterobacter* sp. DMKU-RP206 can be used instead of the synthetic IAA. In addition, other alternative low-cost sources can be used

over a synthetic medium, although few reports have shown the use of alternative low-cost materials for IAA production (Srisuk et al., 2018). Another example was developed for a rhizobacterial strain, *Pseudomonas putida* Rs-198, where corn flour was used (Peng et al., 2014). In our group, we optimized IAA production using corn steep liquor, a sub-product from corn steeping water and glycerol, a product resulted from biodiesel production, as nitrogen and carbon sources, developing a cheap medium composition, and obtained higher IAA productions for *Bacillus megaterium* and *Paenibacillus polymyxa* strains (see Part IV).

Table 1. Indol-3-acetic acid (IAA) production by endophytes bacteria isolated from plants. By the author.

Isolate	Host plant	Quantity ($\mu g.ml^{-1}$)	Analysis method	Medium composition	Culture Conditions	Traits in plant growth	Reference
Bacillus subtilis KC202298 *Paenibacillus sp.*	Tomato (*Solanum lycopersicum L. cv. Nagina)*	4.92±0.64 1.66±0.11	HPLC	100 ml Luria-Bertani (LB) broth supplemented with tryptophan (100 $mg.L^{-1}$)	28 ± 2 °C for 48 h in an orbital shaker at 150 rpm	nitrogen fixation, P-solubilization through organic acid production, synthesis of IAA, ACC deaminase activity, AHLs production and biocontrol potential.	Qbal et al., 2018
Bacillus subtilis B. pumilus B. subtilis B. Subtilis	maize sap, roots, and leaves	3.2 to 61.6	Colorimetric method	TSB medium supplemented with 1.0 mg.mL−1 of l-tryptophan	30◦C for 5 days at 100 rpm in the dark.	Strains with the ability to solubilize Fe-P in vitro and resulted in enhanced shoot and root dry weight and root N P content of pearl millet plants. Positive performance on plant biomass production and accumulation of nutrients N P K in the shoot.	Ribeiro et al., 2018
Sphingomonas sp. LK18 Methylobacterium radiotolerans LK17 B. subtilis LK14 B. subtilis LK15 Sphingomonas sp. LK16	Moringa	25.46±0.13 µM to 165.53±1.01 µM	Colorimetric assay UPLC–MS/MS method	20 mL of LB broth without (0 $g.L^{-1}$) and with 0.1 $g.L^{-1}$ of L-tryptophan	30±2°C in shaking incubator at 200 rpm for 7 d.	Enhanced the development of tomato seedling: shoot length, shoot weight, root length, and chlorophyll contents by *B. subtilis* LK14 culture. Antibacterial effect of *Bacillus* strains	Khan et al., 2016

						against *R. solanacearum.*	
Stenotrophomonas maltophilia BE 25	Banana plant sample (Roots)	13.8 and 21.0 µg ml-1	Colorimetric technique HPLC	Yeast malt dextrose broth containing 250 and 500 µg.mL^{-1} of tryptophan	28 °C for 4 days		Ambawade and Pathade, 2013
Klebsiella pneumoniae PnB 8 Enterobacter cloacae PnB 9 Enterobacter sp. PnB 10 Klebsiella sp. PnB 11 Pa.agglomerans PnB 12	*P. nigrum*	Maximum rates: 869.78±82 755.48±21 719.61±48 642.77±07 686.33 ±5.68	Quantification by Colorimetric and HPTLC methods HPLC for confirmation	10-mL nutrient broth containing 0.2% tryptophan. Tryptophan: 0.2, 0.4, 0.8 and 1.6% Addition of 0.5% of filter sterilized aqueous extract of pepper plant and without extract was used as control.	Best conditions: 28°C on shaking incubator at 120 rpm, 10 days Other different conditions: temperature 4, 28 and 37°C pH 4, 7 and 9 Days: 5, 10 and 15 days With 120 rpm and without shaking.		Jasim et al., 2014
Gram negative and Gram positive	rhizomes of *Gloriosa superba*	52.85 to 89.28	Colorimetric method	Nutrient Broth (10 ml) supplemented with 0.5 g% tryptophan, inoculated with 0.1 ml [1% (v/v)] inoculum	48h, 30°C with a shaking speed of 120 rpm.	Endophytes with multi traits and are strong contenders for bioinoculant production either individually or as consortia	Ogale et al., 2018
Pseudomonas, Paenibacillus, Rhodanobacter, Microbacterium, Azospirillum Pseudacidovorax, Paenibacillus, Janthinobacterium, Rhizobium,	Duckweed tissues	3.48 to 78.16	Salkowski colorimetric assay	LB medium and liquid TSB, with and without 5mM L-tryptophan.	48 h at 28◦C with shaking at 240 rpm		Gilbert et al., 2018

Herbaspirillum, Bosea, Acidovorax							
Serratia marcescens subsp. Marcescens	coconut tree (*Cocos nucifera* L.)	10.36 11.18	colorimetric method	LB broth medium amended with 1% of 5 mM L-tryptophan L-tryptophan (5, 10, 15, 20, and 25 mM)	30ºC on rotary shaker (120 rpm) for 96 hrs. pH (6, 7, 8, and 9), and temperatures (35, 37, 40, and 45ºC).		Hasuty et al., 2018
B. megaterium NMp082	Nodules of *M. polymorpha*	8.82±0.57	Colorimetric method	LB medium with Tryptophan	28◦C at 150 rpm and for 66h.	Displayed ACC deaminase activity. Inoculation with NMp082 remarkably enhanced plant growth in all Medicago species.	Chinnaswamy et al., 2018
Enterobacter sp. DMKU-RP206	rice leaves	5561.7	HPLC	0.85% of lactose as a carbon source, 1.3% of yeast extract as a nitrogen source, 1.1% of L-tryptophan as a precursor, 0.4% of NaCl, an initial pH of 5.8	30 °C, and a shaking speed of 200 rpm	Capability of phosphate solubilization and IAA, ammonia, and siderophore production, including fungal antagonistic activity.	Nutaratat et al. (2017)
E. cloacae	Alfalfa	112 ± 6	Colorimetric method	Bertani broth supplemented with 0.0 and 0.2 mg.mL^{-1} of tryptophan	30 °C for 3 days.	Phosphate solubilization, Antibacterial activity, salt stress tolerance	Khalifa and Saleh, 2016
Gram negatives and Gram positives	*C. rotundus* (nutgrass)	49.91 and 0.13 ppm	Colorimetric method	nutrient broth medium containing 0.1 mg mL-1 L-tryptophan	35± 2ºC for 48 h in a rotary shaker (120 rpm).	Ability of phosphate solubilization	Zega et al., 2018
Pseudomonas stutzeri	saline desert plants	97.5 103.75	Colorimetric method	tryptone yeast extract broth medium supplemented by	incubated at 37°C for 168 hours.		Patel and Patel, 2014

Bacillus sp. *TA_AJ2*				tryptophane 200µg.mL^{-1}			
Acetobacter diazotrophicus	sugarcane variety Co 740 stem	26.28	Colorimetric method	sucrose (12% w/v), yeast extract (0.05 g/l), L-tryptophan (1.2 g/l) and NH4Cl (0.1% w/v), pH=6	incubated in the dark at 30ºC for 7 days on shaker at 200 rpm.	increased root and shoot biomass as well as IAA level of the fresh root tissue of maize plant	Patil et al., 2011
Streptomyces sp. PT2	Saharan spontaneous plants	127.0	Colorimetric method	yeast extract–tryptone broth (YT) (5 g yeast extract/L, 10 g tryptone/L, 5 g NaCl/L, pH 7.2) supplemented with 5 mg/ml of L-tryptophan	cultured on a rotary shaker (200 rpm) at 30 °C for 5 days		Goudjal et al., 2013
Ochrobactrum sp. *MSP9 Enterobactersp. MS10*	Pea plants	0.86 to 16.16	Colorimetric method HPLC	YEM broth supple-mented with tryptophan (100 mg l-1) for 1 week.		Ability to fix nitrogen, solubilize inorganic phosphorus, considerably better potential for colonization on pea roots, biofilm formation	Tariq et al., 2014
P. lentimorbus P. macerans.	meristems collected from *Cymbidium eburneum* orchids	1.50 to 3.63	Colorimetric method	nutrient broth liquid medium, supplemented with 10 mM of NH4Cl2 and 10 mM L-tryptophan.	incubated in the dark at 28 °C, under agitation (120 rev/min), for 24 h.	promoted *Cattleya loddigesii* seedling growth during ex vitro acclimatization.	Faria et al., 2013

IAA-PRODUCING ENDOPHYTES AS PLANT BIOINOCULANTS

IAA has a profound influence on plant growth and development (Sachdev et al., 2009). One of the most prominent features of plants inoculated with auxin-producing plant growth-promoting bacteria is the modification in the root morphology and development (Ahmed and Hasnain, 2014), and indirectly the improved nutrients uptake by increased root growth (Pindi et al., 2013; Ribeiro et al., 2018). In addition, the effects of auxins on plants are concentration-dependent, since as low concentration may stimulate growth while high concentrations might be inhibitory (Ambawade and Pathade, 2013).

Microorganisms are pointed to directly or indirectly modulate seedling morphogenesis, by releasing phytohormones and other biomolecules, during seedling development (Verma et al., 2018). *Pantoea vagans*, *Pseudomonas oryzihabitans* and *Microbacterium* sp. endophytes bacteria from *Leersia oryzoides* seeds were found to promote positive gravitropic response in rice seedlings and further stimulated root growth and improved root architecture, in comparison to bacteria-free controls (Verma et al., 2018). This response was attributed to the modulation by the bacterial auxin. Thus, auxins are responsible to promote root growth by increasing root surface area, which indirectly promotes water and nutrient uptake by increasing soil-plant interface, thereby stimulating plant growth positively (Ahmed and Hasnain, 2014; Verma et al., 2018).

Nevertheless, there are two forms to apply the produced bacterial auxin for seed or plant treatment. The first, using the culture supernatant filtrate with the auxins, and the second by using the entire culture broth, with the bacteria cells and all the fermented broth. Tomato seeds treated with crude IAA from supernatant culture of *Streptomyces* sp. PT2 showed higher seed germination rates and root length of seedlings, similar to seeds treated with 50 $\mu g.mL^{-1}$ of standard IAA solution. Meanwhile, they observed that the presoaking time of the seeds in the IAA solution had an effect on the seedlings response. Presoaking of tomato seeds for 24 h showed better results than seeds treated for 36–72 h, which in turn, showed a considerable decrease in seed germination rates and root seedlings elongation (Goudjal et al., 2013).

Plants response to exogenous IAA differs due to the concentration perceived by tissues, whereas auxin at optimal level promotes root growth, but elevated concentrations, inhibits it (Park et al., 2015) as a consequence for its

activity on the biosynthesis of ACC (amino-cyclopropane carboxylate) increase, an ethylene immediate precursor. Ethylene is required during seed germination but high concentrations after germination, may lead to the inhibition of root growth (Ahmed and Hasnain, 2014; Glick, 2014).

Data from the pot experiments using the culture filtrate of *Piriformospora indica* after producing IAA revealed that the auxin had a prominent effect as compared to control of *S. melongena L.* cultivars. The culture filtrate significantly increased the root and shoot length and biomass of *S. melongena* L. cultivars after 45 days of growth. The roots from treated plants were densely covered with root hairs, with an increased number of lateral roots (Swethaa and Padmavath, 2016). Still, treatment of the cuttings of kidney bean with orchid-associated bacterial supernatants containing IAA, promoted root formation five- to fourteen-fold higher than in the control samples, without inhibiting or suppressing effects (Tsavkelova et al., 2007). Application of a suspension of an IAA producing *B. subtilis* on the surface of the plants, resulted in an increase in stem and root length, increase in fresh weight of the stem and root, an increase in the root: stem ratio and increase in the number of sprouts as compared with non-inoculated plants of the edible tubercle *Dioscorea rotundata* L. (Swain et al., 2007).

However, applying all the fermented broth, with bacterial cells and produced auxins, may have more advantages in some cases. Application of endophytic bacteria that produce and excrete phytohormones, might change the locally endogenous hormone balance of the host, thereby promoting plant cell division, growth, and nutrient release, and supporting their own growth (Ahmed et al., 2010). Even so, after inoculation, the endophytic bacteria may enter into root tissues and colonize, and promote plant growth, either by excretion of more phytohormones or by other direct or indirect mechanisms. It is also well known, however, that the effect of bacterial inoculation is very dependent on the plant genotype, and on many other environmental conditions, biotic and abiotic factors such as soil characteristic and natural microbial population (Taulé et al., 2016). Some endophytes are well adapted only on their own plant host, and are unable to promote an effective colonization and maintain high populations on other plant species (Parnell et al., 2016). In comparison among the endophytic communities isolated from micro-propagated easy-to-propagate and difficult-to-propagate genotypes of *Prunus*, (Quambusch et al., 2014) observed that the community

structures of among them differed significantly. The bacterial genera, which were previously reported to have plant growth-promoting effects, were detected only in the easy-to-propagate genotypes, indicating a possible positive impact of these bacteria on in vitro propagation of *P. avium*. The authors further isolated and inoculated some of these bacteria on the difficult-to-propagate genotypes and observed that the root percentage was significantly enhanced.

The IAA producing *Acetobacter diazotrophicus* L1 endophytic showed physiological implications in the root development of maize plants, which promoted striking growth of roots, that were longer and densely covered by root hairs. Even more, the bacterial inoculation significantly increased root and shoot biomass of maize after 30 days of growth. After that, the evaluation showed that the fresh roots of maize plants inoculated with the bacterium contained a higher amount of IAA than control uninoculated maize roots (Patil et al., 2011).

In respect of abiotic stresses, salinity has become one of the major concerns to plant growth and development, having negative effects on the morphological, physiological, and biochemical levels (Akbarimoghaddam et al. 2011; Abbasi et al. 2015; Tedeschi et al. 2017). Higher concentrations of salts lead to an osmotic potential reduction, disrupting cell´s water efflux, and consequently leading to a rapid cell dehydration (Ashraf 2004; Amjad et al. 2014). Moreover, this affects all the major biological plant processes as seed germination, foliar growth and development, photosynthesis, cell division and elongation, energy and lipid metabolism, and protein synthesis (Ali et al., 2012; Kushwaha et al., 2020). And as expected, this might also affect plant-associated bacteria IAA production. Meanwhile, inoculation with *Bacillus megaterium* overcame the negative effects of salt stress, and improved early seed germination (Chinnaswamy et al., 2018). *B. megaterium* NMp082 also induced tolerance to salt stress in alfalfa and Arabidopsis plants. This ability to salt stress alleviation may have resulted from the produced IAA and the ACC deaminase activity displayed by the endophyte. This isolate was also highly tolerant to water deficit and to the presence of different heavy metals (Chinnaswamy et al. 2018).

Under osmotic-stress conditions, IAA producing endophytes showed that the phytohormones production dropped considerably. The results showed that bacterial cells treated with 25% PEG exhibited a slower growth rate, thus

consequently affecting the IAA production of *P. aeruginosa* and *Bacillus* (Ghosh et al., 2019).

ECOLOGICAL CONSIDERATIONS FOR BIOINOCULANTS FUNCTION

Currently, the bottom-up selection process to identify candidates for a PGPB mainly uses culture-dependent screenings and sometimes, screening of the underlining PGP genes. But these pre-selection methods provide limited information, especially for scale-up the candidate to perform as a bioproduct with acceptable results in the field (Compant et al., 2019). For this reason, it is very important to evaluate the same reproducibility of PGPB in the field as those in the lab and greenhouses, and have a better understanding of the microbial interactions that result in plant growth for the success rate of field applications (Patel and Patel, 2014).

Diversified environmental conditions interfere directly in the efficiency of a bioinoculant survival and propagation rate (Iqbal and Hasnain, 2013). Climatic conditions are a major factor for the constituency of soil biodiversity, soil fertility and soil carbon content, and also the availability of the nutrients, thus the performance of biofertilizers (Schütz et al., 2018). The diversity of the natural adapted microbial community present in the soil, turns the environment more competitive and difficult for the introduced inoculant to establish a safe and successful interaction with the host (Compant et al., 2019). In this case, the competitive ability of an inoculant strain must be evaluated to design greenhouse and field experiments.

Yet, the competitive ability of an inoculant strain is linked with the application dosage of the microbial cells as well as the physiological activity (Samad et al., 2017). Moreover, the key success to assure the effective delivery of a certain dosage of cells is the product formulation and shelf-life (Arora et al., 2016; Compant et al., 2019). The bio inoculants must be formulated in a suitable delivery system to maintain their condition and to provide easier handling and efficiency in field applications (Djaya et al., 2019; Stephens and Rask, 2000). Mishra et al. (2009), evaluating inoculum levels, found that an inoculum dosage of 10^6 c.f.u/mL, was optimal to obtain the desired benefits of co-inoculation of *B. thuringiensis*-KR1 with *R. leguminosarum*-PR1 in pea and lentil plants, while lower inoculum concentrations resulted in decreased cell recovery and plant

growth performance and higher cell densities had an inhibitory effect on nodulation and plant growth.

Another additional important aspect is whether the strain is suited to colonize the respective plant species, genotype or tissue, and establish a compatible interaction with the host, including molecular interaction with the plant immune system (Finkel et al., 2017) and further exhibit the desired function in the receiving environment (Compant et al., 2019; Finkel et al., 2017). In this sense, two strains isolated from tomato, *Bacillus subtilis* NgE3 and *Paenibacillus* sp. NgE4, were evaluated for root colonization efficiency under gnotobiotic conditions. Ultra-structure studies by Transmission Electron Microscope (TEM), showed that both strains could infect and colonized the endosphere tomato root hair cells (Qbal et al., 2018). In another study, two endophytes isolated from sweet pea plant roots, *Ochrobactrum* sp., and *Enterobacter* sp. were evaluated for legume root surface colonization and nodulation. Both endophytes were able to re-infect roots, but did not develop root nodule on their host. Meanwhile, they showed very high colonization on the root surface with high efficiency in biofilm formation (Tariq et al., 2014).

To overcome lab to field hurdles, the application of microbial consortia is an emerging approach (Singh and Singh, 2016). A microbial consortium can result in a combination of close related strains with the same mode of action but with different environmental conditions plasticity, to expand the niche breadth of a certain trait. Or by complementing with distantly related strains with different traits, such as plant growth enhancement and biocontrol of pathogens, thus contributing to an overall additive effect (Compant et al., 2019; Finkel et al., 2017). This last consortium group is more explored, especially using a non-rhizobial PGPB with a nodule strain.

Co-inoculation of *Medicago* spp. plants with a nodule species *Ensifer medicae* and a *Bacillus megaterium,* produced a significant enhancement of all plant growth parameters in comparison with the non-inoculated plant´s treatment and with treatments of individual inoculations. Even so, *E. medicae* was further re-isolated from surface-sterilized root nodules after co-inoculation (Chinnaswamy et al., 2018). Similarly, co-inoculation of *B. thuringiensis*-KR1 with *R. leguminosarum*-PR1 was found to positively influence plant growth and nodulation of pea and lentil plants, when compared to inoculation with R.

leguminosarum-PR1 alone (Mishra et al., 2009). To assess the synergistic effect of a consortium biofertilizer for soybean, diazotroph endophytic bacteria *Pseudomonas aeruginosa* (LSE-2) strain, that has the potential to produce IAA with *Bradyrhizobium sp.* (LSBR-3) was recommended. The dual inoculant with both LSBR-3 + LSE-2 strains improved growth parameters when compared to LSBR-3 alone and un-inoculated control treatments. Higher yields were recorded and resulted in an additional income gain production of 5089/ha over the un-inoculated control treatment (Kumawat et al., 2019).

Today, commercial applications of bio inoculants are being tested worldwide and are frequently fruitful. Successful examples are the officially authorized use of *A. brasilense* Ab-V5 and Ab-V6 strains as inoculants for maize and wheat, which have shown benefits on crop nutrition and productivity (Oliveira et al., 2017; Piccinin et al., 2013). And new eco-friendly approaches are raising with the nanotechnology green synthesis using microorganisms as biofabrics. Bacteria strains are been studied to be used to produce nanoparticles with the ability to antimicrobial and nutritional activity to be used in agriculture (Gupta, 2018; Hassan et al., 2018; Hoseinpour and Ghaemi, 2018; Mazumdar and Haloi, 2011). Despite that more information is necessary to evaluate the real application of these nanomaterials, in case of for risk assessments and effects on human health, this emergence of bio-nanotechnology field brings an enormous prospective to provide superior solutions in agriculture for crop improvement, disease prevention, and environmental protection (Achari and Kowshik, 2018; Thakur et al., 2018).

CONCLUSION AND PERSPECTIVES

Bacteria bioinoculants are readily available commercially to be used for crop yield improvement and protection. And their successful field performance drives even more interest to increase the understanding of the key impact of microorganisms for the growth and development of crops, as a key to the future development of microbial products. However, the substantial research revenues focus mostly on rhizobacteria nitrogen-fixing bacteria, and endophytes receives less attention as a target for microbial product for agricultural applications.

Plant endophytes bacteria can also be exploited as an eco-friendly for many agriculture issues solutions, especially for plant nutrition and protection,

once they perform a remarkable association with the host plant, increasing their benefits. Bio inoculants based on endophytes may benefit over other microorganisms, as they can be far more effective once they live inside the plant tissue, not facing the competition of other soil microbes, which is common in the case of rhizosphere microbes. Moreover, they can live in the host in a closed-circuit system where leakage of metabolites is minimal.

On the other way, they can act as consortia with other plants benefits bacteria to optimize their application on the field, as a combination of endophytes with rhizosphere bacteria, or the combination of bacterial and fungal endophytes. Their exclusively genomic and metabolomic characteristics may provide several important bioactive compounds for endophyte-plant interactions and can be utilized in agriculture sustainable practices.

One way of vision to use endophytes is to explore their ability to produce phytohormones, such as auxins. Auxins are important phytohormones, and can be a powerful additive to bio inoculants, and directly improve plant root growth, and subsequently promote plant nutrient uptake. The bacteria auxins biosynthesis pathways are well known, and easier to reproduce in the laboratory. But, as any bioprocess, it is important to go deeper on the research for process optimization, and only few studies report to seek endophytes IAA optimization process using fermentation process. In our view, more interest must come to increase auxin production by endophytes and use them as bio-product for crop improvement. As discussed above, auxins have primary role in plant physiological processes.

However, studies focusing on the bacterial phytohormones influence on plant growth in terms of hormone sensitivity (concentrations), and synergistic or antagonistic hormone interactions within the host plant are still needed. Beyond that, future research in the bioinoculant field should aim to link root colonization by endophytes bioinoculant, in terms of bacterial quantification in the field, with rhizosphere and also with plant hormone concentrations, to confirm the appropriate dosage and efficiency.

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PART II- METAGENOMIC ANALYSES, ISOLATION AND CHARACTERIZATION OF ENDOPHYTIC BACTERIA ASSOCIATED WITH *Eucalyptus urophylla* IN VITRO PLANT

ABSTRACT

Endophytic bacteria can promote plant growth by nitrogen fixation, phosphate solubilization, phytohormone production, and other traits. They are present in all plant tissues, even in vitro tissue culture. This work aimed to analyze the diversity of endophytic bacteria using a culture-independent method via metagenomic analysis, and further isolate using culture-dependent method and characterize the endophytes toward plant growth promotions abilities, from *Eucalyptus urophylla* clone AEC224 in vitro leaves. Metagenomic analysis revealed the presence of the phyla Firmicutes (35%), Proteobacteria (30%), Actinobacteria (10%), Bacteroidetes (7.5%), Gemmatimonadetes (5%), Crenarchaeota (2.5%), Euryarchaeota (2.5%) and Acidobacteria (2.5%), signed in 16 classes, 14 orders, 19 families and 18 genera. Thirty strains were isolated and eleven produced indole-3-acetic acid (IAA). From those, two isolates were identified as *Enterobacter sp.* and *Paenibacillus polymyxa* by 16S rDNA, and proved also to fix nitrogen, solubilize phosphate and produce ammonium. These isolates can further be explored as bioinoculants or as biocontrol agents in sustainable agriculture.

Key words: Bioinoculants; *Enterobacter sp*.; IAA production; micropropagation; *Paenibacillus polymyxa*;

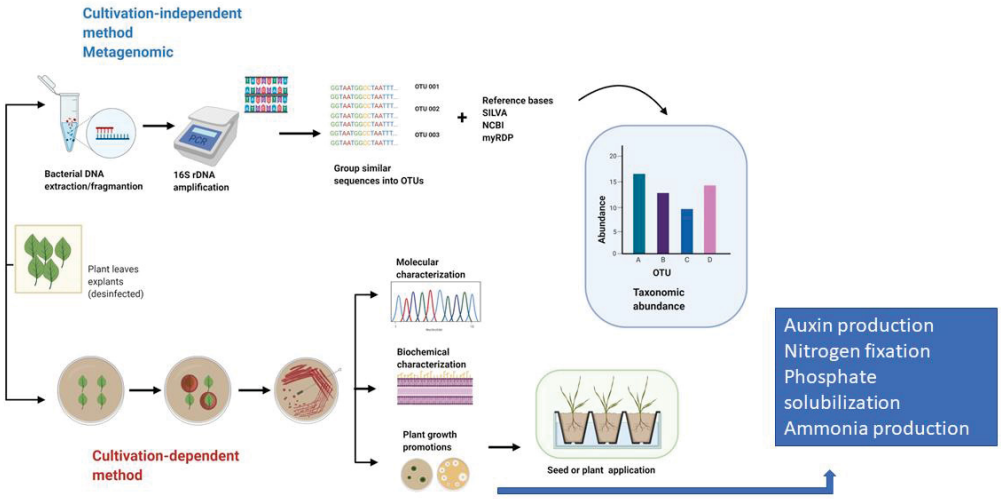

Figure 2. Graphical abstract for the methodology using cultivation independent and dependent methodologies to investigate the endophytic bacteria community from Eucalyptus leaves explants.

INTRODUCTION

The interaction of the endophytic microorganisms with plants can be beneficial, detrimental, and/or neutral (Hung and Annapurna 2004), where they live either in the symplast or apoplast in the plants (Gunson and Spencer-Phillips 1994; Hakizimana et al. 2011), receive nutrients from the host plant while mutually conferring direct or indirect support, increasing plant health and growth without substantively harming it (Dawwam et al., 2013; Haddad et al., 2013; Hakizimana et al., 2011).

Endophytes are beneficial to the growth and health of plants in many aspects, contribute to overall plant metabolism and defense. These benefits include: (1) producing plant growth regulators (PGR), (2) promoting mechanisms in nitrogen fixation, (3) synthesizing particular compounds to facilitate the uptake of certain nutrients as mineral phosphates, (4) overcoming phytotoxic effects caused by environmental contamination, (5) suppressing stress in ethylene synthesis by 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, (6) inducing resistance to phytopathogens by competing for space and nutrients or by producing antibiotics, and others (Ahmad et al. 2014; Dawwam et al. 2013; Hung and Annapurna 2004; Van der Lelie et al. 2009; Zhuang et al. 2007). Due to these characteristics, these microorganisms are increasingly arousing interest for commercial use as bioinoculants for fertilization and biocontrol products

(Parnell et al., 2016), as an alternative to develop a sustainable agriculture henceforward, to boost agricultural production through crop yield increase with nutrient uptake enhancement (inoculants), and reduce crop loss over pests (biocontrol) (Parnell et al., 2016; Uribe et al., 2010). The Agricultural Inoculant Market, including crop protection agents, soil amendment, and bio-fertilizers, is estimated to be valued at USD 292.2 million, as of 2018, and is expected to have a Compound Annual Growth rate (CAGR) of 9.0% during 2019-2024 (Mordor intelligence, 2019).

Two approaches have been used to screen for endophytic bacteria, cultivation-independent, using a metagenomic analysis (Akinsanya et al., 2015; Mashiane et al., 2017), and cultivation-dependent, using classic microbiological methods for microbial isolation on solid media (Miguel et al., 2016). Metagenomic approach allows to understand global microbial composition and diversity, and also helps to understand the interaction among microbes and their hosts (Akinsanya et al., 2015; Strobel and B. Daisy, 2003; Walitang et al., 2017). This technique also allows the prospection of bacteria regarding their economic potential for plant growth purposes. In addition, metagenomic combined with cultivation method can result in successful isolates for biotechnology applications, even that still not possible to recover all microorganisms by medium culturing (Afzal et al., 2019; Akinsanya et al., 2015).

Eucalyptus is one of the most important tree genera from planted forestry in Brazil and other tropical and subtropical regions of the world, due to its rapid growth and adaptability and the economic and social value of its wood, cellulose production, and pulp and paper industry (Brooker, 2000; Castro et al., 2016). All these gains came along with new management techniques and forestry improvements, mainly by genetic breeding programs which contributed to development of eucalyptus species with many important genetic traits of interest (Castro et al., 2016). Today, research and commercial laboratories use *in vitro* tissue culture techniques for eucalyptus plantlets micropropagation and genetic transformation studies (Bettencourt et al., 2018; Futuragene, 2015; Oliveira et al., 2016), and bacteria contamination can be a serious problem for in vitro techniques, such as organogenesis regeneration and genetic transformation. This contamination can occur during handling or because of existing endophytic

bacteria. Therefore, besides the negative impact on in vitro culture, these endophytes can be explored to be used as bioinoculants and biocontrol agents.

In the meantime, the majority of previous studies have focused on soil and root microorganisms in association with *Eucalyptus* plantations (Bonito et al., 2014; Da Silva et al., 2014; Da Silveira et al., 2006; De Araujo Pereira et al., 2017; Fonseca et al., 2018; Lan et al., 2016; Nnadozie et al., 2017). There is a lack of studies regarding to endophytic bacteria community with *Eucalyptus* trees (Ferreira et al., 2008; Miguel et al., 2019, 2016; Procópio et al., 2009), especially from in vitro cultivated plants (Esposito-Polesi et al., 2015). Thereby, the objective of this work was to evaluate the endophytic community from leaves of micro propagated *Eucalyptus urophylla* clone AEC224, and to isolate the endophytic bacteria with plant growth promoter abilities.

MATERIALS AND METHODS

Plant material

Leaves with petiole of in vitro micropropagated plantlets of clone AEC224 of *Eucalyptus urophylla* were used as explants for metagenomic analysis and endophytic bacteria isolation. The clone was introduced *in vitro* using stem explants of a single plant from green-house cultivation. For in vitro establishment, the explants were washed with neutral detergent in running tap water and disinfested with 1% (v/v) Cercobin® for 10 min, and then 5 min in 0.05% (v/v) Hg_2Cl_2 solution, rinsed after with sterile distilled water, three times, inside a laminar flow hood. The micro-propagated plantlets were introduced and subculture every 30 days, on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 30 $g.L^{-1}$ sucrose, 0.88 µM 6-benzylaminopurine, 0.2 $mg.L^{-1}$ myo-inositol, and 7 $g.L^{-1}$ of agar; and kept at a temperature of 23±2°C in a growth room under white fluorescent light with an irradiance of 40 $\mu mol.m^{-2}.s^{-1}$ and 16 hours of photoperiod, and maintained for 2 years at Plant tissue culture from Bioprocess Engineering and Biotechnology Department, of Parana Federal University, Curitiba, Brazil.

Metagenomic analysis of endophytic bacteria

To avoid concomitant bacteria, only shoots with no visible contamination and only leaves from the top of the shoot, which had no contact with the medium

were used. The leaves with the petiole of *Eucalyptus urophylla* AEC224 plantlets were first sterilized as follows: 1 min on ethanol 70 %, then 2 % hypochlorite for 5 min, and rinsed trice with sterile dH_2O. Afterward, DNA purification from 300mg of liquid nitrogen homogenized sample was performed with ZymoBiomics kit (Zymo), according to manufacturer instructions. The quality and quantity of the extracted DNA was evaluated by spectrophotometry using Nanodrop 2000 (Thermo). Twenty nanograms of DNA was utilized as a template for 18 cycles of amplification of the V4 region of the 16SrRNA gene, using the primers 515F and 806R (Caporaso et al., 2012) and GoTaq Master Mix (Promega). The PCR products were quantified using the Qubit dsDNA HS kit (Invitrogen) and sequenced using the 300V2 Sequencing Kit (Illumina) on an Illumina MiSeq (Illumina). The sequences were analysed using the QIIME 1.9 (Quantitative Insights Into Microbial Ecology), considering 97 % similarity with the 16S SILVA database (Quast et al., 2013).

Isolation of endophytic bacteria from in vitro *Eucalyptus urophylla* AEC224

The same procedure with the leave explants as described above was used for endophytic isolation. The leaves with petiole explants were placed on agar plates of solid lysogeny broth (LB) (Sambrook et al. 1989) medium containing 10 $g.L^{-1}$ of NaCl, 10 $g.L^{-1}$ tryptone, and 5 $g.L^{-1}$ yeast extract, 13 $g.L^{-1}$ agar, pH 7.0; and incubated in the dark at 23°C for 3 days for allowing endophytic bacteria growth. To further purify the colonies, bacterial colonies grew around the explants were transferred to a fresh solid LB medium and cultivated for another 3 days in the same conditions. Finally, 30 single colonies were picked and kept on LB with 20% glycerol at -80ºC for further tests.

Sequencing and phylogenetic identification of two isolates

To assign the phylogenetic of two chosen isolates, the sequencing of the 16S rDNA gene was carried out and analyzed. Total genomic DNA extraction was done as described by Vicente et al. (2008). For the 16S rRNA gene sequence analysis, universal forward primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse rD1 (5'-AAGGAGGTGATCCAGCC-3'), corresponding to positions 8 to 27 and positions

1541 to 1525, respectively from *E. coli* strain K12, as described by Weisburg et al. (1991) were used in amplification by Polymerase Chain Reaction (PCR), with expected amplicons of 1500 base pairs (bp). The PCR reactions were performed under the following conditions: PCR mixture (25 µl) containing 1.0 U Taq DNA polymerase, 2.0 mM $MgCl_2$, 1x PCR buffer, 2.0 mM of each dNTP, 0.4 mM each of forward and reverse primer (Invitrogen, Brazil) and approximately 50 ng template DNA, was subjected to PCR cycles: 1 cycle of 94ºC for 2 min, followed by 35 cycles of 94 ºC for 1 min, 58 ºC for 1 min and 72 ºC for 2 min, and 1 cycle of 72 ºC for 6 min. The amplicons integrity was observed on 1% agarose gel electrophoresis, photographed by a Cannon PowerShot S2 IS camera under Syngene Bio Imaging program. Prior to sequencing, the amplicons were purified by PEG purification as following: 50 µL of 20% PEG were pipetted on PCR tubes containing the amplicons, gently mixed by up & down pipetting and rested for 30 min at 37ºC. After that, the tubes were centrifuged for 20 min at 22,000xg and the supernatant was discarded. One hundred and twenty-five microliters (125 µL) of freezer-cold 80% ethanol were added and centrifuged for an additional 2 min, and the supernatant was discarded. This step was repeated with freezer-cold 96% ethanol. The remaining pellet was left to dry at 45ºC for 1 h, in which then resuspended with 15 µL ultrapure water and left at room temperature for 2 h. Sequencing of the amplicons was carried out in ABI3130 sequencer from Biochemistry Department, Paraná Federal University (UFPR) using the same primers with PCR reaction described above with additional components. In the sequencing reaction: 1 µL of mix for sequencing Big Dye, 2 µL sequencing buffer, 0.5 µL of 1.5 pmol of each primer, 3 µL of purified PCR products, and 3.5 µL of purified ultrapure water were used. The resulting products were further purified using Sephadex™ G-50 filtration medium.

Alignment and Phylogenetic construction of the isolates

The obtained sequences were compared with the reference database (NCBI - http://www.ncbi.nlm.nih.gov/) as well as by BLAST program (Altschul et al. 1997) to detect similarity amidst sequences. The sequences alignment was done using Bioedit sequence alignment editor© and Mega software version 7.0.1 (Tamura et al. 2011). The phylogenetic tree was constructed using Bayesian inference with Mrbayes 3.2.6 software. The best-fit model of sequence evolution

was identified by Mega 7.0 software. The matrix for *Enterobacter* genus was built with all valid 36 known species and 5 subspecies (http://www.bacterio.net/enterobacter.html) and the outgroup *Bacillus subtilis subsp. subtilis* NCIB 3610(T) (https://www.ezbiocloud.net). The matrix for *Paenibacillus* genus was built with 31 taxa with representatives from known valid species and subspecies and *Bacillus subtilis subsp. inaquosorum* KCTC 13429 (Type) as the outgroup (https://www.ezbiocloud.net). FigTree program version 1.4.3 was used for tree visualization.

Characterization of the isolates regarding their plant growth promotion potential

Auxin production

The auxin production was determined for the 30 isolates. The ability of the isolates to produce indole acetic acid (IAA) was the first trait of screening for plant growth promotion. Auxin production was determined qualitatively and quantitatively. The isolates were first cultured overnight on LB medium at 23°C to prepare the inoculum. Two hundred microliters of the inoculum were then inoculated into a 50-mL Erlenmeyer flask containing 25 mL of LB medium supplemented with 0.1 % tryptophan (Trp) and incubated in the dark for 4 days at 23ºC, on a shaker circularly rotated at 120 rpm. After 4 days, the cultured cell was centrifuged at 958xg for 15 min and supernatant was collected. Two milliliters of the supernatant were mixed with 4 mL of Salkowski's reagent (a mixture of 50 mL 35% perchloric acid and 1 mL 0.5M $FeCl_3$), and allowed to stand for 25 min on temperature room. Qualitative measurement of the IAA production, production of rose-to-purple color was observed, whereas in quantitative measurement, absorbance was measured at 530 nm using a spectrophotometer (UV Espectrophotometer 1800, Shimadzu). To further quantitatively determine the concentration, a standard curve was developed using a standard solution of pure indole-3-acetic acid (0 - 100 $\mu g.L^{-1}$) (Sigma, Brasil). Based on this IAA production results, two isolates were selected for further analyzes.

Nitrogen fixation

Nitrogen-fixing ability was qualitatively studied by allowing the two chosen isolates to grow in nitrogen-free, bromothymol blue (NfB) medium (all in $g.L^{-1}$: 10

Sucrose, 0.6 K_2HPO_4, 0.2 $MgSO_4$, 0.2 NaCl, 0.1 K_2SO_4, 2.0 $CaCO_3$, 2.0 ml of 0.5% Bromothymol blue (BTB) solution and pH=6.8) (Goswami et al., 2015a). BTB serves as an indicator, the isolates with the ability to fix nitrogen will increase the pH of the medium which is determined by the color change of the medium from blue to green (Goswami et al., 2015a). The cultures were incubated at 30°C for 3 days.

Phosphate solubilizing

In order to determine the phosphate solubilizing potential of the isolates, we used the National Botanical Research Institute's phosphate growth medium (NBRIP) contained (in $g.L^{-1}$): 10 glucose, 5 $Ca_3(PO_4)_2$, 5 $MgCl_2.6H_2O$, 0.25 $MgSO_4.7H_2O$, 0.2 KCl, 0.1 $(NH_4)_2SO_4$. The medium was supplemented with 13 $g.L^{-1}$ agar and adjusted the pH= 7.0, before autoclaving (Shekhar Nautiyal, 1999). A volume of ten microliters of the strains broth was plated in petri dish and incubated for 3 days at 30°C. After incubation, the phosphate solubilizing was evaluated by the presence of a halo surrounding the bacteria grown area and compared to a non-inoculated plate.

Ammonia production

One hundred µL of freshly grown bacterial isolates were inoculated in test tubes with 10 ml of peptone water and incubated for 3 days at 30°C. After incubation, 1 mL of each culture was mixed with 50 µLof Nessler’s reagent [10% HgI2; 28 ± 2◦C. After incubation, 1 mL of each culture was transferred 7% KI; 50% aqueous solution of NaOH (32%)] in a new microtube. The evaluation was done qualitatively, by observing the mixture color change. Small int of yellow color indicates a small amount of ammonia and deep yellow to brownish indicates maximum production of ammonia (Cappuccino and Sherman, 1992; Dutta et al., 2015).

Scanning electron microscopy (SEM)

Leaves of *Eucalyptus urophylla* clone AEC224 were fixed according to Bomblies et al. 2008, for sample preparations to SEM. The dehydrated explants were dried using the critical point technique and coated with gold. The photomicrograph was taken by a JEOL (JSM 6360-LV) scanning electron

microscope (SEM; JEOL Ltd. Japan) at the Electronic Microscopy Centre, Paraná Federal University (UFPR).

Effect of the endophyte isolates culture and the produced auxin non common bean seed germination

Seeds of common bean (*Phaseolus vulgaris*) were first washed with 70% ethanol for 1 min and 2.5% sodium hypochlorite for 5 min and then rinsed with sterile dH2O thrice. The isolates were cultured as described above for auxin production. Thirty milliliters of 4 days old fermented solution were centrifuged at 1800 g for 15 min and the supernatant was separated, filtered to sterilize and diluted until a ~50 $\mu g.mL^{-1}$ of auxin. Twenty seeds were immersed in the bacteria culture supernatant (containing the produced auxin) or in the bacterial culture broth (with bacterial cells and produced auxin) according to the treatments, to determine the influence on plant growth. Water and 50 $\mu g.mL^{-1}$ of standard IAA (Sigma, Brazil) were used as negative and positive controls, respectively. The seeds were submerged in the different treatments for 30 min and planted in the greenhouse (at 28°C±2.0) with vermiculite and irrigated with a mineral mixture (Kristalon™ hydroponic fertilizer + calcinate + micronutrients, in concentrations recommended by manufactures). The plant growth and development was evaluated after 30 days for *Enterobacter sp*. DEBB B-355 and 45 days for *Paenibacillus polymyxa* DEBB B-358 by calculating the germination percentage, plant height, number of leaves, numbers of roots, root size, fresh and dry weight. Each treatment consisted of 20 seeds. Statistical analysis was performed by using the statistical software STATISTICA®, version 8.0. The significance of each treatment was established by one-way ANOVA and means were separated by Fisher's test ($p \leq 0.05$).

RESULTS

Metagenomics analysis

The 16S rRNA analysis of the leaves of *Eucalyptus urophylla* clone AEC224 with Illumina MiSeq gave the averaged numbers of sequences of 241.498 with a total of observed operational taxonomic units (OTUs) of 7.104. In addition, the retained sequences were grouped into 40 OTUs, which one of them was referred to Chloroplast class with 99.9% of the sequences. Though, after

removal of Chloroplast OTU, other sequences were assigned into Archaea and Bacteria kingdom, within 8 known phyla signed into 16 classes, 14 orders, 19 families and 18 genus, and others not assigned according to 16S SILVA database (Quast et al., 2013). OTUs belonging to the phyla Firmicutes (35%) were the most abundant, followed by Proteobacteria (30%), Actinobacteria (10%), Bacteroidetes (7.5%), Gemmatimonadetes (5%), Crenarchaeota (2.5%), Euryarchaeota (2.5%) and Acidobacteria (2.5%), still 2.5% were not assigned (Fig. 1). The results showed a higher presence of Clostridia (21.05%), followed by Gammaproteobacteria (18.42%), Bacilli (10.52%), Actinobacteria (7.8%), Alphaproteobacteria (5.26%), Deltaproteobacteria (5.26%), and Betaproteobacteria, Thaumarchaeota, Acidobacteria-6, Thermoleophilia, Bacteroidia, Cytophagia, Shingobacteriia, Erysipelotrichi, Gemm-1, Gemm-5 and others (all with 2.63 %) (Fig. 4). We observed some assigned genera whose species are known to be plant growth promoters such as *Bacillus, Brevibacterium, Erwinia, Lactobacillus, Streptomyces,* and *Pedobacter* (data not shown).

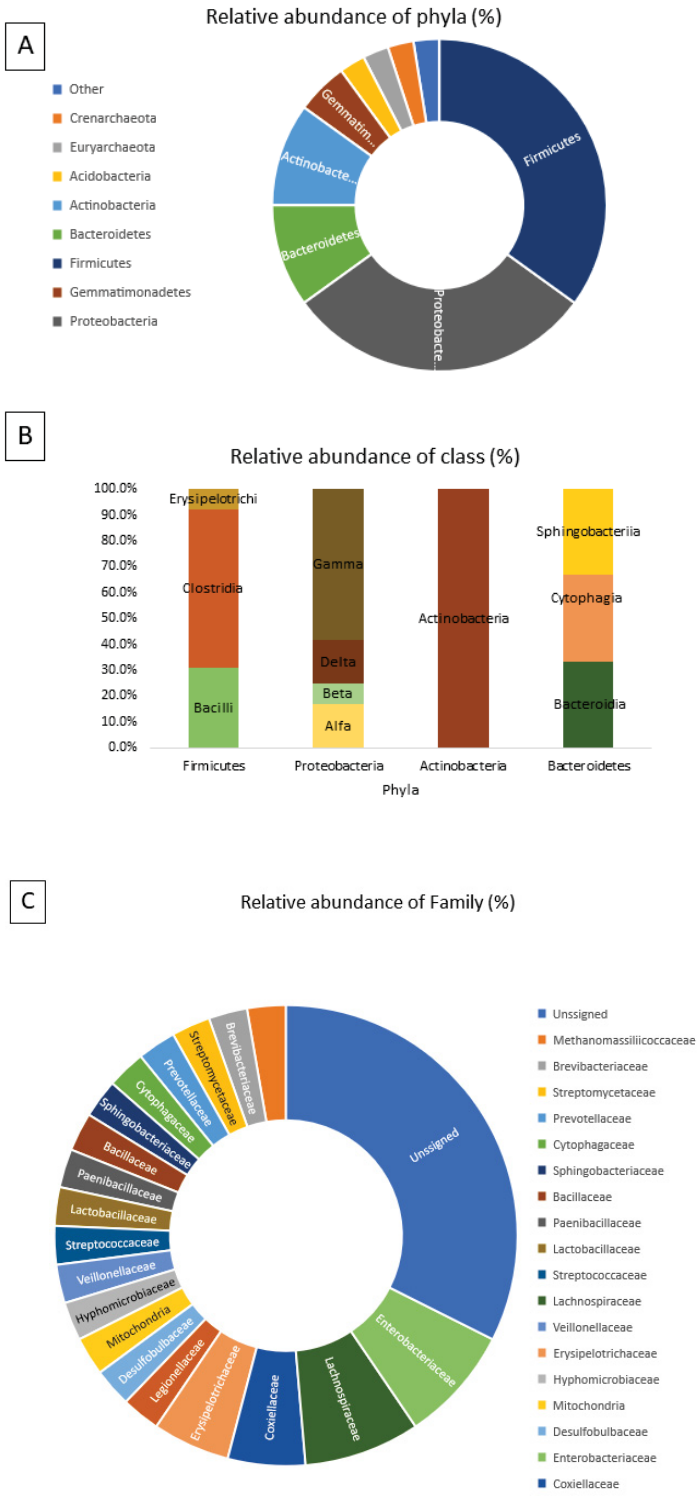

Figure 3. (A) Relative taxonomic distribution of OTUs in *Eucalyptus urophylla in vitro* leaves explants Taxonomic bacterial metagenome for the abundant phyla, class, and family. (B) Showing the taxonomic distribution of the four most abundant phyla in the bacterial metagenome: Firmicutes (35%) were dominant followed by Proteobacteria (30%), Actinobacteria (10%), Bacteroidetes (7.5%), Gemmatimonadetes (5%), Crenarchaeota (2.5%), Euryarchaeota (2.5%) and Acidobacteria (2.5%), with their respective class representants that were described by the analysis. (C) Showing the relative abundance of the family members. The sequences were analysed using the QIIME 1.9 (Quantitative Insights Into Microbial Ecology), considering 97 % similarity with the 16S SILVA database (Quast et al., 2013). Bars designated as “Others” and “Unassigned” include OTUs which failed to align with any reference sequence.

Isolation and characterization of endophytic bacteria from *in vitro Eucalyptus urophylla* plants, capable to produce auxin

After three days of culture on LB medium, we observed endophytic bacteria growing around the leaves of *E. urophylla* clone AEC224. The bacteria were picked and streaked on new LB agar plates, and the formation of several single colonies was observed. It was obtained 30 isolates which were evaluated for auxin production, in order to select ones with higher production ability. Among them, eleven isolates produced auxin in a range of 3.15 – 22.27 $\mu g.mL^{-1}$ according with to Salkowski´s test (Fig. 6 B). From those, two isolates (strains DEBB B-355 and DEBB B-358) that exhibited the higher auxin production, with 13.80 $\mu g.mL^{-1}$ and 22.27 $\mu g.mL^{-1}$ of IAA amount, were selected for further analyses.

Using BLAST program from sequence database GenBank-NCBIthe isolate DEBB B-355 showed 99.85% similarity with *Enterobacter ludwigii*, and isolate DEBB B-358 with 99.64% similarity with *Paenibacillus. polymyxa* strains. Based on the phylogenetic analysis using the Bayesian inference the isolates were identified as *Enterobacter* sp*. DEBB B-355* and *Paenibacillus polymyxa DEBB B-358,* as indicated by cluster shown on clade, which was also well supported by posterior probability (>0.5) (Fig. 5). The nucleotide sequence obtained from both strains has been submitted to GenBank under accession numbers of MT126447 and MT126448, for DEBB B-355 and DEBB B-358 respectively.

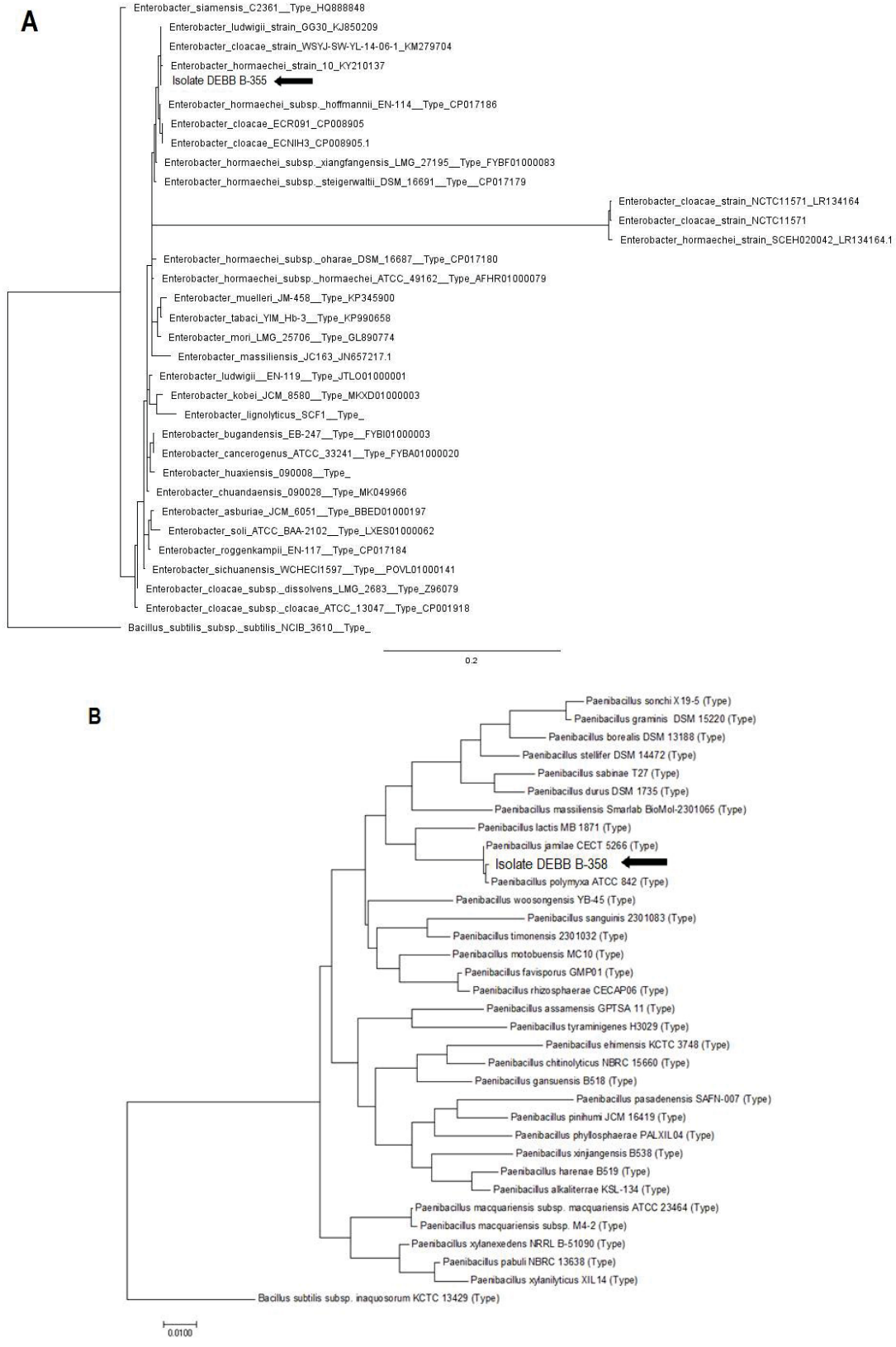

Figure 4. The phylogenetic tree was constructed using Bayesian inference with Mrbayes 3.2.6 software, using the best-fit model of sequence evolution was identified in Mega 7.0. software. The matrixes were built with representatives from the known species and subspecies among (A) *Enterobacter* and (B) *Paenibacillus* genus, and two outgroup species. The analysis was based on the respective characters, using a print frequency of 1000 and a sample frequency of 10. The

number of chains was set as 4 with a heating parameter of 0.2 and discarded 25% of the sampled trees. For tree visualizing, FigTree version 1.4.3 was used.

In vitro plant growth-promotion traits: nitrogen fixation, phosphate solubilization and ammonium production

The ability to fix nitrogen was evaluated using Bromothymol blue (BTB) as an indicator. With the nitrogen fixation, the pH increases and changes the medium color from blue to green (Goswami et al., 2015a), and this was observed for both isolates in which the liquid media turned green, indicating the ability to fix nitrogen (Fig 6 C).

Appearance of a halo surrounding bacteria growth confirmed the ability to solubilize phosphate surrounding bacteria growth, when using NBRI phosphate growth medium (Fig. 6 D). Both isolates showed small ammonia production, once when we mixed the bacterial culture with the Nessler's reagent the medium turned yellow (Fig 6 E).

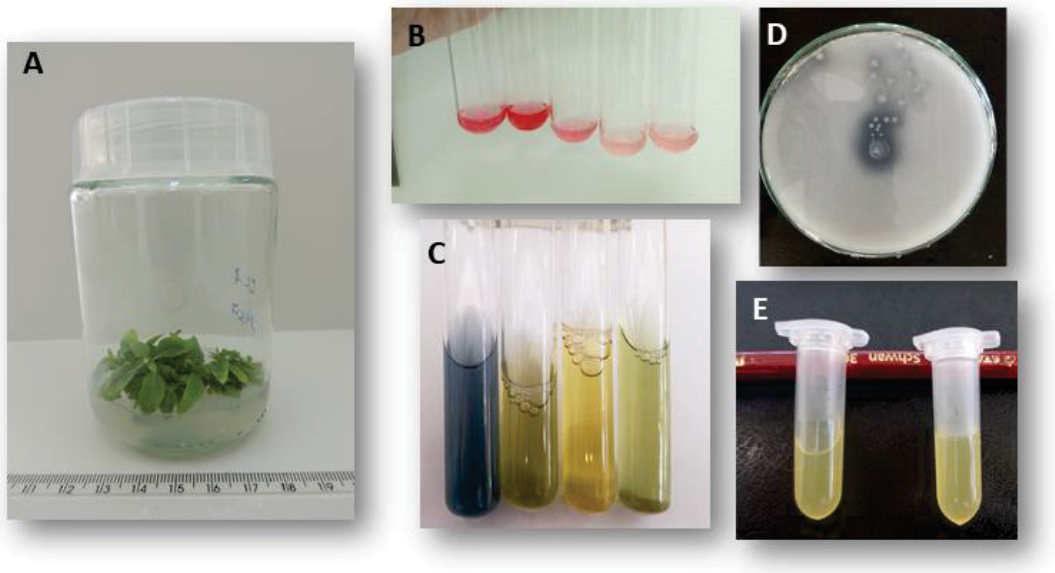

Figure 5. A) micropropagated *Eucalyptus urophylla* AEC224 plantlets; B) Auxin production of the endophytic isolates showing positive with the presence of pink colour; C) Nitrogen fixation assay, control tube with blue staining, yellow and green color confirm nitrogen fixation from the strains of *Enterobacter* sp DEBB B-355 and *Paenibacilus polymyxa* DEBB B-358. D) Clear halo surrounding the bacteria growth represents phosphate solubilization for DEBB B-355 and E) Yellow color formation exhibits positive for ammonium production when mixed with Nessler's reagent.

Scanning electron microscopic image from *Eucalyptus urophylla* leaves (SEM)

The SEM analyses were done in order to demonstrate the presence of endophytic bacteria in leaves from micro-propagated eucalyptus. It was possible to observe the presence of a high concentration of bacteria associated with the micro-propagated plant tissues even after two years on *in vitro* culture. The SEM image showed micro-colonies of bacteria present at high density on leaves and in the intercellular space (Fig. 7).

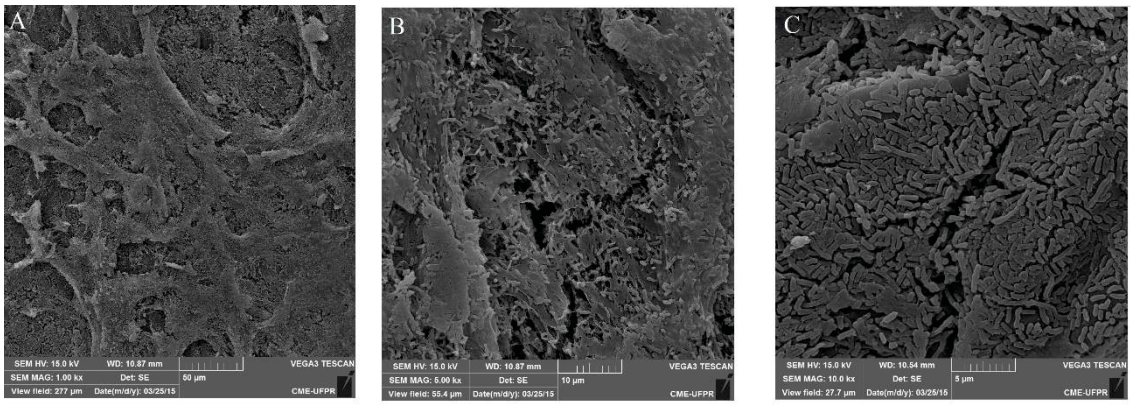

Figure 6. SEM images showing the presence of bacteria micro-colonized, with bacillus shape and the presence of a biofilm matrix around the interface of leaf tissue of the *E. uropylla* clone AEC224. A, B, and C images correspond to 50, 10, and 5 µm bars, respectively.

Inoculation of seeds with the endophytes

The experiment with plants showed that the endophytic bacterium extract with auxin can promote plant growth. Immersing the seeds on the *Enterobacter* sp. DEBB B-355 bacterial culture supernatant with the produced auxin (CS) increased plant height and root length, leaves and root number and fresh weight, followed by bacteria extract (BC) and standard IAA treatments (Table 2). Bacterial extract increased seed germination (67%) compared to other treatments (47%). When compared to the negative control treatment, bacteria auxin showed 1.4 times increase in the plant height and leaves number, and 1.7 times on root length. Bacterial culture decreased root number and dry weight, even they looked more robust after harvesting (Fig. 8).

The percentage of seed germination after inoculated with *P. polymyxa* DEBB B-358 culture supernatant showed a higher germination rate (53%), followed by bacteria culture (47%), positive control (40%), and negative control

(33%) (Table 2). The produced auxin increased plant height and leaves number. Even if with no statistical difference, seeds inoculated in bacteria culture supernatant and the bacterial culture broth increased plant and root lengh, fresh and dry weight, similar to the standard IAA (positive control). However, seeds treated with the isolate bacteria broth and the supernatant showed more root growth (Figure 9).

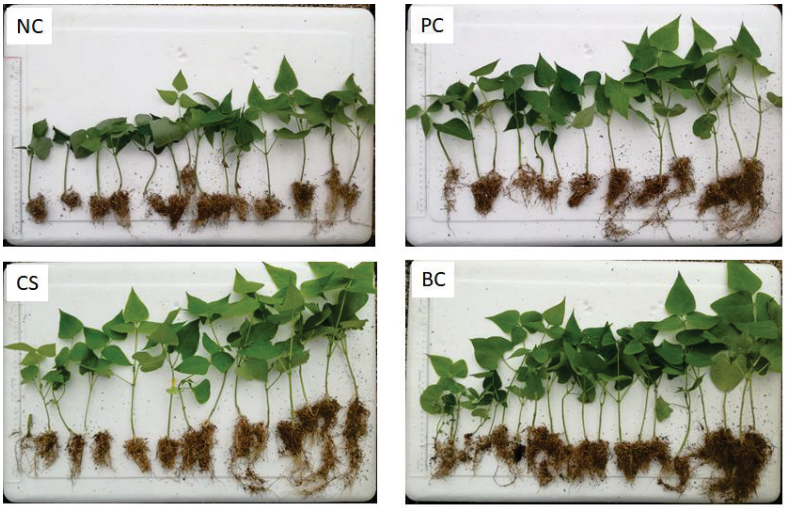

Figure 7. Common beans (*Phaseolus vulgaris*) seed inoculated with *Enterobacter* sp. DEBB B-355 on greenhouse after 30 days. Beans seeds were inoculated on NC - Negative control (water), PC – positive control (IAA = 50.0 µg.mL^{-1}) , CS- culture supernatant (IAA = 50.0 µg.mL^{-1}), BC- bacteria culture (UFC/mL = 1.6^{10}, IAA = 50.0 µg.mL^{-1}).

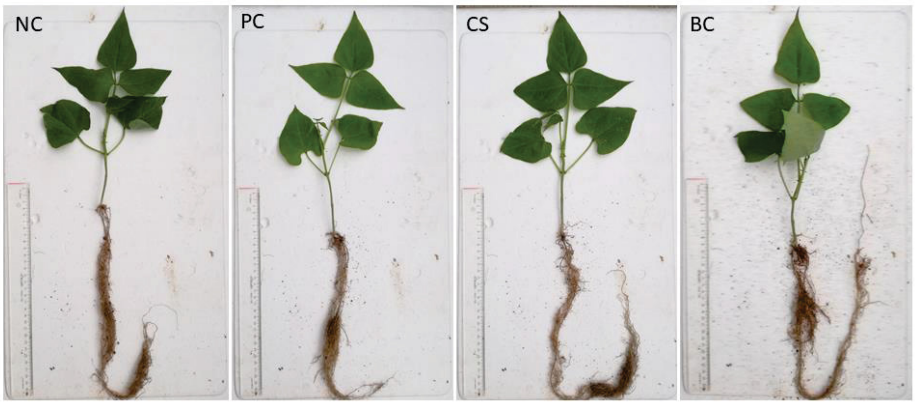

Figure 8. Common beans (*Phaseolus vulgaris*) seed inoculated with *Paenibacillus polymyxa* DEBB B-358 on greenhouse after 45 days. Seeds were inoculated on NC - Negative control (water), PC – positive control (IAA = 50.0 µg.mL^{-1}), CS- culture supernatant (IAA = 50.0 µg.mL^{-1}), BC- bacteria culture (UFC/mL = 2.10^{10}, IAA = 50.0 µg.mL^{-1}).

Table 2. Plant biostimulation by *Enterobacter* sp. DEBB B-355 and *Paenibacillus polymyxa* DEBB B-358 and its produced auxin supernatant on beans seeds. Evaluations were after 30 days of harvest for DEBB B-355 and 45 days for DEBB B-358 strains.

***Enterobacter sp.* DEBB B-355**							
Treatment	**Germination (%)**	**Height (cm)**	**Leaves**	**Root number**	**Root lenght (cm)**	**Fresh weight**	**Dry weight**
NC	47^{b}	17.84 ± 2.33^{b}	4.38 ± 0.82^{b}	12.29 ± 2.41^{a}	10.59 ± 3.93^{b}	1.33 ± 0.35^{b}	0.19 ± 0.055^{b}
PC	47^{b}	21.97 ± 2.91^{ab}	5.71 ± 0.86^{ab}	12.00 ± 2.57^{a}	14.35 ± 5.84^{ab}	1.71 ± 0.37^{ab}	0.23 ± 0.06^{ab}
CS	47^{b}	24.98 ± 3.98^{a}	6.54 ± 1.42^{a}	12.69 ± 2.14^{a}	18.05 ± 5.64^{a}	1.59 ± 0.36^{ab}	0.23 ± 0.07^{ab}
BC	67^{a}	21.06 ± 4.77^{ab}	5.95 ± 1.24^{a}	9.85 ± 2.77^{a}	13.12 ± 4.72^{ab}	1.44 ± 0.56^{ab}	0.19 ± 0.08^{b}
***Paenibacillus polymyxa* DEBB B-358**							
Treatment	**Germination (%)**	**Height (cm)**	**Leaves**	**Root number**	**Root length (cm)**	**Fresh weight**	**Dry weight**
NC	33^{c}	24.45 ± 5.34^{a}	6.5 ± 1.8^{b}	10.4 ± 1.4^{ab}	32.25 ± 12.38^{a}	2.959 ± 1.29^{a}	0.454 ± 0.24^{a}
PC	40^{bc}	28.1 ± 5.51^{a}	7.45 ± 1.34^{ab}	10.45 ± 2.31^{a}	34.65 ± 10.00^{a}	3.62 ± 1.21^{a}	0.57 ± 0.26^{a}
CS	53^{a}	29.28 ± 4.79^{a}	9.07 ± 1.66^{a}	9.07 ± 1.66^{bc}	33.47 ± 8.39^{a}	3.61 ± 1.24^{a}	0.55 ± 0.2^{a}
BC	47^{b}	27.58 ± 4.66^{a}	7.46 ± 0.89^{ab}	7.77 ± 1.79^{c}	35.6 ± 10.65^{a}	3.72 ± 1.59^{a}	0.51 ± 0.25^{a}

Legend: NC - Negative control, PC – positive control, CS- culture supernatant, BC- bacteria culture

DISCUSSION

Tissue culture has been used as an effective technique for plant genetic transformation procedures and to obtain clonal plantlets, especially for species belonging to the *Eucalyptus* genus (Brondani et al., 2012; Oliveira et al., 2016). Normally, the starting material for in vitro plant tissue culture is the shoot tip meristems or embryos. However, even with the aseptic procedures to introduce explants under *in vitro* conditions, endophytes microorganisms can still maintain latent inside the plant and continue their association with the host with no harm for the plant tissue (Esposito-Polesi et al., 2015).

The in vitro *Eucalyptus* clone AEC224 presented the growth of concomitate bacteria surrounding the plantlets in contact with the medium, especially when leaf explants where cut and used for genetic transformation protocol (Bettencourt et al., 2018). This happened even after two years of the clone *in vitro* multiplication. Probably, this phenomenon might be a reaction over the stress caused by excessive multiplication of plants or due to changes in the micro-environmental conditions (Esposito-polesi, 2011; Thomas, 2004). This phenomenon caught our attention, and we proposed to study the endophytic bacterial community and search for plant growth promotion traits.

According to Miguel et al. (2016), the prevalence of endophytes bacteria can be strain- and host-specific, organ and stage-specific, and also be influenced by environmental conditions, nutritional conditions such as composition and concentration of sugars and amino acids. The endophytic bacterial community can shift according to several variables (Esposito-Polesi et al., 2015). The diversity and distribution of the endophytic bacterial community can change from plant organs, stage of development, and environmental stimulus (Pirttila, 2011). Poor conditions maintain the bacterial community in latency, while contrary, under favorable conditions the bacteria endophytic community, started to multiply rapidly (Miguel et al., 2016). As it is known, the in vitro culture medium is rich in sugars, mostly sucrose, vitamins, and amino acids. This can turn to be a perfect environment for bacteria overgrowth, as observed with eucalyptus explants used in this study in which the endophytic bacteria started to migrate from the internal plant host tissues to the medium. Higher sucrose concentration was indicated to benefit diazotrophic bacteria, as they require carbon as an energy sources (Miguel et al., 2016; Rodionov et al., 2010; Velázquez-Hernández et al., 2011).

SEM images showed endophytic bacteria growing in the in vitro eucalyptus leaves at high concentrations within the tissues, as well as forming biofilm (Fig. 6). As defined by Costerton et al. (1995), “biofilms are a matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces”. It functions as a cooperative consortium, in a complex and coordinated manner, that require collective bacterial behavior (Davey and O’toole 2000), which confers protection to the bacterial communities from any adverse environmental conditions, as well as from biological and chemical threats i.e. antibacterial agents (Costerton et al. 1995; Silva et al. 2011). Ulrich et al. (2008) observed high densities of endophytic bacteria on micro-propagated poplar plantlets, as we did on our *E. urophylla* clone, confirming that endophytes seemed to accumulate under in vitro conditions, remain covert or latent, without visible negative influences on the plant’s development.

In order to confer a symbiotic relationship with the host, while the endophytic bacteria benefit from the plant nutrient and safe environment, they help them with their plasticity to produce several bio-compounds such as plant growth regulators, antibiotic and improve nutrients uptake, that is essential for plant physiological processes, and protection over biotic and abiotic stresses (Ahsan et al., 2019; Orlikowska et al., 2016). Whence, due to their potential to produce the substance of interest, endophytic bacteria are receiving more attention towards their benefits for agricultural use as bioinoculants or biocontrol agents (Ahmed and Hasnain, 2014; Brader et al., 2014; Puri et al., 2018; Venugopalan and Srivastava, 2015; Zinniel et al., 2002).

Metagenomic analysis turns to be a fast semiquantitative method to provide more information on the endophytic bacterial community and its interaction with the plant host. And, by adding cultivation-dependent methods for isolation can be more effective to find strains of interest. One approach is to screening isolates by one or more trait of plant growth-promotion, as auxin production, nitrogen fixation, phosphate solubilization, or biocontrol activity to provide an effective plant growth promoting bacteria, with an exceptional value in agricultural biotechnology (Erdelyi et al., 2014; Fonseca et al., 2018). Here, in order to select the strains for further analysis to be identified and applied as bioinoculants, we focused on screening for auxin production of the isolates, as many plant-associated bacteria are capable to produce and secrete phytohormones as auxins

(Cardinale et al., 2015; Olanrewaju et al., 2017; Paque and Weijers, 2016). Auxins are indole derivatives compounds responsible to promote several plant physiologic responses such as root formation, regulation of fruit ripening, and stimulation of cell division, extension, and differentiation (Pirttila, 2011; Spaepen and Vanderleyden, 2011a).

However, when the bacteria community evaluated are plant-associated, the results from metagenomic analysis are interference from plant organelles, such as mitochondria and plastids, which are also amplified by the usual 16S rNRA gene, resulting in abundant PCR products derived from the plant (Puri et al., 2019). This was observed on our total assembly OUT´s which chloroplast represented up to 99.9% of the sequence's coverage.

Nevertheless, after the removal of dominant plant amplified products, our findings with metagenomics analyses revealed a prevalence of the phyla Firmicutes (35%), Proteobacteria (30%), Actinobacteria (10%), Bacteroidetes (7.5%), Gemmatimonadetes (5%), Crenarchaeota (2.5%), Euryarchaeota (2.5%), and Acidobacteria (2.5%). These findings were similar to those reported in other studies, showing that these phyla are predominant in the wood communities (Nnadozie et al., 2017). Proteobacteria, Firmicutes, and Actinobacteria were reported to dominate the endophytic bacteria community on leaves from different stages of *Eucalyptus* development by (Miguel et al., 2016). Bacteroidetes (15%) were present in the *Eucalyptus* spp. woodchips, also with Proteobacteria (77%), Acidobacteria (3%), and Actinobacteria (2%) (Nnadozie et al., 2017). These evidences enforce that the bacterial endophytic communities are host-specific and changes according to natural conditions, such as pH and oxygen, and plant development stage.

Firmicutes and Proteobacteria, particularly, are topmost phyla found on wood plants at higher concentrations, probably due to their cellulolytic and lignin-modifying activities (Bugg et al., 2011; Sharmin et al., 2013), and encompass several plant growth promoters bacteria, incorporating both gram-negative and gram-positive bacteria (Velázquez et al., 2014). Many of these have shown to be able to catabolize simple and complex carbohydrates, facilitating their adaptation as endophytic bacteria (Li et al. 2014).

Within the Firmicutes, the presence of Clostridia, Bacilli and Erysipelotrichi classes by metagenomic analysis was observed, and some known PGPB genre demonstrating a

rich environmental inside eucalyptus leaves, such as *Bacillus* (Đorđević et al., 2017), *Erwinia* (Jha et al., 2011; Tsavkelova et al., 2007) and *Lactobacillus* (Limanska et al., 2013).

Further on, one of the chosen isolates for plant growth promotion analysis was a gram-positive strain, identified as *Paenibacillus polymyxa* species. The genus *Paenibacillus*, was established by Ash and collaborators (Ash et al., 1994, 1993). It is a facultative gram-positive, rod-shaped bacteria, changing between the free-living and endophytic stages, and aerobic endospore-forming bacteria which are essentially ubiquitous in agricultural systems to their survival traits (McSpadden Gardener, 2004), allowing their adaptation to extreme abiotic conditions, such as extreme temperatures, pH, or pesticide (Dodd et al., 2010; Ribeiro et al., 2018; Sözer Bahadir et al., 2018). Members of the genus are widely distributed in diverse ecological niches from soil, rhizosphere and plant tissues. As our best knowledge this is the first report of the existence of *P. polymyxa* as endophytic bacteria on in vitro tissue culture of eucalyptus. Most of the reports isolated this species from rhizosphere samples (Erturk et al., 2012; Petersen et al., 1996; Ryu and Park, 1997), and from different plant tissues and organs (Anand and Chanway, 2012; Bhore et al., 2010; Xin et al., 2017). As in our study, Bent and Chanway (2002) and Ulrich et al. (2008) have also isolated endophytic *P. polymyxa* from wood plants, in pine and poplar.

So far, most studies associate *P. polymyxa* species with biocontrol activity and with different antibiotic production (Timmusk et al., 2005). Our *P. polymyxa* isolate was capable to produce auxin, fix nitrogen, solubilize phosphate and produce ammonium. These results are in accordance with other reports that revealed outstanding growth-promoting effects on several plants species by *P. polymyxa* (Grady et al., 2016; Ryu and Park, 1997), related to phytohormones production (Bhore et al., 2010; Park et al., 2008; Timmusk et al., 2005; Xin et al., 2017), nitrogen fixation (Lindberg and Granhall., 1984; Timmusk et al., 2005), soil phosphorus solubilization (Singh and Singh, 1993), iron acquisition (Grady et al., 2016) and pathogen biocontrol (Lai et al., 2012), by antimicrobial and enzymes production, an extracellular polysaccharide with antioxidant activity (Haggag, 2007; He et al., 2007; Raza et al., 2011), production of volatile compounds that elicit priming plant immunity (Jeong et al., 2019). All these wide ranges of properties with

ecological and biotechnological importance, have turned this species, in these two last decades one of the most attractive for industrial processes and sustainable agriculture practices (Daud et al., 2019; Lal and Tabacchioni, 2009).

Today, most of the reports involving plant growth promoters bacteria or bioinoculants products are based on Proteobacteria phylum, which covers the legume nodule endophytes as well as rhizobia endosymbionts, gram-negatives, aerobic or facultative anaerobic bacteria from alpha, beta, delta and gamma classes (Singh and Singh, 2016; Suprapta et al., 2014; Velázquez et al., 2014). To mention, some commercial products use *Bradyrhizobium japonicum* (SEMIA 5079) and *Bradyhrizobium diazoefficiens* (SEMIA 5080) from BIAGRO™ HC (Agro Bayer Brasil), *Bradyrhizobium sp from CTS 500® (Bayer Co.), Azospirillum brasiliense* strain *AbV5 and AbV6* UFPR from *AZOKOP* (Koppert Biological Systems), *Rhyzobium tropici SEMIA 4077* strain from BIOMARHYZO BEANS- PEATY (BIOMA, Brazil), *Sinorhizobium meliloti* RF14, indicated for alfafa (Andermatt Biocontrol, Swizerland) in general, all for seed treatment and the key benefits is nitrogen fixation.

In this study, proteobacteria were the phyla with the second most prevalent in the metagenomic analysis, mostly represented by gammaproteobacteria, involving Enterobacteriales and Legionellales orders, followed by alpha, delta and beta-proteobacteria classes. Gammaproteobacteria seems to be a constant group in Eucalyptus endophytic community (Miguel et al., 2016; Nnadozie et al., 2017). Members of this class are usually responsible for cellulose degradation, and have the ability to hydrolyze the hemicellulose and degrade lignin, which can be the response of their higher dominance on wood trees such as eucalyptus (Nnadozie et al., 2017; Scully et al., 2013; Yu et al., 2007). Following our cultivated-dependent isolation, a gram-negative strain showed higher auxin production, and later on was identified as *Enterobacter* sp. This isolate was also positive for auxin production, nitrogen fixation, phosphate solubilization and ammonium production.

Species from the genus *Enterobacter* were also reported to be isolated from various environments in association with plant rhizosphere and as endophytic organisms (Camila et al., 2018; Carvalho et al., 2014; Haichar et al., 2008; Mirza et al., 2001; Van der Lelie et al., 2009), exhibiting similar plant-growth-promoting effects as production of

phytohormones (Koga et al., 1991), nitrogen fixation, P solubilization (Mukhtar et al., 2017) and biocontrol activity (Koga et al., 1991; Madhaiyan et al., 2010) by the production of siderophore and other bio-compounds that confers plant protection against bacterial and fungal infections (Srisuk et al., 2018; Taghavi et al., 2010). More specific to phytohormones production, *Enterobacter* species were therefore indicated to be a potent IAA-producing bacteria, reaching up to 5561.7 $mg.l^{-1}$ of IAA production (Nutaratat et al., 2017).

In this study, Actinobacteria and Bacteroidetes were present at the analysis results with 10 and 7.5 %, respectively. Actinobacteria are among the classes most consistently found as endophytes, whereas Bacteroidetes, are less commonly found as endophytes (Santoyo et al., 2016), and are mentioned to be abundant on the roots and rhizosphere (Qiao et al., 2017). Probably due to a phylogenetic signal associated with the presence of complex biopolymers in their root exudates, as they hold a diverse set of carbohydrate processing enzymes, that turns them capable to degrade complex biopolymers (Berlemont and Martiny, 2015; Pérez-Jaramillo et al., 2018). Actinobacteria were identified in the *Eucalyptus urograndis* endophytic bacterial community (Fonseca et al., 2018; Miguel et al., 2016). Therefore similar to our findings, other studies revealed less percentage of the presence of Actinobacteria around 5-10% in *Aloe vera* plant tissues (Akinsanya et al., 2015; Bulgarelli et al., 2013), and endosphere and rhizosphere of *Populus deltoides* roots (Gottel et al., 2011). In contrast, Fonseca et al. (2018) reported the occurrence of this phylum in approximately 59% of the sequences analysis, followed by Proteobacteria (28%), Firmicutes (7.5%), Bacteroidetes (1.5%) Acidobacteria (1.2%), TM7 (<1%), and Chloroflexi (<1%), with five lineages classified into *Kineococcus*, *Microbacterium*, *Nocardia*, and *Rhodococcus*. Metagenomic analysis could reveal two genera, *Brevibacterium* and *Streptomyces* into Actinobacteria, both known as PGPB (Noordman et al., 2006; Olanrewaju and Babalola, 2019), and *Prevotella* and *Pedobacter* from Bacteroidetes.

When the produced auxin and bacteria culture broth were evaluated, separately on common beans seeds, the results with *Enterobacter* sp. DEBB B-355 showed that seeds inoculated only with the auxin solution had more positive effects when compared to other treatments. Primary root length increased over 41.33 % and plant height was

28.58 % higher when compared to the negative control (only water). Indeed, roots system are the most sensitivity organ to IAA supplementation and levels (Hussein et al., 2016), and by increasing overall root development, allows better uptake of water and minerals for the plant (Gilbert et al., 2018; Spaepen and Vanderleyden, 2011a). However, bacteria cells and the auxin in the culture, stimulated seed germination. Similarly, Srisuk et al., (2018) reported that jasmine rice supplemented with IAA produced by *Enterobacter* sp. DMKU-RP206 had significantly increased length and dry weight of shoot compared with the negative control, after 2 weeks plantation, indicating that the IAA in culture filtrates of DMKU-RP206 could be used instead of the synthetic IAA. Another example of application, was the inoculation of bacterial supernatant containing IAA, from *Halomonas desiderata* culture, in Mung bean cuttings (Ali and Hasnain, 2007), where the bacterial supernatant improved the number of roots and root length similar to standard IAA, and also showed higher root elongation.

Although no statistically difference was observed among the treatments, seeds inoculated with *Paenibacillus polymyxa* DEBB B-358 culture and its produced auxin showed higher enhancement on germination percentage and all plant growth parameters, apart from root numbers, when compared to the control treatment. Bacterial IAA was also reported to be responsible for *Brassica oleracea* L. seeds shoot elongation increase (over 110% and 130%) (Ali and Hasnain, 2007). Moreover, *P. polymyxa* E681 was isolated from *A. thaliana* and incremented plant growth as foliar fresh weight and total leaf area (Jeong et al., 2019, 2006). As endophytic bacteria, they have the capacity to colonize various plant compartments such as roots, stem, leaves, flowers, fruits, and seeds and improve plant growth, improve plant development and protection (Da Mota et al., 2008; Puri et al., 2018). *P. polymyxa* was reported to efficiently colonize some important agricultural crops (Puri et al., 2018) and wood plant seedlings (Anand and Chanway, 2012). Alike, endophyte *Paenibacillus* species isolated from the meristem tissues of *Cymbidium eburneum*, identified as *P. lentimorbus* and *P. macerans*, and revealed to be able to produce indole compounds and, subsequently were able to improve the shoot and root biomass, root length and increased plant survival during the acclimatization process in micropropagated *Cattleya loddigesii* (orchids) plants (Faria et al., 2013). Therefore, the

responsiveness to plant growth promotion by bacteria or auxin are highly dependent on the particular auxin applied and of the plant genotype (Dodd et al., 2010).

In conclusion, the community of endophytic bacteria from in vitro micro-propagated *Eucalyptus urophylla* evaluated, presented endophytic bacteria isolates from leaves with plant growth-promoting traits, such as auxin production, nitrogen fixation, solubilization of phosphate and ammonium production, which can indirectly be acting as a growth promoter and as an adjuvant in the development of the E. urophylla clone. Therefore, as none of the identified strains had shown a detrimental effect on common beans seed development, and improved plant growth parameters, this study highlight that both endophytes strains, DEBB B-355 and DEBB B-358 from eucalyptus, can be useful as inoculants for others plant species. Howsoever, more evaluations must be done to optimize IAA production and to test their plant promotions abilities for a long period in green-house or even in the field.

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PART III -AUXIN PRODUCTION BY A PLANT ENDOPHYTE *Enterobacter* sp. DEBB B-355 ISOLATE AND ITS BIOSTIMULATION ON PLANT GROWTH

ABSTRACT

This study reports auxin production evaluation, optimization, and scale-up process from endophyte bacteria *Enterobacter* sp. DEBB B-355. The production of auxin was optimized by statistical methods. First, pH, carbon, and nitrogen sources were evaluated. Afterward, a central composition design was used to seek optimal ranges among C:N ratio, and days of incubation, inoculum percentage and tryptophan concentration interaction for auxin optimization. For a higher amount of auxin an initial pH of 5±0.2, 5.11 $g.L^{-1}$ of CSL and 10.11 C:N ratio equivalent to 28.0 $g.L^{-1}$ of glycerol were defined as nitrogen and carbon sources, respectively. The results showed that L-tryptophan and inoculum size had a significant importance in terms of auxin production. Higher production of 824.09 $\mu g.mL^{-1}$ was observed after 4 days of culture with 4% of inoculum and 1000 $\mu g.mL^{-1}$ Trp. In addition, the statistical model was validated and the on optimized conditions, the production increased up to 910.27 $\mu g.mL^{-1}$ when cultured on 10 L STR bioreactor. The auxin produced and bacterial fermented culture promoted lettuce seed germination and development. The optimized medium composition with CSL and glycerol for IAA production by an *Enterobacter* sp. isolate was reported for the first time in this study.

Key words: IAA, phytohormones, root elongation, rhizobacteria, plant growth

INTRODUCTION

Biosynthesis of phytohormones is one of the mechanisms for plant growth stimulation by plant-associated microorganism, either by acting directly as a chemical signaling to impact on plant process and functioning or by indirect mechanisms to improve water and nutrients uptake (Dodd et al., 2010; Egamberdieva et al., 2017). The auxins from the phytohormones family, having indole-3-acetic acid (IAA) as the main member, plays a key role in a number of plant processes such as cell division, cell elongation, cell differentiation, root initiation, lateral root formation, phototropism and geotropism,

including the regulation of falling leaves and fruit ripening, etc (Ahmed and Hasnain, 2014). IAA is also related to enhance cellular defense system and improve plant tolerance over abiotic and biotic stresses (Egamberdieva et al., 2017; Etesami et al., 2015), and have an essential paper on other phytohormones modulation, such as abscisic acid and ethylene (Dodd et al., 2010).

Microbial technology can act as an environmental strategy for a safe and secure food production and supply to the rising global population. Microorganisms can be used as biofertilizers and increase crop yield and quality, especially in hostile environments with less use of pesticides and N fertilizers (Erdelyi et al., 2014; Park et al., 2015). Therefore, plant-associated bacteria capable to produce auxin have been receiving more attention from agronomists and plant scientists (Liu et al., 2017; Mashiane et al., 2018; Rybakova et al., 2016; Santoyo et al., 2016). They have emerged as a desirable trait to prospect an environmentally sustainable agricultural method to be used as bioinoculant/biofertilizer to improve crop resources, (Dodd et al., 2010; Pirog et al., 2018).

From most of the plant-associated bacteria from rhizosphere, phyllosphere, or endophytic, over 80% are reported to possess the ability to produce IAA (Jalgaonwala et al., 2011; Singh and Singh, 2016). Endophyte plant-associated microorganisms are those with a mutualistic relationship living in internal plant tissue, better protected from abiotic stresses such as extreme variations in temperature, pH (Puri et al., 2018; Rosenblueth and Martínez-Romero, 2006). They also take advantages from the relative water and nutrient-rich environment from the internal tissues, with amino acids, sugars, fatty acids and other organic compounds (Brader et al., 2014; Dutta et al., 2014). In return, they synthesize biologically active compounds, including phytohormones, antifungal compounds, enzymes, and volatile compounds, for plant growth development stimulation by improving nutrient acquisition, and assigning systemic plant tolerance over various abiotic stress factors and defend plants from pathogenic microorganisms (Egamberdieva et al., 2017).

Enterobacter species are gram-negative, non-spore-forming, aerobic and motile bacteria, reported to have a symbiotic relationship with plants. They have direct and indirect mechanisms traits such as phosphate and zinc oxide solubilization, ammonia and polyamine production, and ACC deaminase activity, phytohormones, and siderophore

production, able to promote plant growth development and antagonistic activity against phytopathogenic (Khalifa and Saleh, 2016; Nutaratat et al., 2017; Park et al., 2015). Thus, *Enterobacter* species appears as a high potential bacterial resource to be used as a plant bioinoculant for agricultural use (Nutaratata et al., 2017). *Enterobacter* species were found endophytically in maize stems and leaves (Mashiane et al., 2018), rice leaves (Nutaratat et al., 2017), non-nodulating roots of alfafa (*Medicago sativa*) (Khalifa and Saleh, 2016), poplar (Taghavi et al., 2009b) and on many other rhizosphere environments with plant growth promotions (Khalifa and Saleh, 2016).

In this study, we isolated an endophyte bacterium from *Eucalyptus* sp. in vitro plantlets leaves and evaluated different factors of auxin production, optimization and process scale-up, and its effects on plant growth promotion.

MATERIALS AND METHODS

Microorganism

The endophytic bacteria used in this study was isolated from aseptic *Eucalyptus* sp. plantlets, which were maintained under in vitro conditions at Tissue and culture laboratory of Bioprocess engineering and Biotechnology Department – UFPR (Curitiba-Brazil). The isolate was identified by 16S gene sequencing as *Enterobacter sp*. DEBB B-355 (described on PART II).

Evaluation of auxin production by *Enterobacter sp*. DEBB B-355

First we evaluated pH varying from 5, 6, 7 and 8 using Luria Broth (LB) medium (5.0 $g.L^{-1}$ yeast extract, 10 $g.L^{-1}$ tryptone, 10 $g.L^{-1}$ NaCl) and 100 $\mu g.mL^{-1}$ of Trp. For inoculum, an aliquot of 100 µL of the isolate maintained in glycerol at -80°C was incubated at 30°C, in a shaker with 120 rpm for 16 h in LB medium. Then 1% of the inoculum was transferred into 250-mL Erlenmeyer flask with 50 mL of LB medium supplemented with 100 $\mu g.mL^{-1}$ of Tryptophan (Trp) and cultured for 3 days, at 30ºC and 120 rpm. With the chosen pH, we followed to evaluate nitrogen sources (5 $g.L^{-1}$): corn steep liquor (CSL), peptone, urea, yeast extract (YE) and ammonium sulphate (AS), using as basal media composition ($g.L^{-1}$): K2HPO4: 0.5; KH_2PO_4: 0.3; $MgSO_4 \cdot 7H_2O$: 0.1; NaCl: 0.75; $CaCl_2 \cdot 6H_2O$: 0.03; glucose: 15.0, 1 mL of trace element solution (in gL^{-1}: $FeCl_3$: 0.02, KI:

0.083, $Na_2MoO_4.2H_2O$: 0.124, H_3Bo_3: 0.03, $ZnSO_4.7H_2O$: 0.287, $MnSO_4.4H_2O$: 0.223, $CuSO_4.5H_2O$: 0.249) (Blinkov et al., 2014) and compared to LB medium. Therefore, after choosing the nitrogen source we evaluated glucose, sucrose, and glycerol as carbon sources (with 15 $g.L^{-1}$), using the same basal media. For both, nitrogen and carbon sources experiments, the medium was supplemented with 300 $\mu g.mL^{-1}$ of Trp and pH of 5±0.2. All the experiments were conducted using 1% of fresh inoculum culture (16h) at 30°C, 120 rpm and cultured for three days (72h). For auxin detection and quantification, bacterial culture was centrifuged at 958 g for 15 min and the supernatant was collected and evaluated qualitatively by Salkowski's reagent [one milliliter of cell-free supernatant was mixed with 2 mL of (a mixture of 50 mL 35% perchloric acid and 1 mL 0.5M $FeCl_3$) and allowed to stand for 30 min on temperature room in dark]. The reaction was evaluated in a spectrophotometer at 530 nm. A standard curve was developed with a pure indole-3-acetic acid solution (0 - 100 $\mu g.mL^{-1}$, R^2= 0.98).

Experimental design for auxin production optimization using response surface methodology (RSM)

Carbon and Nitrogen ratio evaluation

Response surface methodology including central composite design (CCD) was employed to study the interaction among the carbon and nitrogen concentration to find out their optimum levels (Table 3). Yeast extract (YE) and corn steep liquor (CSL) were evaluated as nitrogen sources, combined with glycerol, as carbon source. The culture was performed using 1% inoculum, 500 $\mu g.mL^{-1}$ of Trp, pH of 5±0.2, 30°C and 120 rpm. We evaluated auxin and culture growth (OD) after three days of fermentation.

The influence of days of incubation, inoculum and tryptophan concentration on auxin production

Having the results of the nitrogen source and the best C:N ratio, the next step was to evaluate the interaction of incubation days, inoculum percentage and tryptophan concentration for auxin production (Table 4), using RSM and CCM. The conditions pH, temperature and rotation were maintained as mentioned above.

RSM model validation for auxin production in flasks and scale-up

After optimization of IAA production, the better conditions were used to validate the RSM model in shaking flask culture, and then a scale-up production was performed on a stirred tank fermenter, with 1.5L and 6L of working volume. The medium optimized composition was 5.11 $g.L^{-1}$ CSL, 28.09 $g.L^{-1}$glycerol, 1250 $\mu g.mL^{-1}$ of Trp, pH = 5±0.2 at 30 °C, using 4 % of inoculum, under agitation. Scale-up was performed using 2 L STR bioreactor (BIOSTAT B, B. Braun Biotech International, Germany) and 10 L bioreactor (New Brunswick's Bioflo 110 Fermenter/Bioreactor, GMI, USA). The 2 L STR fermenter was operated with 1.5 L working volume, with agitation speed of 400 rpm, aeration at 1 vvm (3 $L.min^{-1}$). For 10 L bioreactor, the working volume was 6 L, agitation speed varied from 750 to 400 rpm over the fermentation, aeration of 1 vvm, pH and oxygen rate were controlled during the fermentation. Samples were taken daily and analyzed for IAA production and bacterial biomass weight. Glycerol and tryptophan consumption, and pH variation were also controlled in 10 L fermentation. Total volumetric of IAA productivity (r_{Pmax}), global volumetric IAA productivity ($r_{Pglobal}$) and IAA yield relation with glycerol ($Y_{P/S}$) and Trp ($Y_{P/T}$) was calculated using 10 L bioreactor results, as follows:

Total volumetric of IAA productivity: $$r_{Pmax} = \frac{\Delta P}{\Delta t}$$

Global volumetric IAA productivity: $$rPglobal = rPtotal\ x\ V$$

IAA yield: $$Y\ P/S = \frac{\Delta \mathrm{P}}{\Delta S} =$$

$$Y\ P/T = \frac{\Delta \mathrm{P}}{\Delta T}$$

Considering 124 h of fermentation, and total volume of 6 L.

Auxin identification by HPLC and FTIR identification

For auxin compounds extraction, the fermented bacterial culture was first centrifuged for 10 min 4000 rpm, then the culture supernatant was adjusted to pH=2.8 using 1N HCl and was extracted with an equal volume of ethyl acetate (EtOAc). After vigorous shaking, it was allowed to stand for 10 min, and the supernatant was recovered. This extraction procedure was repeated 3 times. The EtOAc was then evaporated on a rotary evaporator (Vacucell, MMM Group) at 35°C. The indole compounds were dissolved

in 50% methanol (MeOH) and kept at 4°C for further analysis (Sudhapriyadharsini et al., 2016). For HPLC analysis, indole compounds were analyzed in a PrepStar 218 Preparative HPLC System (Varian Inc., Palo Alto, CA) equipped with a 250×21.4 mm Microsorb 60-8 C18 column (Varian). The column was eluted with 1% acetic acid as a mobile phase at a flow rate of 0.5 mL/min. The indole compounds were detected at 254-280 nm (Lim and Kim, 2009). The infrared spectrum of the purified compound was obtained using Fourier Transform Infrared (FTIR) spectrophotometer (Shimadzu FTIR 8400) and the spectra was recorded in the nujol mull using KBr cells and expressed in wavenumber (cm^{-1}).

Plant growth promotion by the endophytic bacteria and the produced auxin on lettuce seed

We screened for the effect of the bacterial fermented broth concentration on lettuce *(Lactuca sativa)* seed germination and plant growth. Before seed treatment with bacterial culture, seeds were washed with 70% of ethanol for 1 min and 2.5% of sodium hypochlorite for 5 min, and then rinsed with sterile dH2O thrice. And then, forty seeds were inoculated on petri dish with a filter paper and immersed with the treatment solutions (table 3): 0.5, 1, 2, 5 and 10 % of bacterial broth (4 days old bacterial broth). The explants were left in dark to allow seed germination, and were then maintained on a 16 h photoperiod in a growth room at 25°C. The plantlets were evaluated after 14 days and transplanted to trays containing vermiculite in a greenhouse for 30 more days. The plantlets were irrigated regularly with water and a mineral mixture (with macro and micronutrients) in alternated days. After 30 days the plant growth promotion activity was evaluated: plant height, root length, number of leaves, fresh and dry weight.

Statistical analysis

Statistical analysis was performed by using the statistical software STATISTICA®, version 8.0. The data obtained from RSM on auxin production were subjected to analysis of variance (ANOVA). The relationship between the coded values and actual values, independent variable, and the response, were calculated according to a second-order quadratic model. The relative effects of the variables on response were examined from

three-dimensional contour plots, to represent the effect of the independent variables on IAA production and bacteria growth (OD). These response curves were then used to predict the optimum level of the factors. All experiments evaluating auxin production were conducted using 250 mL Erlenmeyer's with 50 mL of medium, in triplicate and were repeated twice. For plant promotion experiment, the significance of each treatment was established by one-way ANOVA and means were separated by Fisher's test ($p \leq 0.05$).

RESULTS

Auxin production was better on acidic pH = 5 (10.04±1.71 μm.mL^{-1}) followed by pH 8 (8.93±1.27 μm.mL^{-1}), 6 (8.61±1.02 μm.mL^{-1}) and 7(6.39±0.85 μm.mL^{-1}), although no statistical difference where observed among the treatments (Fig 10). Yeast extract stimulated auxin production compared to all the other nitrogen sources, reaching 71.37 ±10.59 μm.mL^{-1}, followed by CSL (28.88±5.45 μm.mL^{-1}). Glycerol increased auxin production (57.56±3.04 μm.mL^{-1}) three-fold when compared to glucose and sucrose, with 18.23±2.77 μm.mL^{-1} and 19.23±2.77 μm.mL^{-1}, respectively (Fig. 10). In this order, we choosed pH = 5±0.2, glycerol as carbon source, and tested yeast extract and CSL as nitrogen sources for the carbon:nitrogen ratio experiment.

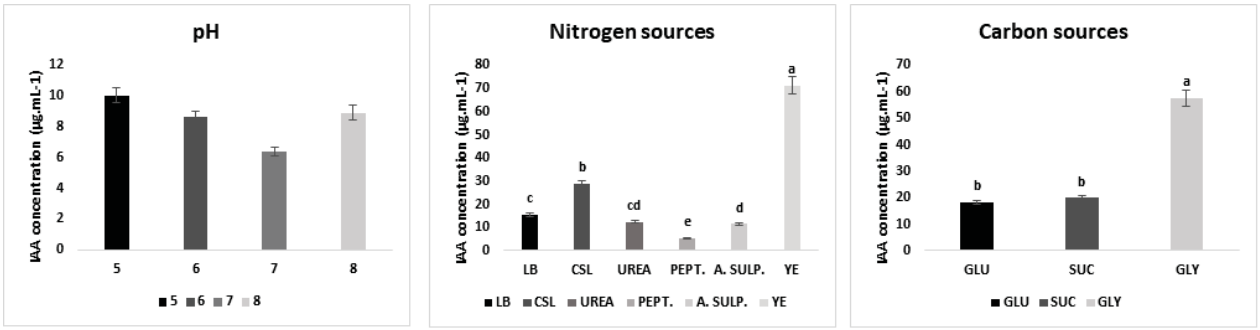

Figure 9. Screening for auxin production by the endophytic *Enterobacter sp.* DEBB B-355 over different ranges of pH (5, 6, 7 and 8), nitrogen sources: LB medium, Corn steep liquor (CSL), urea, peptone (Pept.), ammonium sulphate (A. Sul.) and yeast extract (YE), and carbon sources: glucose (GLU), sucrose (SUC) and glycerol (GLY). Values expressed in μm.mL^{-1}.

By only changing the carbon and nitrogen ratio, CSL increased the auxin production, when compared to YE (Table 3). The highest auxin concentration was 143.25

μm.mL^{-1}, obtained using the center points of 5.0 g.L^{-1} of CSL and C:N ratio equal to 8. Both variables had a significative effects on auxin production (Fig. 11), and RSM showed that the critical levels were around 5.11 g.L^{-1} of CSL and C:N of 10.28, which corresponds to 28.09 g.L^{-1} of glycerol. The statistical analysis showed that the linear variables were significative for auxin production with YE, although the quadratic terms were not significative, and the analysis failed to map the surface response for auxin production. New variables ranges could give a better response. However, the peak auxin amount with YE was 111.25 μm.mL^{-1}, also with 5 g.L^{-1} but with the lower C:N ratio (2.36). In general, higher nitrogen concentration, on both CSL and YE, increased bacterial growth. Based on these results, we further used CSL as the nitrogen source.

Using the optimized values of CSL and glycerol for medium composition, we further evaluated days of incubation, inoculum percentage and tryptophan concentration. These optimized conditions led us to increase the auxin production over 82 times since we first analyzed this isolate for auxin production with LB media. We obtained up to 824.09 μm.mL^{-1} when the bacteria were cultured for 4 days, with 4% inoculum and 1000 μm.mL^{-1} Trp (Table 4). The statistical analysis revealed that tryptophan and inoculum had a significative effect on auxin production (Fig. 12). Auxin decreased after the 5 and 6th day, and drastically on the lowest Trp concentration. The opposite was observed on bacteria growth (OD), which increased mostly after 5 and 6 days. Pareto analysis for bacterial growth has shown that days and Trp concentration had a significative effect ($p<0.5$, data not shown). Hence, the surface response showed that the critical factor values were 3.3 days, 4.45% inoculum and 1248.45 μg.mL^{-1} Trp, for a maximal IAA production.

The model obtained in these three factors experiment showed the determination coefficient value (R^2) of 0.77 for IAA production, and the resulted RSM equation of the model using the three variables for auxin production was:

$$IAA\ (\mu g.mL^{-1}) = -513 + 375Days + 109Inoculum + 0.252Trp - 62.2\ Days^2 - 14.9Inoculum^2 - 0.000422Trp^2 + 4.5Days\ x\ Inoculum + 0.087Days\ x\ Trp - 0.0006\ Inoculum\ x\ Trp$$

We used this data to validate the model, to set up the kinetical experiment and to evaluate the auxin production over 6 days (144h) in Erlenmeyer flasks and

used the same conditions for scale-up in Bioreactors. By using 4 % of inoculum with 1250 µg.mL^{-1}, the highest auxin production was around 770.49 µg.mL^{-1} (Fig. 13 A), and the expected concentration was 803.11 µg.mL^{-1}, an error of 4.06 % from practical to theoretical amount. This result indicates a good degree of correlation between the observed and predicted values, and supports the significance of the developed model.

Using the same optimized medium and inoculum conditions for scale-up, the experiment using 2 L bioreactor had the same behavior as observed in the flasks. We observed an increasing production, especially after 24h of culture, until reaching the highest amount at 96h, and then started to decline. Yet, the maximum production was about 750.05 µg.mL^{-1} of IAA (Fig. 13 B).

Table 3. Central Composite Design matrix of corn steep liquor (CSL) and Yeast Extract (YE) as nitrogen source and carbon and nitrogen ratio, using glycerol as carbon source for auxin (IAA) production by *Enterobacter sp.* DEBB B-355. Values are expressed in $\mu g.mL^{-1}$.

LEVELS		-1.41	-1	0	1	1.41
	X1: CSL ($g.L^{-1}$)	2.18	3	5	7	7.82
	X2: C:N	2.36	4	8	12	13.64

			Corn Steep Liquor			*Yeast Extract*		
Repetition	X1	X2	IAA	Expected	OD	IAA	Expected	OD
1	-1	-1	36.61	42.00	4.28	38.98	74.64	2.88
2	-1	1	117.59	125.89	2.34	41.80	50.82	2.69
3	1	-1	116.48	93.12	4.66	30.92	48.45	4.03
4	1	1	141.99	121.55	4.03	31.14	22.03	4.07
5	-1.41	0	90.66	77.84	3.70	91.58	65.37	3.39
6	1	0	82.87	110.82	4.65	27.11	26.61	3.81
7	0	-1.41	55.45	65.53	4.65	111.25	88.60	5.06
8	0	1.41	122.78	127.23	3.08	36.28	42.89	3.88
9	0	0	141.44	138.96	3.20	33.62	30.78	3.88
10	0	0	143.25	138.96	2.91	36.17	30.78	3.94
11	0	0	131.76	138.96	3.20	32.91	30.78	3.98

Experiments were carried out in triplicate. Data represent the mean value. Standard deviation for estimated values is 0.05. R^2 for CSL-IAA was 0.74 and YE-IAA was 0.46.

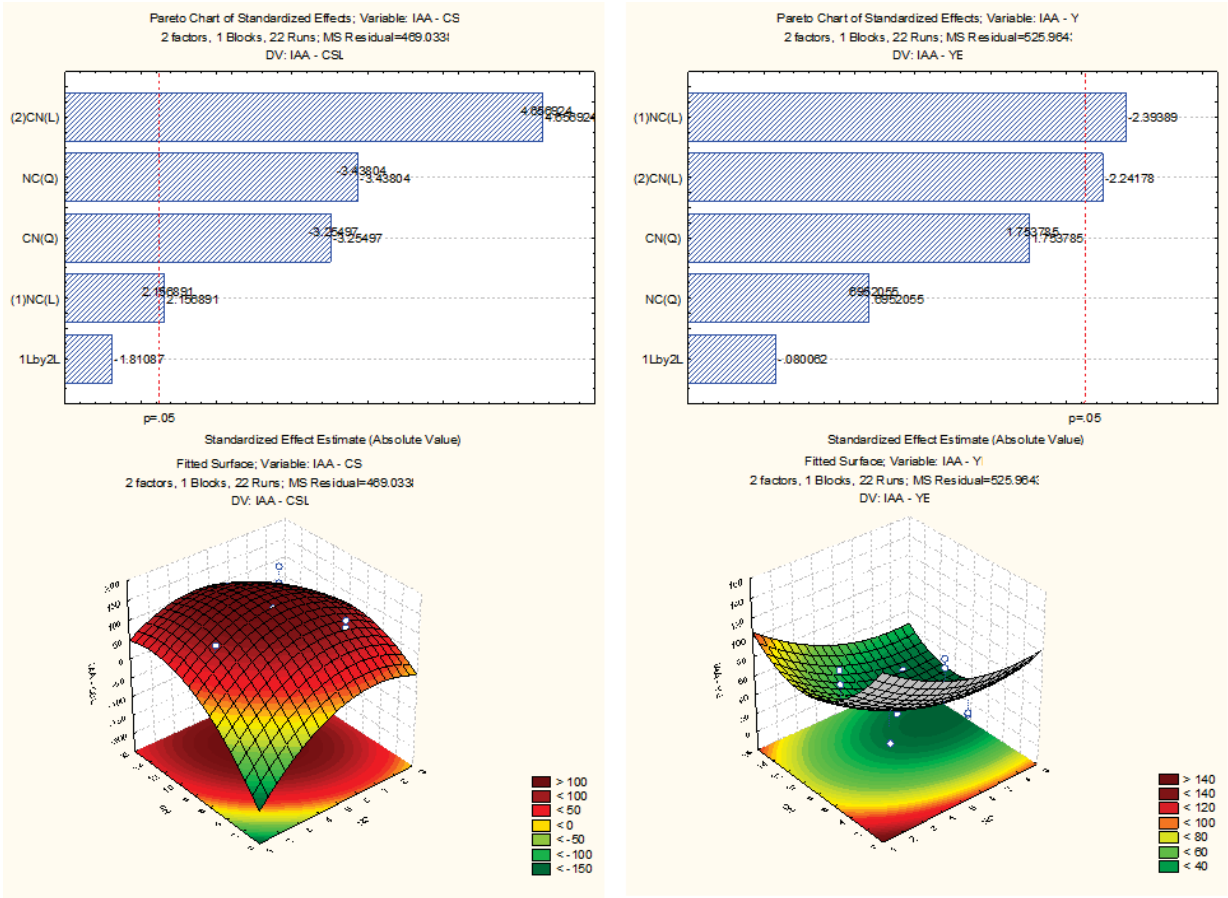

Figure 10. Pareto chart standardized effects with absolute values and fitted surface graph from two factors: nitrogen source concentration and Carbon: Nitrogen ratio interaction for auxin production. On left (up and down) the analysis using corn steep liquor as nitrogen source, and on right yeast extract. Legend: CN – carbon:nitrogen ratio; NC – nitrogen source.

Table 4. Full factorial Central Composite Design (CCD) of three variables on auxin production and optic growth by *Enterobacter sp.* DEBB B-355. Values are expressed in $\mu g.mL^{-1}$.

Levels		-1.68	-1	0	1	1.68
X1- Days		2	3	4	5	6
X2 - Inoculum %		0.641	2	4	6	7.36
X3- Tryptophan ($\mu g.mL^{-1}$)		60	500	1000	1500	1840
Repetition	X1	X2	X3	IAA	Expected	OD
1	-1	-1	-1	317.46	208.21	3.50
2	-1	-1	1	698.12	586.92	4.65
3	-1	1	-1	635.14	455.25	3.59
4	-1	1	1	718.56	722.63	4.82
5	1	-1	-1	186.91	246.94	5.61
6	1	-1	1	284.97	427.31	5.95
7	1	1	-1	357.07	294.25	5.93
8	1	1	1	313.70	363.29	6.42
9	-1.68	0	0	381.93	601.07	3.26
10	1.68	0	0	462.04	280.46	5.37
11	0	-1.68	0	373.81	471.68	2.89
12	0	1.68	0	336.24	453.91	3.39
13	0	0	-1.68	96.30	198.47	4.74
14	0	0	1.68	461.66	574.59	3.46
15	0	0	0	674.36	782.19	3.49
16	0	0	0	810.83	782.19	3.42
17	0	0	0	824.09	782.19	3.26

Experiments were carried in triplicates. Data represent the mean value. Standard deviation for estimated value is 0.05. R^2 for IAA was 0.77 and OD = 0.60

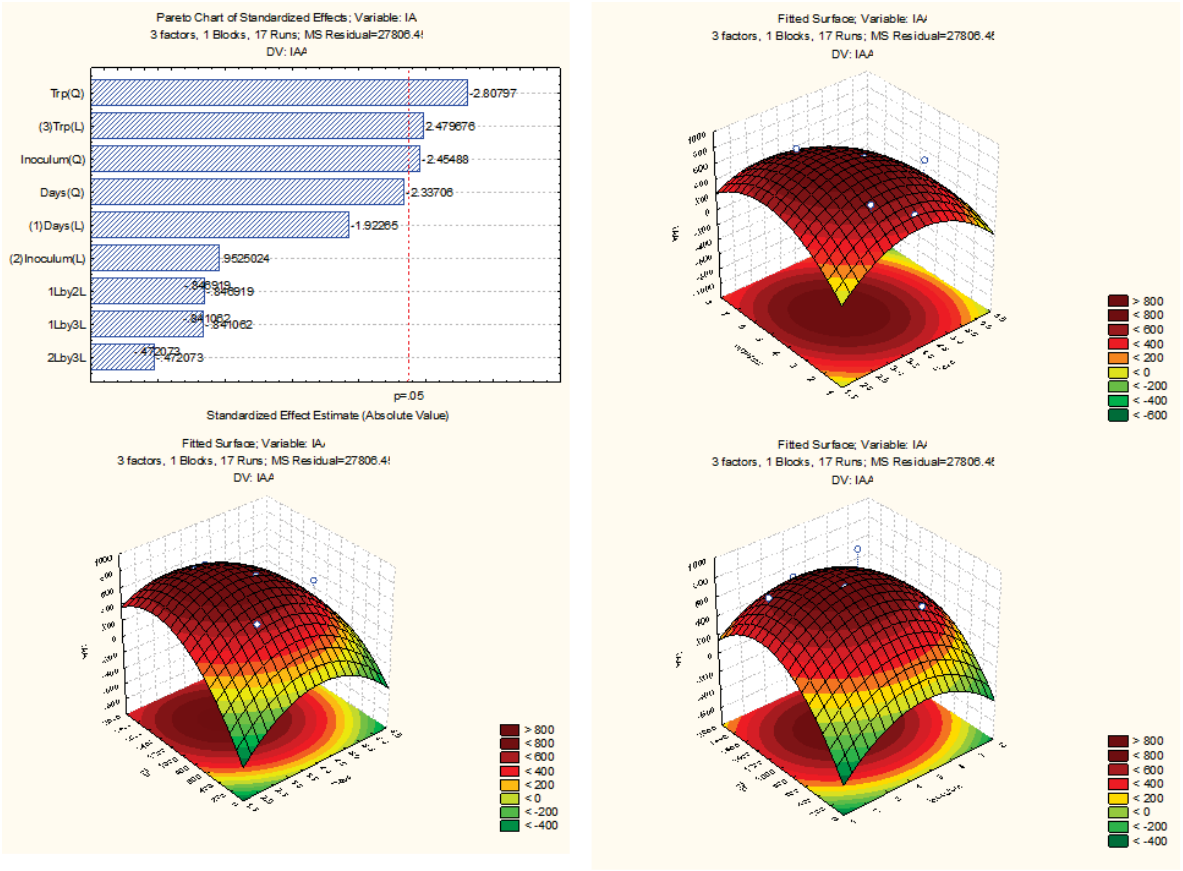

Figure 11. Pareto-plot for CCR parameter estimates for three factors and interaction effect of days of fermentation, inoculum percentage and tryptophan concentration for *Enterobacter sp.* DEBB B-355 auxin production.

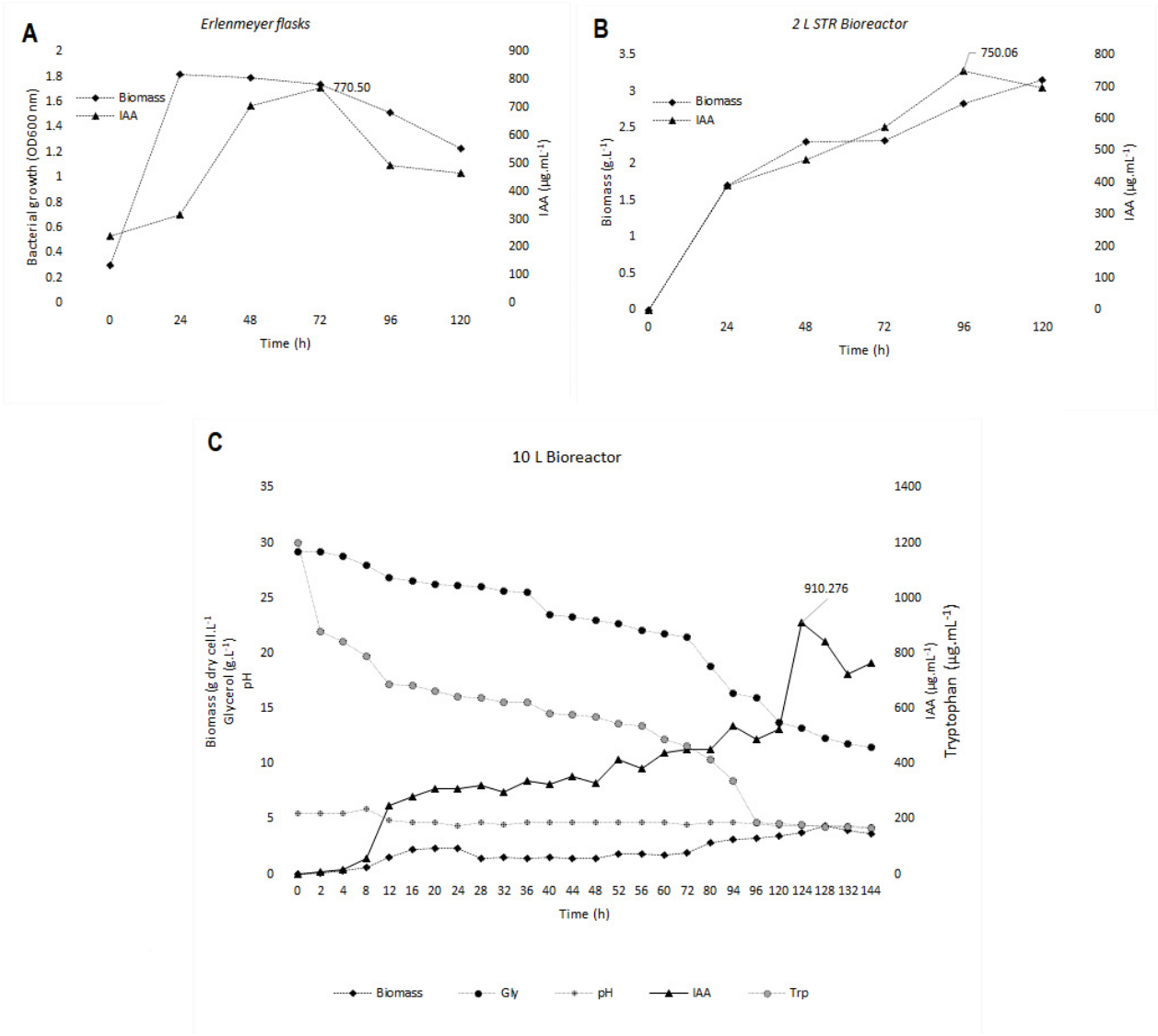

Figure 12. Kinetical profile of auxin production in 250 mL Erlenmeyer flasks (A), 2 L STR Bioreator (B) and 10 L Bioreactor (C) by endophytic bacteria Enterobacter sp. DEBB B-355. The graphic indicates the values for IAA yield (µg.mL-1) production and bacteria growth (OD) or Biomass weight (g of dry cell per litter), glycerol and tryptophan consumption, and pH status over the fermentation time. Culture conditions: temperature: 30 °C, pH = 5±0.2, 120 rpm on flask, 400 rpm and 1VVM in bioreactor.

When scaled-up in 10 L bioreactor, IAA production considerably increased between 120 and 128 h, reaching up to 910.27 µg.mL^{-1}, and started to decline thereafter. This represented an increase of 18.14 % of IAA biosynthesis compared to the flask experiment, although with an additional of 32h of fermentation, from 96h to 128h. This IAA yield corresponded to 7.34 mg/L.h of total volumetric IAA productivity (considering 124 h of fermentation) and a global volumetric IAA productivity of 44.04 mg.L^{-1}, in relation

with the total volume (6 L). The IAA yield in relation to glycerol ($Y_{P/S}$) and Trp ($Y_{P/T}$) consumption was 53.79 mg of IAA/g of glycerol and 0.85 mg of IAA/mg of Trp, respectively. Gradual pH change could be observed, turning the culture more acid, throughout the process of cultivation (Fig. 13 C). The IAA compound produced by the endophytic was identified by HPLC and FTIR as showed on figure 14.

The experiment with plants showed that the bacterial extract with bacterial auxin can promote plant growth. Although no dose dependence was observed, lower concentrations of bacterial extract showed to be appropriate among the first 14 days to plantlets growth, especially to plantlets height, root length and plant weight (Table 5). Different, 10% of bacterial extract decreased plant height and root growth, similar to positive control when used IAA. After transplanting the plantlets from growth room to greenhouse, the negative and positive control treatments did not respond well. Generally, bacterial extract improved all evaluated parameters. The plant´s height increased using 1, 5 and 10% of bacterial extract, differing from other treatments. Higher root growth was observed with the highest bacterial extract's concentration of 5 and 10% (30.16 and 28.36 cm, respectively) when compared to negative and positive control. One and 5 percent of the bacterial extract resulted on 16.71 and 15.14 gr of fresh weight, but the concentration of 5% showed higher dry weight, different from all the other treatments. Hitherto, it seems that lower bacterial extract (also lower auxin concentration) was appropriate for the first days of plant growth, and then a concentration 10 times higher (5%) showed to be appropriate for the bacterial inoculation on lettuce plantlets (Fig. 15 and 16).

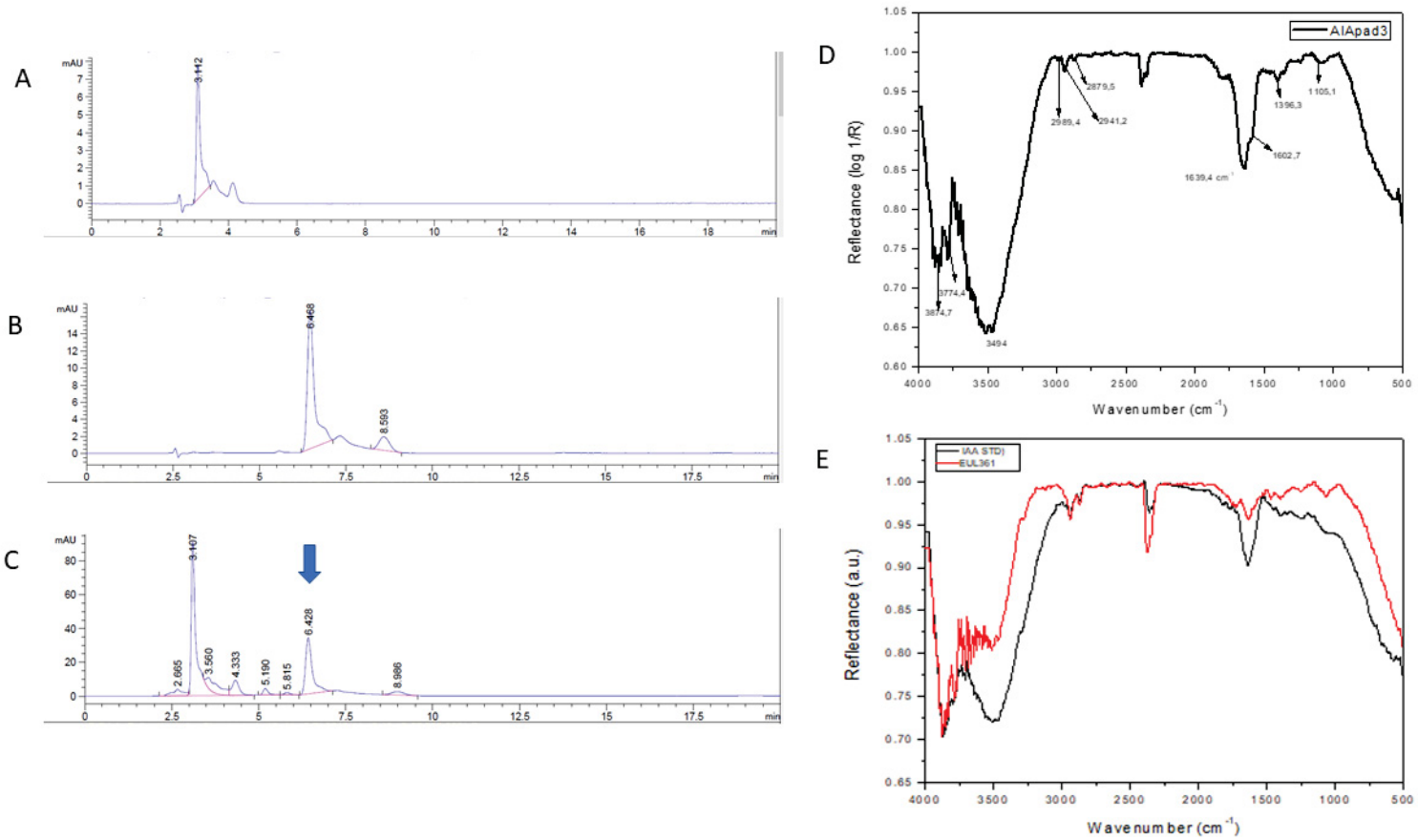

Figure 13. HPLC graphic of indol-3-acetic acid compounds and tryptophan. A: Tryptophan standard peak at 3.112 min; B: IAA standard peak at 6.468 min; C: confirmation of IAA production and tryptophan compounds on *Enterobacter sp*. DEBB B-355; D: standard IAA FTIR profile (on top and black line); E: IAA FTIR produced by the isolate *Enterobacter sp*. DEBB B-355 (at bottom and red line).

Table 5. Plant growth properties of the endophyte bacteria *Enterobacter* sp. DEBB B-355 on lettuce seeds. Evaluations were done after 14 and 45 days for plant height, root length, root number, number of leaves, fresh and dry weight.

			14 days					45 days				
Treat. (%)	IAA ($\mu g.mL^{-1}$)	UFC/ mL^{-1}	Height (cm)	Root length (cm)	Root number	Leaves	Fresh weight (gr)	Height (cm)	Root length (cm)	Leaves	Fresh weight (gr)	Dry weight (gr)
NC	0	0	1.22±0.46^{ab}	6.44±2.01^{ab}	2.40±0.74	2.80±0.64	0.126±0.05^{b}	6.83±1.4^{d}	14.24±3.3^{c}	6.33±0.5^{c}	3.21±0.8^{e}	0.31±0.1^{d}
PC	3	0	0.98±1.38^{bc}	3.26±2.2^{c}	2.60±0.93	2.80±0.74	0.148±0.01^{ab}	7.03±0.9^{d}	15.81±3.6^{c}	7.27±0.5^{b}	3.98±1.4^{e}	0.36±0.2^{d}
0.5	1.78	$1.52x10^{08}$	**1.34±0.41^{a}**	**6.3±2.44^{ab}**	2.80±0.97	3.00±0.72	**0.173±0.02^{a}**	10.83±1.5^{c}	26.73±6.2^{ab}	8.39±1.5^{a}	11.22±9.9^{cd}	1.93±1.^{c}
1	3.99	$2.12x10^{8}$	**1.08±0.23^{ab}**	4.8±1.63^{bc}	1.80±0.96	3.00±0.4	0.16±0.02^{ab}	**12.01±1.5^{a}**	23.91±5.0^{b}	8.5±1.5^{a}	16.71±6.0^{a}	3.04±1.0^{b}
2	8.85	$4x10^{8}$	0.98±0.28^{bc}	3.54±1.73^{c}	2.40±0.76	2.80±0.31	0.163±0.01^{ab}	10.37±1.8^{c}	27.73±5.^{ab}	7.72±1.0^{ab}	9.27±3.8^{d}	1.92±1.7^{c}
5	38.08	$2x10^{9}$	**1.2±0.6^{ab}**	**7.44±3.03^{a}**	2.60±1.21	3.00±1.23	0.154±0.04^{ab}	11.54±1.7^{ab}	**30.16±6.6^{a}**	**8.22±1.5^{a}**	15.14±6.7^{ab}	**4.27±3.3^{a}**
10	76.16	$4x10^{9}$	0.7±0.46^{c}	3.06±2.9^{c}	2.60±1.21	2.60±1.0	0.142±0.02^{ab}	11.28±1.2^{abc}	**28.36±10.1^{a}**	**8.28±0.3^{a}**	13.01±3.3^{bc}	2.96±0.8^{b}

Data represent the mean ± SD from the repetitions. Different letters are significantly different from each other ($P < 0.05$). Legend: NC – negative control (water), PC – positive control (IAA = 3 $\mu g.mL^{-1}$).

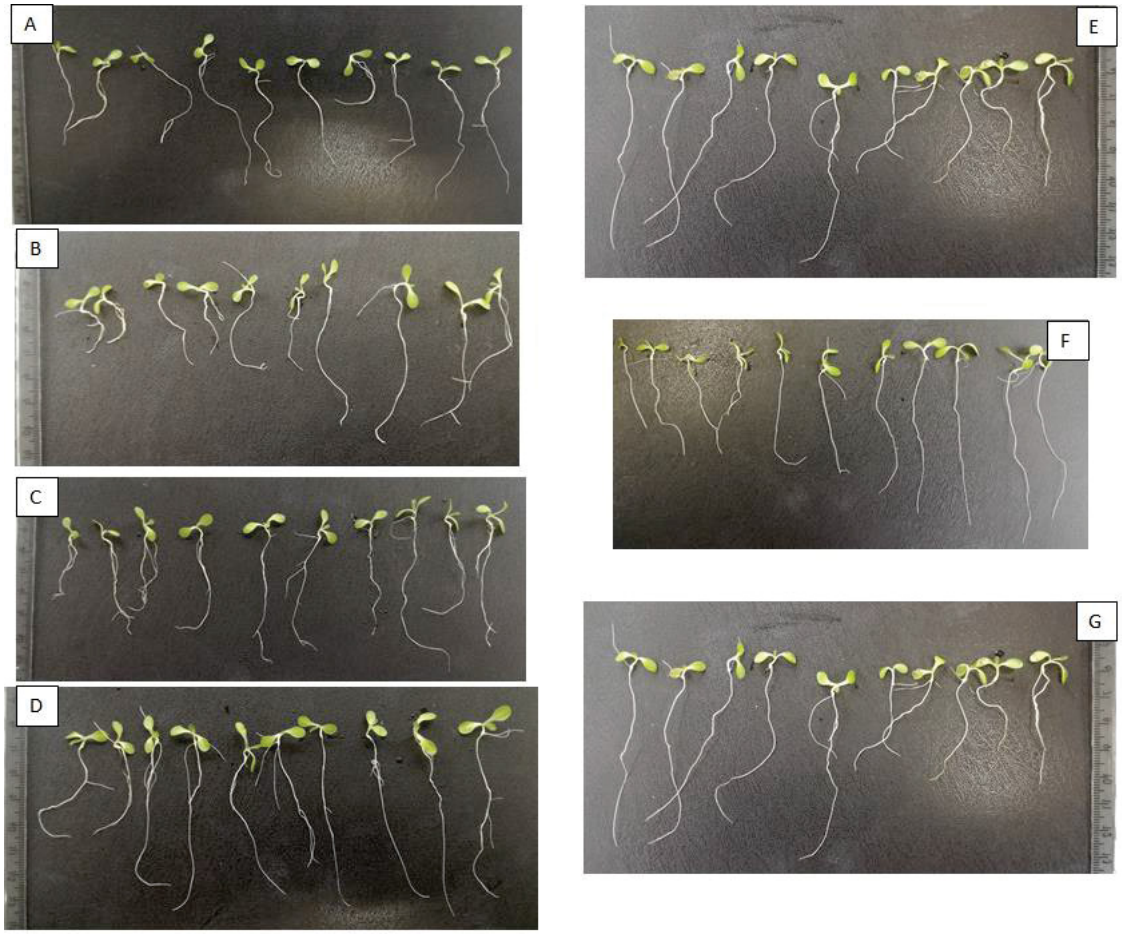

Figure 14. Lettuce seed growth after 14 days. Seeds were inoculated on A) negative control (water), b) positive control (IAA = 3 µg.mL^{-1}), C – G) 0.5, 1, 2, 5 and 10% of Bacterial extracts with IAA.

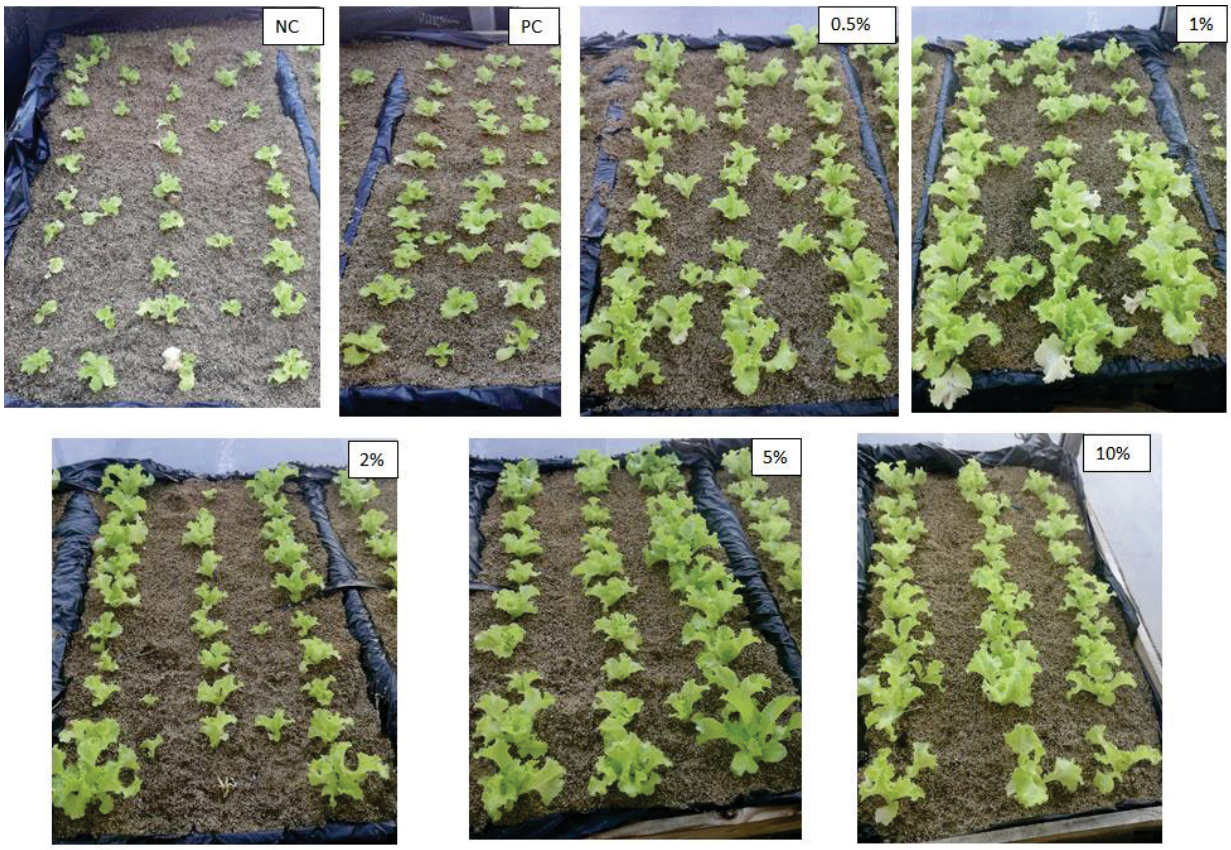

Figure 15. Lettuce planted cultured on greenhouse for 45 days. Seeds were inoculated on A) negative control (water), b) positive control (IAA = 3 µg.mL^{-1}), C – G) 0.5, 1, 2, 5 and 10% of Bacterial extracts with IAA. The seeds were first germinated and planted on a growth room for 14 days, and then transplanted to greenhouse, using vermiculite.

DISCUSSION

Most research on plant growth promotion (PGP) by microorganisms are focused on rhizobacteria, since they exist in the majority rather than to the endophytic or phyllospheric bacterial population (Ahmed and Hasnain, 2014; Ribeiro et al., 2018). However, endophytic form can result in a more close and stable relationship between bacteria and plant and turns more direct and intense their effect on plant growth (Hardoim et al., 2015, 2008; Ribeiro et al., 2018, Yadav and Yadav, 2017) with less competition with other microorganisms (Omer, 2017). Most endophytes with PGP is facultative, changing between the free-living and endophytic stages due to internal and external factors such as the soil, microbial competitors, and plant nutrients and capable to multi-

traits on PGP as phytostimulation through hormone production, biofertilization by nitrogen fixation and P solubilization, and biocontrol by competition or inhibition of phytopathogenic microorganisms (Gaieiro et al., 2013; Ramanuj and Shelat, 2018; Rosenblueth and Martínez-Romero, 2006).

Thereby sustaining their application in the agricultural system as biofertilizer, an environmental sustainable method that affects positively soil quality and agronomic productivity (Buragohain et al., 2018; Mukherjee et al., 2017), and reduces chemical fertilizer inputs which are used nowadays by the farmer´s to increase crop productivity (Qbal et al., 2018), and also reducing production costs and environmentally risks such as nitrate accumulation and leaching (Buragohain et al., 2018).

Many species from the genus *Enterobacter* were isolated from various environments and in association with plant rhizosphere and endophytically (Camila et al., 2018; Carvalho et al., 2014; Haichar et al., 2008; Van der Lelie et al., 2009), exhibiting plant-growth-promoting effects including production of phytohormones (Koga et al., 1991), nitrogen fixation, P solubilization (Mukhtar et al., 2017) and biocontrol activity (Koga et al., 1991; Madhaiyan et al., 2010) by the production of siderophore, 2-phenylethanol and 4-hydroxybenzoate that confers plant protection against bacterial and fungal infections (Srisuk et al., 2018; Taghavi et al., 2010). More specific to phytohormones production, *Enterobacter* species were therefore indicated to have potent IAA-producing bacteria (Nutaratat et al., 2017).

Auxins are naturally occurring substances possessing growth-promoting activity and Indole-3-acetic acid (IAA) is one of the main physiologically active auxins (Mitra et al., 2016). IAA is related to many plant promotion mechanisms (Etesami et al., 2015), especially to root morphology and development, which increases the root surface area promoting more plant nutrient uptake (Spaepen et al., 2007), and to act as signaling molecule and regulation of a group of genes related to many plant processes (Ahmed and Hasnain, 2014). IAA activates the transcription of 1-aminocyclopropane-1-carboxylate-deaminase (ACC), increasing their concentrations and subsequently inhibiting levels of ethylene (Etesami et al., 2015). Phytohormones, as auxins are also related to mitigate abiotic stresses (Omer, 2017). Indeed screening endophytic bacteria with the potential of IAA production appears to be a very effective procedure and less

time consuming to discover and selection of effective PGP bacteria (Etesami et al., 2015). This study focused on to optimize auxin (IAA) production by and endophytic bacteria *Enterobacter* sp. DEBB B-355, scale-up its production, and evaluate as a bioinoculant for plant growth promotion.

Acid pH values are reported to increase some organic acid production (Marra et al., 2015). In this study, the endophytic bacterium produced more auxin on more extreme acid pH (5) followed by basic pH (8). Thus soils chemical properties are in majority more acidity, which can be in accordance to the fact that most media used to produce some organic compounds are more acid (Marra et al., 2015).

The nitrogen and carbon sources that are used in the medium for secondary metabolite productions have a profound effect on the overall efficiency of biosynthesis (Chandra et al., 2018). Plant-associated bacteria can utilize both organic and inorganic nitrogen sources, varying from each isolate and species (Dat et al., 2015). When evaluated the different nitrogen sources, the endophytic bacterium preferred yeast extract and produced the highest auxin, followed by CSL. This result is in accordance with many reports that revealed YE as the best choice for auxin production for an *Enterobacter* sp. DMKU-RP206 isolate (Nutaratat et al., 2017), *Bacillus subtilis* TIB6 (Dat et al., 2015), and *Pseudomonas sp*. (Balaji et al., 2012).

Yeast extract is an organic nitrogen that contains vitamins and growth factors that benefits bacterial growth and some biomolecules biosynthesis, in this case, may contribute to increase tryptophan availability that can be converted to IAA production (Nutaratat et al., 2017). Another organic nitrogen source, meet extract was reported to give higher IAA production for *Pantoea agglomerans* strain PVM (2.104 $mg.ml^{-1}$) (Apine and Jadhav, 2011). Nitrogen inorganic source lowered auxin production for our isolate, this result was in accordance to (Nutaratat et al., 2017), that reported that inorganic nitrogen compounds (mono-sodium glutamate, ammonium sulfate, ammonium chloride, urea, potassium nitrate, and sodium nitrite) did not support IAA production of *Enterobacter* sp. DMKU-RP206. However, some reports showed higher IAA production with some inorganic nitrogen sources as $(NH_4)_2SO_4$ (Bharucha et al., 2013). Even though, we still optimize the C:N ratio with both YE and CSL, and subsequently, CSL showed to improve auxin production when the C:N ratio was optimized.

Carbon sources provide energy and improves co-factor recycling in the cells, thus contribute to the global efficiency of secondary metabolites biosynthesis (Kumari et al., 2018). In this study, presence of glycerol as C-source in the medium had a profound effect on the overall efficiency for auxin biosynthesis (57.56±3.04 $\mu g.mL^{-1}$) followed by sucrose (19.23±2.77 $\mu g.mL^{-1}$) and glucose (18.23 $\mu g.ml^{-1}$). Glycerol was mentioned before to be used as a carbon source by a *E. cloacae,* including D-xylose, D-maltose, and esculin melibiose (Khalifa and Saleh, 2016). Differently, glucose and sucrose showed to be suitable to auxin production for *Bacillus subtilis* TIB6 isolate (Dat et al., 2015) and sucrose showed to suitable perform better for IAA production for *Enterobacter* sp. A3CK and *E. cloacae* A7CK isolates (Ghosh et al., 2015). Notwithstanding, monosaccharides were pointed out to improve more IAA production by rhizosphere bacteria than polysaccharides (Chandra et al., 2018). Recently Nutaratat et al., (2017) reported a 13.4-fold improvement in IAA production by *Enterobacter* sp. DMKU-RP206 optimizing a medium with 0.85% of lactose as a carbon source and 1.3% of yeast extract as a nitrogen source, in similar pH conditions (5.8), temperature (30°C) and using higher shaking speed (200 rpm).

Once we have optimized the pH condition, nitrogen and carbon sources we followed to optimize the medium using central composite design to find the best carbon and nitrogen ratio and interactions of days, inoculum size, and tryptophan concentration. The results using response surface methodology (RSM) had shown that lower CSL concentration was better for auxin production, and contrary, at higher concentrations, it may influence bacteria growth instead of auxin production. In response, glycerol was needed at higher concentrations to maintain a quite high C:N ratio (around 10.61). We could not find an optimal region using YE as a nitrogen source for this experiment, may be due to the concentration range used. It turned necessary to perform another experiment with different carbon and nitrogen concentrations and ratio to optimize the medium using YE.

Therefore, we decided to continue the evaluations using only CSL, as it is an agricultural waste and will save production costs. And hitherto, most IAA production reports with bacteria have used synthetic medium and so far, there is no report of an optimized medium for IAA production using CSL. Only a few reports have shown the use of alternative low-cost substrates for bacterial growth and IAA production (Peng et al.,

2014; Srisuk et al., 2018; Sudha et al., 2012; Swain and Ray, 2008). Recently, Srisuk et al. (2018) succeeded in using sweet whey as a feedstock instead of lactose for *Enterobacter* sp. DMKU-RP206, and produced 3,963.0 $\mu g.mL^{-1}$ of IAA, which decreased the need for NaCl addition into the fermentation medium. Corn flour and soybean meal were used as a carbon and nitrogen source to study IAA production by *Pseudomonas putida* Rs-198 (Peng et al., 2014). Other agro-industrial residues such as cassava fibrous and chickpea have also been used to produce and optimize the IAA production (Sudha et al., 2012; Swain and Ray, 2008).

Tryptophan and inoculum percentage had significative interaction on auxin production (p-value < 0.05). Higher auxin biosynthesis was after 4 days of culture, using 1000 $\mu g.mL^{-1}$ of Trp and 4 of inoculum. The RSM showed that the optimal Trp concentrations were about 1248.45 $\mu m.mL^{-1}$ and 4.45 % inoculum. Tryptophan is the main precursor for the IAA biosynthesis in plants and bacteria (Patten and Glick, 1996), and the mechanism to convert Trp to IAA varies according to each species or strain of the microorganisms (Hasuty et al., 2018). Koga et al. (1991) first reported the detection of the indole-3-pyruvate (*IPyA*) and indole-3-acetaldehyde (*IAAld*) biosynthetic pathway via Trp for IAA production in *E. cloacae*. And they also pointed out that IAA biosynthesis increases under more aerobic conditions.

Many reports suggested and confirmed that higher Trp concentrations stimulated IAA production (Apine and Jadhav, 2011; Balaji et al., 2012; Bharucha et al., 2013; Dat et al., 2015). Similar to our results, exactly 1000 $\mu g.mL^{-1}$ of Trp gave the maximum IAA production 158 % (1.577 $mg.ml^{-1}$) in *P. agglomerans* strain PVM (Apine and Jadhav, 2011). They observed that 78 % Trp was converted to IAA biosynthesis. We used a 16 h old inoculum (stills at exponential phase) to perform the experiments, and 4 % gave the highest auxin productivity. The optimal inoculum size for IAA productivity varies from 1.65 % from *B. subtilis* TIB6 (Dat et al., 2015) to 3.5 % of *Enterobacter sp.* DMKU-RP206 (Nutaratat et al., 2017).

As all secondary metabolites, IAA production behavior is attainment stationary growth phase (Ali et al., 2017). We studied the effect of incubation time under the optimized cultural conditions and the results showed that the production arises after the 2^{nd} day until it reaches the maximum at 4^{th} day (96h) of incubation, and started do decline,

and kept nearly constant between 120 and 144h. However, the auxin peak production varies among each isolate growth rate. Evaluating IAA production over different rhizobacteria from sugarcane soil samples, Inui-Kishi et al., (2012) observed that each isolate showed to produce maximum auxin at an OD around 3.70, although at different times: after 48h *Enterobacter* sp. (FJ890899) accumulated 136.91 $\mu g.mL^{-1}$, 60 h the isolate *E. homaechei* subsp*. verschuerenii* (FJ890898) produced 152.63 $\mu g.mL^{-1}$, 72h *Burkholderia sp.* (FJ890895) showed 58.34 $\mu g.mL^{-1}$ IAA and after 96h *Labrys portucalense* (FJ890891) produced 17.63 $\mu g.mL^{-1}$ of IAA. Similar to our results, after reaching the maximum production of 382.23 $\mu g.mL^{-1}$ at 72 h, IAA produced by *E. cloacae* SN19 declined in the growth medium during the late stationary phase of growth at 84 h and remained constant up to 96 h (Bose et al., 2016). In the meantime, Mirza et al. (2001) reported a significant amount of IAA produced by *Enterobacter* strain SR12 during later growth stages, after 14 days of growth (2211 $\mu g.L^{-1}$). The explanation behind high IAA production on stationary phase may be due to the increase of expression of key genes for IAA biosynthesis in the presence of Trp with the cell density that reaches its maximum at the stationary phase growth (Nutaratat et al., 2017), as indole-3-pyruvate decarboxylase gene (Vande Broek et al., 1999) and tryptophan side chain oxidase gene (Oberhänsli et al., 1991).

Generally, L-tryptophan had the most significant effect in regard to auxin production, followed by inoculum size and culture days (Fig. 3). Validation of the experimental model was confirmed when the influencing factors were kept at 1250.0 $\mu g.mL^{-1}$ of Trp, 4% of inoculum, 4 days, 5.0±0.2 of initial pH, 5.11 $g.L^{-1}$ of CLS, 28.21 $g.L^{-1}$ of glycerol, 30 °C and 120 rpm. Under these conditions, the observed and predicted values of auxin production were 770.49 and 803.11 $\mu g.mL^{-1}$, respectively, with 4.06 % of relative error between the observed and predicted values. We did get the same auxin production using a 2L STR bioreactor (750.05 $\mu g.mL^{-1}$), and an increase of 18.14% culturing in 10 L bioreactor, producing up to 910.27 $\mu g.mL^{-1}$ of IAA. This result showed that our productivity increased when scaled-up to a volume 120 times higher. However, we still have to evaluate and optimize the rotation and aeration conditions, as it seems that aerobic conditions have a significant influence on IAA biosynthesis (Koga et al., 1991).

According to the results of this study the *Enterobacter* sp. DEBB B-355 isolate showed higher IAA biosynthesis when compared to some previous reports with *Enterobacter* isolates, such as *E. ludwigii* BNM 0357 (~ 30 μg.mL^{-1}) (Shoebitz et al., 2009), *E. asburiae* PS2 (32.0 μg.mL^{-1}) (Ahemad and Khan, 2010), *E. arachidis* Ah-143 (5.6 μg.mL^{-1}) (Madhaiyan et al., 2010), *E. homaechei* subsp. *verschuerenii* FJ890898 (152.63 μg.mL^{-1}) and *Enterobacter* sp. FJ890899 (136.91 μg.mL^{-1}) (Inui-Kishi et al., 2012), *E. cloacae* PnB 9 (282.4 μg.mL^{-1}), *Enterobacter* sp. PnB 10 (273.6 μg.mL^{-1}) (Jasim et al., 2014), *Enterobacter* sp. I-3 (~ 200 μg.mL^{-1}) (Park et al., 2015), *E. cloacae* SN19 (382.23 μg.mL^{-1}) (Bose et al., 2016), *Enterobacter cloacae* MSR1 (112.0 μg.mL^{-1}) (Khalifa and Saleh, 2016), *E. hormaechei* A-2 (131.4 μg.mL^{-1}) and *E. cloacae* subsp. *dissolvens* AnA-10 (119.8 μg.mL^{-1}) (Arraktham et al., 2016). In the meantime, *Enterobacter sp*. DMKU-RP206 showed higher IAA production in a fermenter (5561.7 μg.mL^{-1}) (Nutaratat et al., 2017), using YE and lactose, and 3963.0 μg.mL^{-1} using sweet whey as the nitrogen source, instead of lactose after 7 days of inoculation (Srisuk et al., 2018).

The produced auxin was identified as IAA by HPLC and FTIR. The bacteria supernatant sample showed a peak at 6.4 min, the same retention time as the standard IAA on HPLC. The same pattern was observed on FTIR analysis, for both samples of standard IAA and the isolate fermented culture. It showed to have the same molecular components.

In addition, it was evaluated the plant growth promotion on lettuce (*Lactuca sativa*), and the bacteria inoculation with the auxin solution showed to positively improve plant growth, especially on plant height, root growth, number of plant leaves, fresh and dry weight. Hitherto, statistical analysis had shown few differences among the bacterial extract concentrations on lettuce seed germination and growth, and no dose dependence was observed. It seems that lower bacterial extract (also lower auxin concentration) was appropriate for the first days of plant growth, and then a concentration 10 times higher (5%) showed to be appropriate for the bacterial inoculation on lettuce plantlets. Hence, the auxin concentration among the inoculation determines and regulates the plant developmental patterns (Ahmed and Hasnain, 2014). Ultrahigh quantities of IAA can lead to a disruption of the plant's hormonal status, which is one of the mechanisms of some phytopathogens (Pirog et al., 2018). And, a higher concentration can promote growth

inhibitory effects on plant roots, a consequence of increased ethylene synthesis stimulated by auxin levels via enhanced biosynthesis of ACC, immediate precursor of ethylene, and high concentration of ethylene, after germination, may lead to inhibition of root growth (Ahmed and Hasnain, 2014; Ali et al., 2017; Patten and Glick, 1996).

When transplanted from the in vitro growth room to the greenhouse, the lettuce plantlets from negative and positive control did not developed, as well as those on the treatments with the bacterial broth. After 45 days, we observed a relative interaction between the bacterial growth concentration and the plant growth development, especially on plant height, fresh and dry weight, although with no statistical difference. Meanwhile, these findings of the endophytic DEBB B-355 isolate are in general agreement with previous studies on rhizobacteria and especially *Enterobacter* isolates, that substantially enhanced the plant growth. Inoculation of *Enterobacter cloacae* MSR1 significantly improved the length of the primary, the number of secondary roots, and root dry weight on *P. sativum,* also belong to Fabaceae family. The isolate increased roots length when compared to the control (Khalifa and Saleh, 2016). On another study, five *Enterobacter cloacae* strains significantly increased the plant height by 26.71% to 30.62%, root length by 47.95% to 52.39%, and the root number by 38.25% to 47.82%, when compared to control, and consequently, rised the macronutrients content as of N by 9.20% to 12.93%, P by 15.09% to 17.30%, K by 20.63% to 23.32%, when compared to the control. All these effects caused an increase in chlorophyll content in the leaf of plants at 30, 45, and 60 days after planting (Suprapta et al., 2014).

Indeed, the root system is more sensitive to IAA supplementation and levels (Hussein et al., 2016), and by increasing overall root development, allows better uptake of water and minerals for the plant (Gilbert et al., 2018; Spaepen and Vanderleyden, 2011). Yet, bacteria cells and the auxin in the culture, stimulated seed germination. Similarly, Srisuk et al., (2018) reported that jasmine rice supplemented with IAA produced by *Enterobacter* sp. DMKU-RP206 had significantly increased length and dry weight of shoot compared with the negative control (without IAA), after 2 weeks plantation, indicating that the IAA in culture filtrates of DMKU-RP206 could be used instead of the synthetic IAA.

Therefore, the responsiveness to plant growth promotion by bacteria or auxin is highly dependent on the particular auxin applied and of the plant genotype (Dodd et al., 2010). In conclusion, promoting plant growth of the isolate DEBB B-355 can be used as a bioinoculant to improve crop productivity by direct and/or indirect plant promotion traits. The isolate is capable to produce IAA, and we could optimize the medium for IAA production, using agricultural low-cost byproduct as carbon and nitrogen sources, glycerol and corn steep liquor, and improved auxin production up to 910.27 $\mu g.mL^{-1}$ in 10 L bioreactor.

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SUPPLEMENTARY DATA

Table 6. Analysis of variance (ANOVA) of Central composition design for IAA production, evaluating the interaction of Yeast extract and corn steep liquor concentrations, as nitrogen source, and carbon:nitrogen ratio, using glycerol as carbon source, by *Enterobacter sp*. DEBB B-355.

	Yeast extract				Corn steep liquor			
Factors	Effect	Std.Err.	*P*	-95.%	Effect	Std.Err.	P	-95.%
Mean/Interc.	34.3551	9.36262	0.002072	14.5073	138.7591	8.84141	0.000000	120.0162
(1)CSL (L)	-27.4916	11.48405	0.029273	-51.8367	23.3909	10.84474	0.046570	0.4011
CSL (Q)	9.5267	13.70346	0.496900	-19.5233	-44.4902	12.94059	0.003378	-71.9231
(2)CN (L)	-25.7447	11.48405	0.039502	-50.0898	50.5031	10.84474	0.000263	27.5133
CN (Q)	24.0329	13.70346	0.098599	-5.0171	-42.1213	12.94059	0.004969	-69.5541
1L by 2L	-1.2983	16.21672	0.937181	-35.6763	-27.7315	15.31394	0.088978	-60.1956
Mean/Interc.	34.3551	9.36262	0.002072	14.5073	138.7591	8.84141	0.000000	120.0162
(1)CSL (L)	-27.4916	11.48405	0.029273	-51.8367	23.3909	10.84474	0.046570	0.4011

Yeast extract - R^2= 0.44; Corn steep liquor - R^2= 0.74

Table 7. Analysis of variance (ANOVA) of Central composition design for IAA production, evaluating the interaction of days of fermentation, inoculum percentage and tryptophan concentration, by *Enterobacter sp*. DEBB B-355.

Factors	Effect	Std.Err.	t(7)	*P*	-95.%
Mean/Interc.	782.188	95.1141	8.22368	0.000076	557.279
(1)Days (L)	-160.304	83.3763	-1.92265	0.095954	-357.458
Days (Q)	-170.711	73.0452	-2.33706	0.052070	-343.435
(2)Inoculum(L)	91.510	96.0735	0.95250	0.372566	-135.668
Inoculum(Q)	-287.094	116.9485	-2.45488	0.043793	-563.634
(3)Trp (L)	223.879	90.2857	2.47968	0.042230	10.387
Trp (Q)	-280.370	99.8480	-2.80797	0.026222	-516.473
1L by 2L	-99.862	117.9119	-0.84692	0.425045	-378.679
1L by 3L	-99.171	117.9119	-0.84106	0.428105	-377.989
2L by 3L	-55.663	117.9119	-0.47207	0.651237	-334.480

R^2= 0.77

PART IV - AUXIN PRODUCTION BY ENDOPHYTES *Bacillus megaterium* DEBB B-353 AND *Paenibacillus polymyxa* DEBB B-358 BACTERIA USING AGROINDUSTRY SUB-PRODUCTS AS LOW-COST MEDIA AND PLANT BIO-STIMULATION PROPERTIES

ABSTRACT

Auxin is one of the major phytohormone class, and can be also produced by microorganisms. Factors affecting IAA production were evaluated for two endophytes *B. megaterium* DEBB B-353 and *P. polymyxa* DEBB B-358. Among the evaluated parameters we were able to select the pH at 5±0.2, 9.09 and 8.27 $g.L^{-1}$ of corn steep liquor (CSL) as the nitrogen source, 48.23 and 46.45 $g.L^{-1}$ of glycerol as carbon source, respectivelly. And choose the best range of tryptophan concentration (1030 and 1100 $\mu g.mL^{-1}$, inoculum percentage (4%) and days of fermentation (3-4). The optimized medium and process produced 672.15 $\mu g.mL^{-1}$ of IAA in flasks and 750.61 $\mu g.mL^{-1}$ and 638.45 $\mu g.mL^{-1}$ in 2L and 10L bioreactors using *B. megaterium* DEBB B-353, and 725.01 $\mu g.mL^{-1}$ in flasks and 567.18 $\mu g.mL^{-1}$ and 812.49 $\mu g.mL^{-1}$ using bioreactor with *P. polymyxa* DEBB B-358. The peak IAA production on 10 L bioreactor was recorded after 32h of fermentation. IAA production was confirmed by HPLC and FTIR. Both bacteria culture and its produced auxin showed to promote soy seed germination, plant and root development. This is the highest auxin production amount reported for this species at the moment, using a low-cost medium.

Key words: IAA, plant growth promoting bacteria (PGPB), bioinoculants, biofertilizer

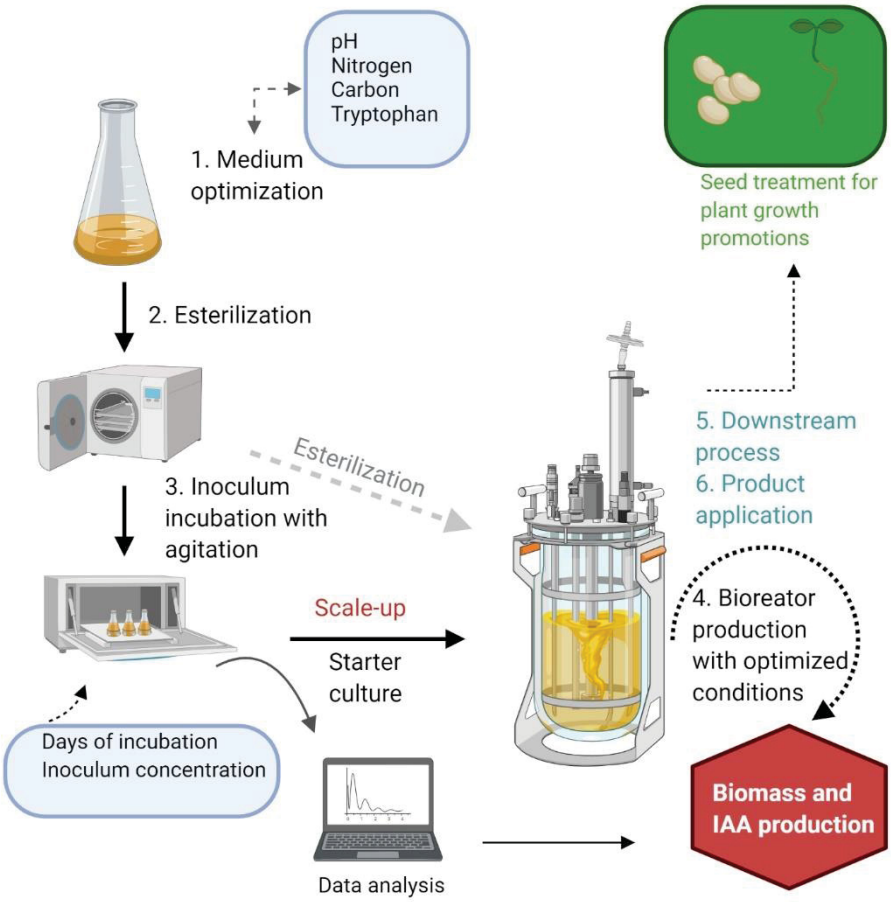

Figure 16. Graphical abstract for medium and culture conditions optimization for IAA production by endophytes bacteria. By the author.

INTRODUCTION

Agriculture has been since the beginning of the most important activity for humanity evolution. And today, we have superior crop yields resulting from genetic breeding, new cultivations methods, biotechnology and the use of agrochemical fertilizers. However, the indiscriminate use of chemical fertilizers is leading to environmental deterioration, resulting in loss of soil quality and fertility, desertification, nitrate pollution, and eutrophication (Buragohain et al., 2018; Erdelyi et al., 2014).

In the last two decades, a new approach has been used in order to overcome these problems using biological solutions and attend the worldwide food demand (Buragohain et al., 2018; Pérez-Montaño et al., 2014). Among these methods, microorganisms are

being studied as plant growth promoters (Afzal et al., 2019; Nehra and Choudhary, 2016). Microorganisms play a vital role in agriculture by promoting plant development and defense. Their potential to produce biologically active metabolites and substances are well known, hence by engineering/manipulating the Phyto-microflora, they can surge as a valuable alternative as biofertilizers to enhance crop productivity and reduce the application of chemical fertilizers (Dutta et al., 2014; Farrar et al., 2014; Khan et al., 2016b; Swethaa and Padmavath, 2016).

From the group of plant growth-promoting bacteria (PGPB), we can evince those inhabiting in the rhizosphere region of soil and those which can establish an intra or extracellular colonization, as endophytes and epiphytes, maintaining a neutral or positive relationship with the host plant, exhibiting a reciprocal interaction (Kawaguchi and Minamisawa, 2010; Saikkonen et al., 2004; Xia et al., 2015). PGPB are capable to afford nitrogen fixation and phosphate-solubilizing, which lowers the need for inorganic N fertilizers and increases available forms of phosphate for plant uptake (Buragohain et al., 2018). They also act directly in plant life vital processes by phytohormones production, siderophores release for iron sequestration, by producing various types of extracellular enzymes such as 1-aminocyclopropane-1-carboxylate (ACC), which is related to ethylene levels lowering (Gaieiro et al., 2013; McSpadden Gardener, 2004), phosphatase, zylanase, cellulases, etc (Khan et al., 2016a; Schulz and Boyle, 2005). Even so, they can indirectly act suppressing disease-causing microbes by synthesis of substances with biocontrol properties of competition for sites on plant tissues, reduction of iron (Lucy et al., 2004; Pérez-Montaño et al., 2014; Swethaa and Padmavath, 2016), by ensuring their microorganisms symbionts lifestyle and so extending greater benefits to the host plant during normal and stressful environmental conditions (Khan et al., 2016a).

PGPB with one or more abilities mentioned above can act as a biofertilizer or biocontrol agent. Yet, screening for phytohormone production such as auxin, cytokinin, and gibberellin, which are one of the most important phytohormones (Davies, 2004) can be a useful start point to find a competent PGPB (Etesami et al., 2015). Auxin was the first-identified phytohormone (Davies, 2004; Normanly et al., 1995), produced mainly by plants but it is also well known to stand as one of the biologically active metabolites produced widespread among various classes of PGPB. Auxins plays a crucial role in plant

growth and development, regulating a range of cellular and physiological process (Spaepen et al., 2007; Spaepen and Vanderleyden, 2011), such as cell division, elongation and differentiation to tropic responses, tissue differentiation, fruit development, and senescence, lateral root initiation, apical dominance, embryo development, leaf abscission, parthenocarpy and stimulation of the of new leaves at the apical meristem (Ahmed and Hasnain, 2014; Chapman et al., 2012; Giehl et al., 2012; Tanimoto, 2005), responses to light and gravity (Swethaa and Padmavath, 2016). Yet, indole-3-acetic acid, the naturally occurring auxin has also demonstrated to be a anti-bacterial diffusible component, acting to suppress phytopathogen attack (Wang et al., 2016).

For this reason, the present study aimed to use two endophytic bacteria from *in vitro Eucalyptus urophylla* and *Illex paraguariensis* capable to produce auxin and optimize their potential for auxin production and their plant growth-promoting ability as biofertilizers.

MATERIALS AND METHODS

Isolation and identification of endophytic bacteria

The endophytes bacteria used in this study were isolated from aseptic plantlets of *Eucalyptus urograndis* and *Illex paraguariensis*, which were maintained in vitro conditions at Tissue and culture laboratory of Bioprocess Engineering and Biotechnology Department – UFPR (Curitiba-Brazil), identified as *Paenibacillus polymyxa* DEBB B-358 and *Bacillus megaterium* DEBB B-353, respectively.

Evaluation of pH, nitrogen and carbon sources for auxin production

The endophytes had shown earlier the ability to produce IAA qualitatively. In this study, we first evaluated the medium pH, in a range from 5-8. For inoculum preparation, an aliquot of 100 μL of the isolate maintained in glycerol at -80°C was incubated at 30°C, in a shaker with 120 rpm for 16 h in LB medium. Then 1% of the inoculum was transferred into 250-mL Erlenmeyer flask with 50 mL of LB medium supplemented with 100 $\mu g.mL^{-1}$ of Tryptophan (Trp) and cultured for 3 days, at 30ºC and 120 rpm. In the second experiment we evaluated the effect of nitrogen sources: corn steep liquor (CSL), peptone, urea, yeast extract (YE) and ammonium sulphate (AS), all at 5 $g.L^{-1}$. A basal medium

described by Blinkov et al. (2014) with some modifications was used with (gL^{-1}): 0.5 K_2HPO_4; 0.3 KH_2PO_4; 0.1 $MgSO_4 \cdot 7H_2O$; 0.75 NaCl; 0.03 $CaCl_2 \cdot 6H_2O$; 15 glucose, 1 mL of trace elements solution (in gL^{-1}: 0.02 $FeCl_3$, 0.083 KI, 0.124 $Na_2MoO_4.2H_20$, 0.03 H_3Bo_3, 0.287 $ZnSO_4.7H_2O$, 0.223 $MnSO_4.4H_2O$, 0.249 $CuSO_4.5H_2O$). LB medium was used as control. After defining the nitrogen source, the next step was to evaluate the effect of carbon source: glucose, sucrose and glycerol (all at 15 $g.L^{-1}$). The medium consisted of the basal media described above using 5 $g.L^{-1}$ of the nitrogen source (CSL). For the experiments that evaluated nitrogen and carbon sources 300 $\mu g.mL^{-1}$ of Trp was used and 1% of fresh inoculum culture (16h), pH of 5±0.2, 30°C and 120 rpm and cultured for three days. After three days the culture cells were centrifuged at 958 g for 15 min and the supernatant was collected to evaluate quantitatively the auxin production by colorimetric method using Salkowski's reagent (Glickmann and Dessauxm, 1995): one milliliter of cell-free supernatant was mixed with 2 mL of (a mixture of 50 mL 35% perchloric acid and 1 mL 0.5M $FeCl_3$). The solution was allowed to stand for 30 min on the temperature room in dark and read in a spectrophotometer (UV Spectrophotometer 1800, Shimadzu, Japan) in 530 nm. A standard curve was developed using a standard solution of pure indole-3-acetic acid (IAA) (0 - 100 $\mu g.mL^{-1}$, R^2= 0.98) for IAA evaluation.

Optimization of auxin production using response surface methodology (RSM)

Two experimental designs were performed according to the Central Composite Design (CCD) and analyzed by RSM using STATISTICA 8.0 (USA) software. First, we analyzed two factors interactions for nitrogen source concentration (CSL) and carbon:nitrogen ratio (using glycerol) (Table 8). For this experiment, 500 $\mu g.mL^{-1}$ of Trp, 1% of inoculum culture, pH of 5±0.1, 30°C and 120 rpm were used, and the auxin and culture growth (OD) values were evaluated after three days of fermentation. With the best C:N ratio, we proceeded to the second experiment with three factor design to analyze the interaction of incubation days, inoculum percentage, and tryptophan concentration (Table 9). The conditions of pH, temperature and rotation were maintained as mentioned above. The relationship between the coded values and actual values, independent variable and the response were calculated according to a second-order quadratic model. The relative

effects of the variables on response were examined from three-dimensional contour plots, to represent the effect of the independent variables on auxin production and bacteria growth (OD). These response curves were then used to predict the optimum level of the factors. The culture growth assessment was carried out by the optical density (OD) after the fermentation time and read at 600 nm.

Kinetics analysis and Scale-up to auxin production

To confirm the RSM model with the optimized conditions for auxin production, both endophytes were cultured using the optimized medium and culture parameters on 250 mL Erlenmeyer and scale-up was performed using 2 L STR bioreactor (BIOSTAT B, B. Braun Biotech International, Germany) and 10 L bioreactor (New Brunswick's Bioflo 110 Fermenter/Bioreactor, GMI, USA). The 2 L STR fermenter was operated with 1.5 L working volume, with agitation speed of 400 rpm, aeration at 1 vvm (3 $L.min^{-1}$). For 10 L bioreactor, the working volume was 6 L, agitation speed varied from 750 to 400 rpm over the fermentation, aeration of 1 vvm, pH and oxygen rate were controlled during the fermentation. Samples were taken daily and analyzed for IAA production and bacterial biomass weight.

Extraction and separation and HPLC and FTIR identification

The bacteria culture supernatant was adjusted to pH 2.8 using 1N HCl and was extracted with an equal volume of ethyl acetate (EtOAc). After vigorous shaking, it was allowed to stand for 10 min, and the supernatant was recovered. The extraction procedure was repeated 3 times. The EtOAc was then evaporated on a rotary evaporator (Vacucell, MMM-Group) under vacuum at 35^{o}C. The indole compounds were dissolved in 50% methanol (MeOH) and kept at 4^{o}C for further analysis (Sudhapriyadharsini et al., 2016). For HPLC analysis, indole compounds were analyzed in a PrepStar 218 Preparative HPLC System (Varian Inc., Palo Alto, CA, USA) equipped with a 250×21.4 mm Microsorb 60-8 C18 column (Varian). The column was eluted with 1% acetic acid as a mobile phase at a flow rate of 0.5 $mL.min^{-1}$. The indole compounds were detected at 254-280 nm (Lim and Kim, 2009).

The infrared spectrum of the purified compound was obtained using Fourier Transform Infrared (FTIR) spectrophotometer (Shimadzu FTIR 8400) and the spectra were recorded in the nujol mull using KBr cells and expressed in wavenumber (cm^{-1}).

In vivo plant growth promotion of the strains and the produced auxin on soy seed germination

The endophytic bacteria used in this study had shown plant growth promotion over common bean seeds previously (data not shown). Here we evaluated their effect on soy seed germination and growth, using the individual strains or as consortia, and as an additive for a commercial organic fertilizer TITAN ADVANCEFS recommended for soy culture (http://www.baic.com.br/baic_produto/soja/). Seeds were treated for 30 seconds with the solutions of each treatment: 1) negative control with water; 2) 10% *Paenibacillus polymyxa* DEBB B-358 (PP); 3) 10% *B. megaterium* DEBB B-353 (BM); 4) bacteria consortia with 5% *Paenibacillus polymyxa* DEBB B-358 and 5% *B. megaterium* DEBB B-353; 5) 10% TITAN ADVANCEFS; 6); 5% TITAN + 5% *Paenibacillus polymyxa* DEBB B-358; 7) 5% TITAN + 5% *B. megaterium* DEBB B-353; 8) 5% TITAN + 5% *Paenibacillus polymyxa* DEBB B-358 + 5% *B. megaterium* DEBB B-353. Ten seeds per treatment were used, and planted on pots containing vermiculite in green-house. For bacterial treatment, a 4-day old fermented culture were used, which had approximately $1x10^{10}$ UFC.mL^{-1} and around 600-700 μg.mL^{-1} of produced auxin. The growth promotion activity was evaluated after 12 weeks by calculating the germination percentage, plant height, number of leaves, root size, fresh and dry weight, and chlorophyll a and b, and total content. The chlorophyll were estimated according to the method of Lichtenthaler (Khan et al., 2016b). Leaves were used to extract the pigments with 80% acetone (v/v) and the absorbance at 663 and 645 nm for chlorophylls a and b, respectively was read. Chlorophyll content was calculated using the following formulae:

$$\text{Chlorophyll a (mg/g FW)} = [\{(12.7 \times A_{663}) - (2.69 \times A_{663})\} / 1000 \times W] \times V$$

$$\text{Chlorophyll b (mg/g FW)} = [\{(22.9 \times A_{645}) - (4.68 \times A_{645})\} / 1000 \times W] \times V$$

Where W is the fresh weight and V is the extraction volume.

Statistical analysis

All five experiments mentioned above evaluating auxin production were conducted using 250 mL Erlenmeyer's with 50 mL of medium in triplicate, and repeated twice. Statistical analysis was performed by using the statistical software STATISTICA®, version 8.0. The significance of each treatment was established by one-way ANOVA and means were separated by Fisher's test ($p \leq 0.05$). The data obtained from RSM on IAA production was subjected to analysis of variance (ANOVA) and the regression model was determined for its adequacy using the coefficient of determination (R2). The model and values obtained were validated in triplicate.

Cost of medium components for IAA production

A bioproduct production is composed of three steps: upstream, fermentation, and downstream, as demonstrated in figure 17. As we developed and optimized the medium composition for IAA production, we decided to estimate the total cost of the medium components and concentrations to produce 1 L of bioinoculant products. Medium components prices were used by available costs from two suppliers of chemistry material for laboratory and industry in Brazil (https://www.lojaquimica.com.br/ and https://www.lojasynth.com/) (observed in April 2020, using Real and the USD quotation of 5.327 from 4/3/2020, https://br.advfn.com/moeda/dolar/2019).

RESULTS

The evaluation of pH range for the two endophytes showed that acidic medium was more appropriate for auxin biosynthesis, producing 17.33 ± 0.58 $\mu m.mL^{-1}$ of auxin by *P. polymyxa* DEBB B-358 at pH=5, differing from values of pH = 7 and 8, and 12.86±0.02 $\mu m.mL^{-1}$ in pH = 5 for *B. megaterium* DEBB B-353, which was similar to alkaline pH= 8 (11.78±1.32 $\mu m.mL^{-1}$). With these results, pH = 5±0.2 was adopted for both bacteria for the next variable evaluation (Table 8).

Ammonium sulphate decreased the auxin production among the nitrogen sources. Therefore, CSL stood out as the best choice. Auxin production reached up to 77.68±2.47 $\mu m.mL^{-1}$ for *P. polymyxa* DEBB B-358 differing from the other nitrogen sources, and 69.76±2.46 $\mu m.mL^{-1}$ for *B. megaterium* DEBB B-353 which did not differ from YE result

(68.46±1.22 μm.mL^{-1}). Thus, CSL was chosen as the nitrogen source for both endophytes.

Glycerol increased auxin production for both bacteria. *Paenibacillus polymyxa* DEBB B-358 produced 72.11±3.76 μm.mL^{-1} differing considerably from glucose and sucrose concentrations (Table 8). Moreover, *B. megaterium* DEBB B-353 auxin production by glycerol was higher (72.81±4.19 μm.mL^{-1}) but similar to glucose (69.60±3.82 μm.mL^{-1}). Based on the former results we could select these three variables to optimize auxin production by both bacteria, the pH value of 5±0.2 and, glycerol and CSL as carbon and nitrogen sources, respectively.

The experiment with CSL concentration and carbon: nitrogen ratio using glycerol, showed significant interactions ($p \leq 0.05$) between them for auxin production (Fig. 18), for both endophytes. For both, auxin concentration increased at central levels with CSL concentration around 8 g.L^{-1} and carbon:nitrogen ratio over 12, reaching 274.98 μg.mL^{-1} for *B. megaterium* DEBB B-353 and 320.99 μg.mL^{-1} for *P. polymyxa* DEBB B-358. And, even if there was no significant effect observed on bacteria optical density (OD) (data not shown), we could observe that bacteria growth (OD) were higher when CSL was used at maximum concentrations, suggesting a contrary sense of balance for bacteria biomass and auxin production in respect of nitrogen source (Table 9). Accordingly to RSM we could determine the optimal levels of the factors: CSL concentration at 9.09 and 8.27 g.L^{-1}, and C:N at 10.27 and 10.79 for *B. megaterium* DEBB B-353 and *P. polymyxa DEBB B-358*, respectively. These C:N values correspond to 48.23 and 46.45 g.L^{-1} of glycerol for the endophytes, in the respective order. These C and N concentrations were used to perform the next CCD to evaluate the interaction of days of incubation, inoculum percentage and tryptophan concentration for auxin production.

Table 8. Auxin production by *Bacillus megaterium* DEBB B-353 and *Paenibacillus polymyxa* DEBB B-358 over different ranges of pH (5, 6, 7 and 8), nitrogen sources (LB medium, CSL, urea, peptone, ammonium sulphate and yeast extract), and carbon sources (glucose, sucrose and glycerol).

			pH			
Strain		5	6	7	8	
DEBB B-353		12.86±1.32^{ab}	9.49±0.30^{b}	10.87±0.96^{b}	11.78±0.02^{a}	
DEBB B-358		17.33±0.58^{a}	13.46±1.24^{ab}	11.17±0.11^{b}	10.90.2±1.60^{b}	
			NITROGEN SOURCES			
	LB	Corn Steel	Urea	Peptone	Ammonium Sulphate	Yeast Extract
DEBB B-353	15.35±1.65^{c}	69.76±2.46^{a}	38.51±4.83^{b}	44.16±1.14^{b}	6.78±1.89^{c}	68.46±1.22^{a}
DEBB B-358	13.92±1.93^{de}	77.68±2.47^{a}	16.62±7.69^{d}	27.69±9.15^{c}	6.64±1.47^{e}	45.60±4.42^{b}
			CARBON SOURCES			
		Glucose	Sucrose	Glycerol		
DEBB B-353		69.60±3.82^{a}	30.29±3.82^{b}	72.81±4.19^{a}		
DEBB B-358		13.58±3.43^{b}	21.95±3.43^{b}	72.11±3.76^{a}		

Legend: *Bacillus megaterium* DEBB B-353; *Paenibacillus polymyxa* DEBB B-358
Data represent the mean ± SD from triplicate. Different letters are significantly different from each other ($P < 0.05$). Values expressed in $\mu g.mL^{-1}$.

Table 9. Central Composite Design matrix of corn steep liquor (CSL) as nitrogen source and glycerol as carbon source for auxin (IAA) production by *B. megaterium* GB711 and *P. polymyxa* DEBB B-358.

Levels			-1.41	-1	0	1	1.41
X1: CSL (gL^{-1})			2.95	5	8	11	13.05
X2: C:N			4.72	8	12	16	19.28
			DEBB B-353			DEBB B-358	
Replicates	X1	X2	IAA	Expected	OD	IAA	Expected	OD
1	-1	-1	108.88	134.26	4.51	172.58	173.07	5.51
2	-1	1	68.58	105.42	5.28	236.21	198.11	4.36
3	1	-1	214.07	239.00	6.03	176.78	235.32	6.50
4	1	1	117.45	153.82	4.56	135.57	155.51	5.65
5	-1.41	0	43.97	21.90	3.87	52.07	79.34	5.79
6	1.41	0	172.33	150.80	6.16	137.58	95.88	6.09
7	0	-1.41	192.38	180.0	4.24	251.71	224.33	6.19
8	0	1.41	101.22	76.28	5.09	159.45	174.49	4.86
9	0	0	274.97	257.72	3.89	320.99	287.91	5.21
10	0	0	267.13	257.72	2.08	273.77	287.91	4.44
11	0	0	273.69	257.72	4.12	283.07	287.91	4.72

Experiments were carried out in triplicate. Data represent the mean value. Standard deviation for estimated values is 0.05. *B. megaterium* DEBB B-353 IAA $R^2 = 0.82$. for *P. polymyxa* DEBB B-358 IAA $R^2 = 0.80$ and OD $R^2 = 0.68$.
Values are expressed in $\mu g.mL^{-1}$.

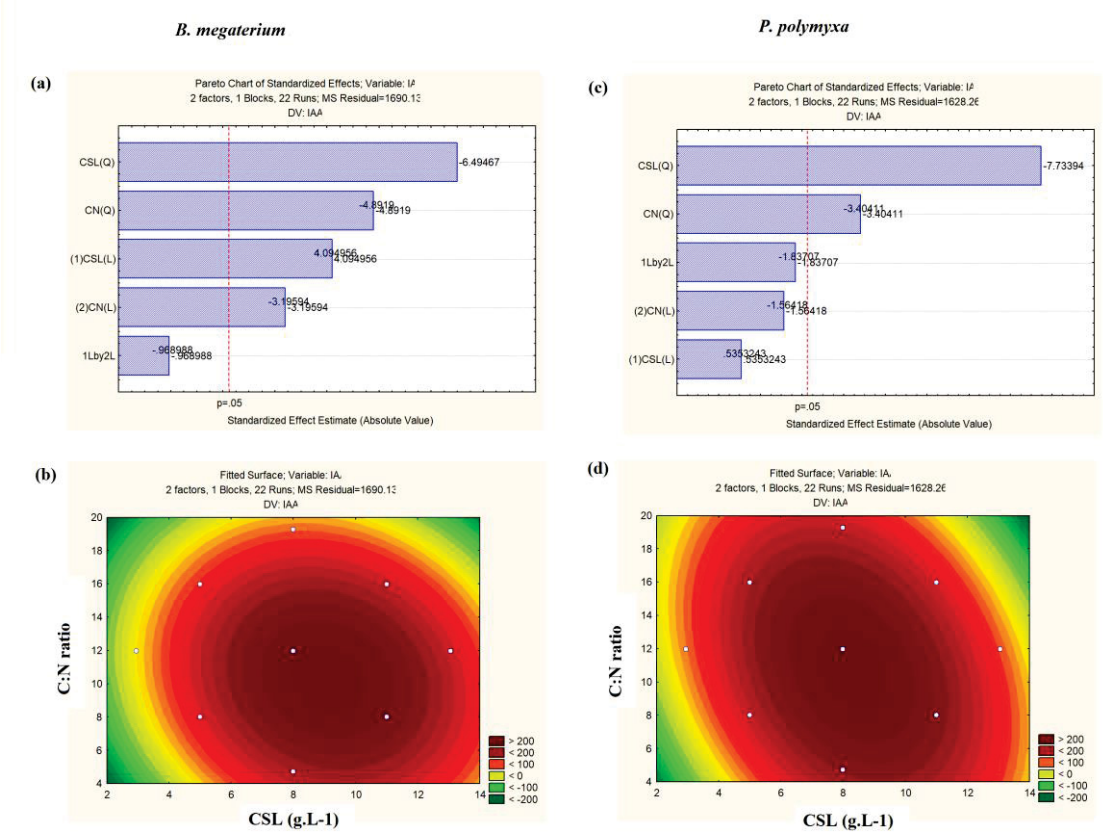

Figure 17. Pareto chart standardized effects with absolute values and fitted surface graph from two factors: CSL as nitrogen source concentration and Carbon: Nitrogen ratio interaction for auxin production by two isolates *B. megaterium DEBB* B-353 (a-b) and *P. polymyxa* DEBB B-358 (c-d) (p-value ≤ 0.05).

Days of incubation and tryptophan concentration had significant effects on *B. megaterium* DEBB B-353 auxin production. Higher auxin concentration 511.77 μg.mL^{-1} was observed after 4 days using 4% inoculum and 900 μg.mL^{-1} of Trp (Table 10). All factors showed effects on bacteria growth (data not shown), although the inverse was also observed in this experiment, better conditions for auxin production decreased bacteria OD. Therefore, statistical analysis revealed the variables optimal points as: 4.03 % inoculum, 1039.33 μg.mL^{-1} of Trp, and 4 days of culture for this isolate. The higher auxin rate for *P. polymyxa* DEBB B-358 was also observed at the center points (4 days using 4% inoculum and 900 μg.mL^{-1} Trp) with 661.10 μg.mL^{-1} and bacteria growth was moderately similar at all treatments, with no statistical difference. Pareto chart showed that all evaluated variables had a significant effect on auxin production (Fig. 19-e). And statistical analysis revealed the variables optimal points as 3.95 % inoculum, 1119.72 μg.mL^{-1} of Trp, and 3.4 days for *P. polymyxa* DEBB B-358.

Table 10. Full factorial Central Composite Design (CCD) of three variables on auxin production and bacteria growth (OD) by *B. megaterium* DEBB B-353 and *P. polymyxa* DEBB B-358.

	LEVEL	-1.68	-1	0	1	1.68
Days	X1	2	3	4	5	6
Inoculum %	X2	1.36	2	4	6	7.36
Tryptophan (µg.mL^{-1})	X3	272	500	900	1300	1572

				B. megaterium DEBB B-353			*P. polymyxa* DEBB B-358		
Replicate	X1	X2	X3	IAA	Expected	OD	IAA	Expected	OD
1	-1	-1	-1	221.44	234.63	3.504	258.73	154.47	5.661
2	-1	-1	1	329.72	338.47	4.647	581.10	531.99	5.301
3	-1	1	-1	249.06	240.91	3.594	339.67	258.83	5.535
4	-1	1	1	381.38	389.87	4.818	441.05	503.55	5.508
5	1	-1	-1	352.10	360.31	5.613	206.24	198.37	6.249
6	1	-1	1	333.59	358.55	5.952	189.39	324.85	5.739
7	1	1	-1	292.98	300.99	5.925	144.64	248.33	6.195
8	1	1	1	340.77	344.35	6.417	83.16	242.01	5.727
9	-1.68	0	0	123.09	120.34	3.258	300.99	416.08	5.262
10	1.68	0	0	213.98	200.5	5.373	365.08	198.44	5.37
11	0	-1.68	0	470.33	435.07	2.889	351.82	411.73	5.031
12	0	1.68	0	404.31	402.96	3.387	415.36	304.88	5.844
13	0	0	-1.68	295.47	291.51	4.743	89.70	180.51	5.772
14	0	0	1.68	412.87	392.92	3.456	593.15	439.90	5.775
15	0	0	0	511.77	474.7	3.492	631.82	617.26	5.961
16	0	0	0	485.80	474.7	3.42	661.10	617.26	5.049
17	0	0	0	414.81	474.7	3.261	599.23	617.26	5.445

Experiments were carried in triplicates. Data represent the mean value. Standard deviation for estimated value is 0.05. R^2 for *B. megaterium* DEBB B-353 IAA was 0.85 and OD = 0.78. R^2 for *P. polymyxa* DEBB B-358 IAA was 0.65. Values of IAA are expressed in µg.mL^{-1}

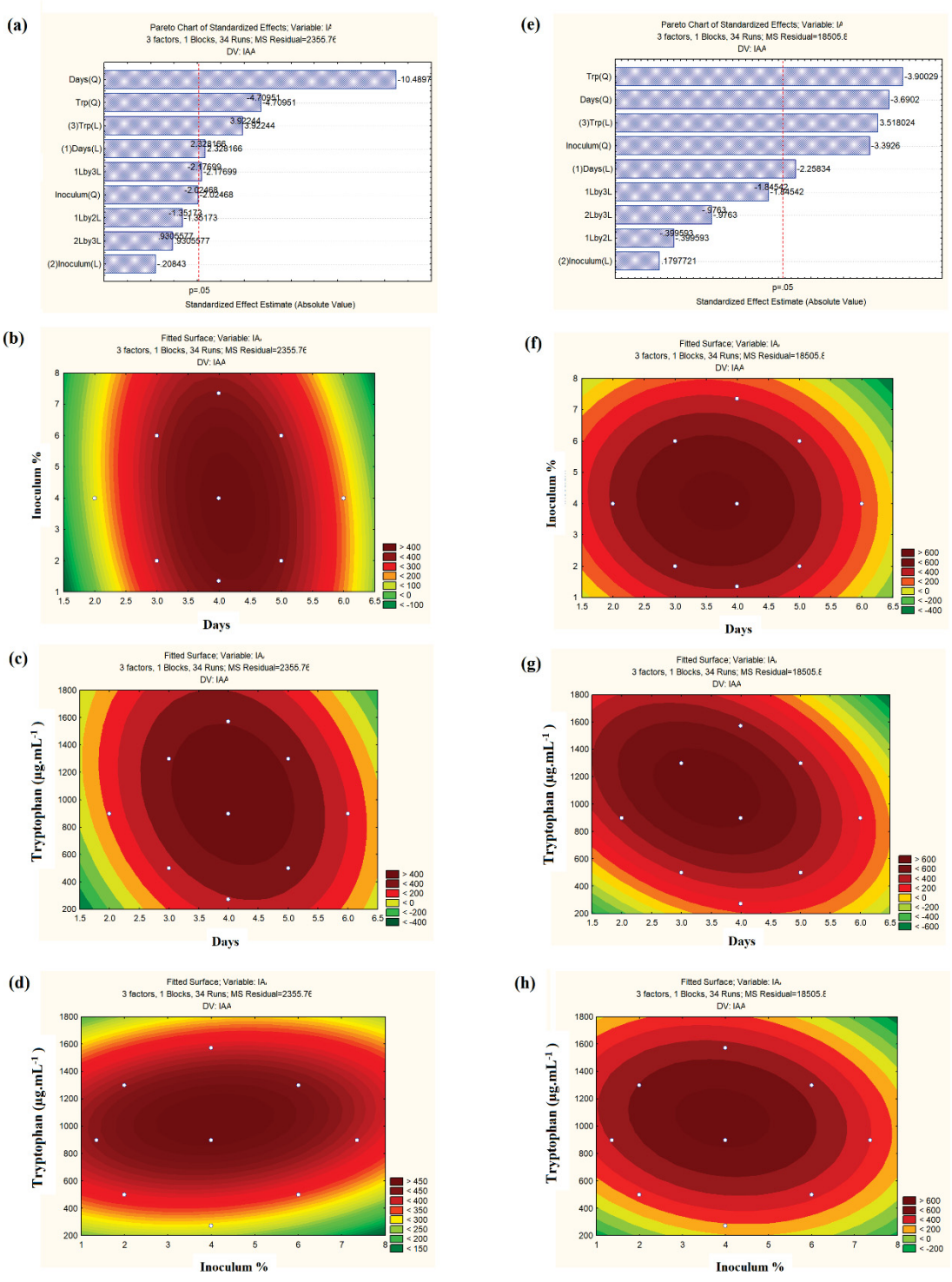

Figure 18. Pareto-plot for CCR parameter estimates for three factors and interaction effect of Days of fermentation, Inoculum percentage and Tryptophan (Trp) concentration (µg.mL-1) for *Bacillus megaterium* DEBB B-353 (a-d) and *Paenibacillus polymyxa* DEBB B-358 (e-h) on auxin production.

By optimization with RSM the resulted equation of the models using the three significant variables were as described as follow described:

Bacillus megaterium DEBB B-353: IAA ($\mu g.mL^{-1)}$ = -1614 + 740.8Days + 67.6Inoculum + 0.872Trp - 78.57 $Days^2$ - 6.06 $Inoculum^2$ - 0.000318Trp^2 - 8.20 Days*Inoculum - 0.0660 Days*Trp + 0.0141 Inoculum*Trp

Paenibacillus polymyxa DEBB B-358: IAA ($\mu g.mL^{-1)}$ = -2501 + 734Days + 295Inoculum + 2.354Trp - 77.5 $Days^2$- 28.47 $Inoculum^2$ - 0.000738 Trp^2 - 6.8 Days*Inoculum - 0.1569 Days*Trp - 0.0415 Inoculum*Trp

To validate the model we used the predicted optimal points by RSM analysis: 4% inoculum and 1030.0 $\mu g.mL^{-1}$ Trp for *B. megaterium* DEBB B-353 and 1100.00 $\mu g.mL^{-1}$ Trp *P. polymyxa* DEBB B-358 and set up the kinetical experiment to evaluate the IAA production over 144 h (6 days) of culture in Erlenmeyer flasks and in STR Bioreactors. Using these optimized conditions, IAA production by *B. megaterium* DEBB B-353 could reach up to 672.15 $\mu g.mL^{-1}$ of IAA in flasks after 72h, an increase of 9.37% with the predict auxin production by the model (614.51 $\mu g.mL^{-1}$). When scaled-up, the production in bioreactors was 750.61 $\mu g.mL^{-1}$ after 96h in 2 L STR and, 638.45 $\mu g.mL^{-1}$ after 32h in 10 L STR (Fig. 20). Production on 10 L bioreactor showed a significant yield production gain in fermentation time from 72 h to 32 h. With *P. polymyxa* DEBB B-358, in flasks we produced 725.01 $\mu g.mL^{-1}$, 12.85 % higher than the expected value of 642.43 $\mu g.mL^{-1}$. The production on STR bioreactors were around 567.18 $\mu g.mL^{-1}$ using 2 L STR after 72h of culture, and 812.49 $\mu g.mL^{-1}$ with 10 L STR, after 32h of fermentation (Fig. 21). The obtained yield from 10 L STR was 12.03 % higher than flask productivity and reduced over 40 h of process, with a productivity of 25.39 mg/L.h. All IAA peak production was related with the stationary phase of bacterial growth.

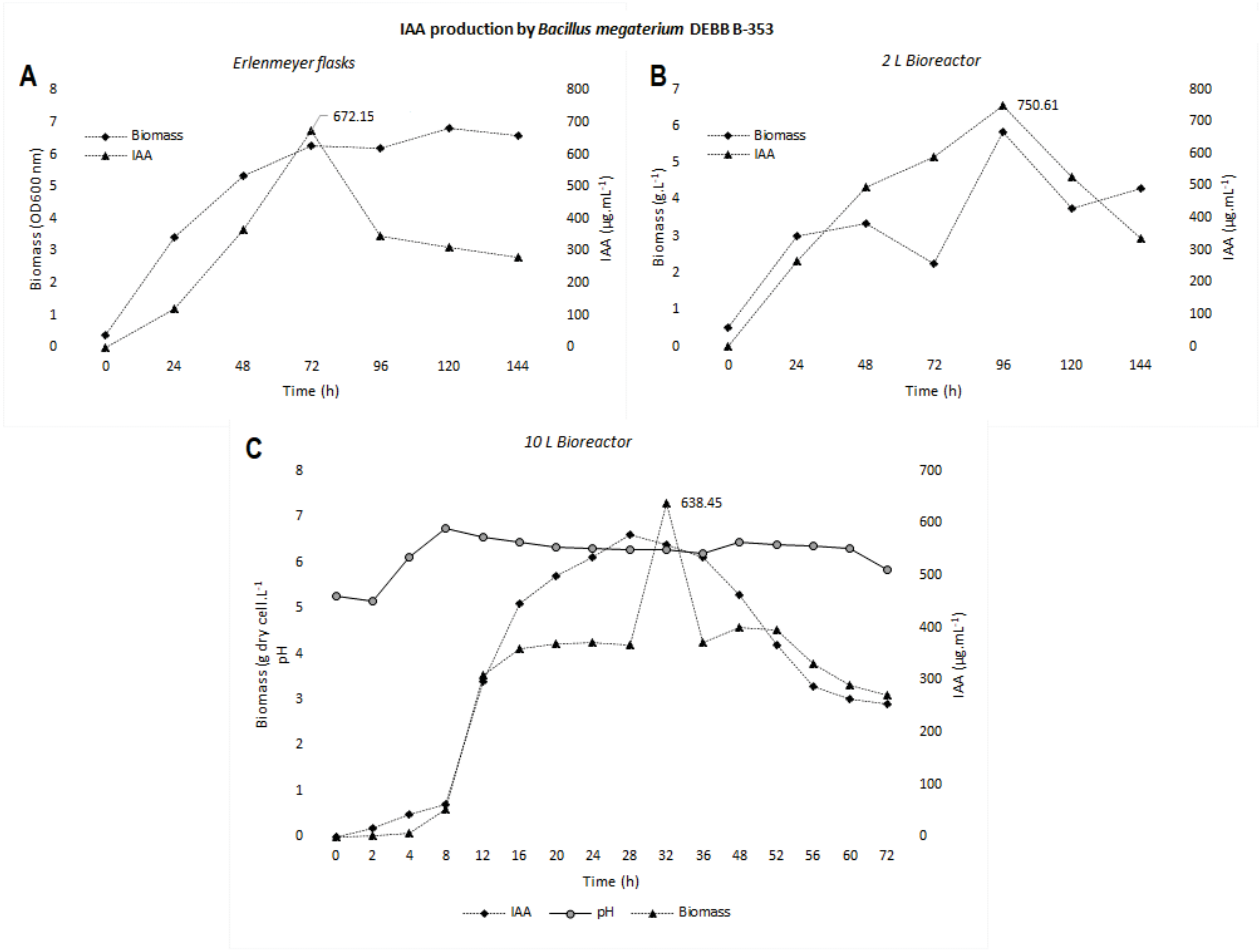

Figure 19. IAA production with the optimized parameters on Erlenmeyer flasks (A), 2 L Bioreator (B) and 10 L Bioreactor (C) Bioreactor by *B. megaterium* DEBB B-353. The graphic indicates the values for IAA yield (µg.mL-1) production and bacteria growth (OD) or Biomass weight (g of dry cell per litter) over the fermentation time. pH was also controlled in 10 L fermenter. Culture conditions: temperature: 30 °C, pH = 5±0.2, 120 rpm on flask, 400 rpm and 1VVM in bioreactor.

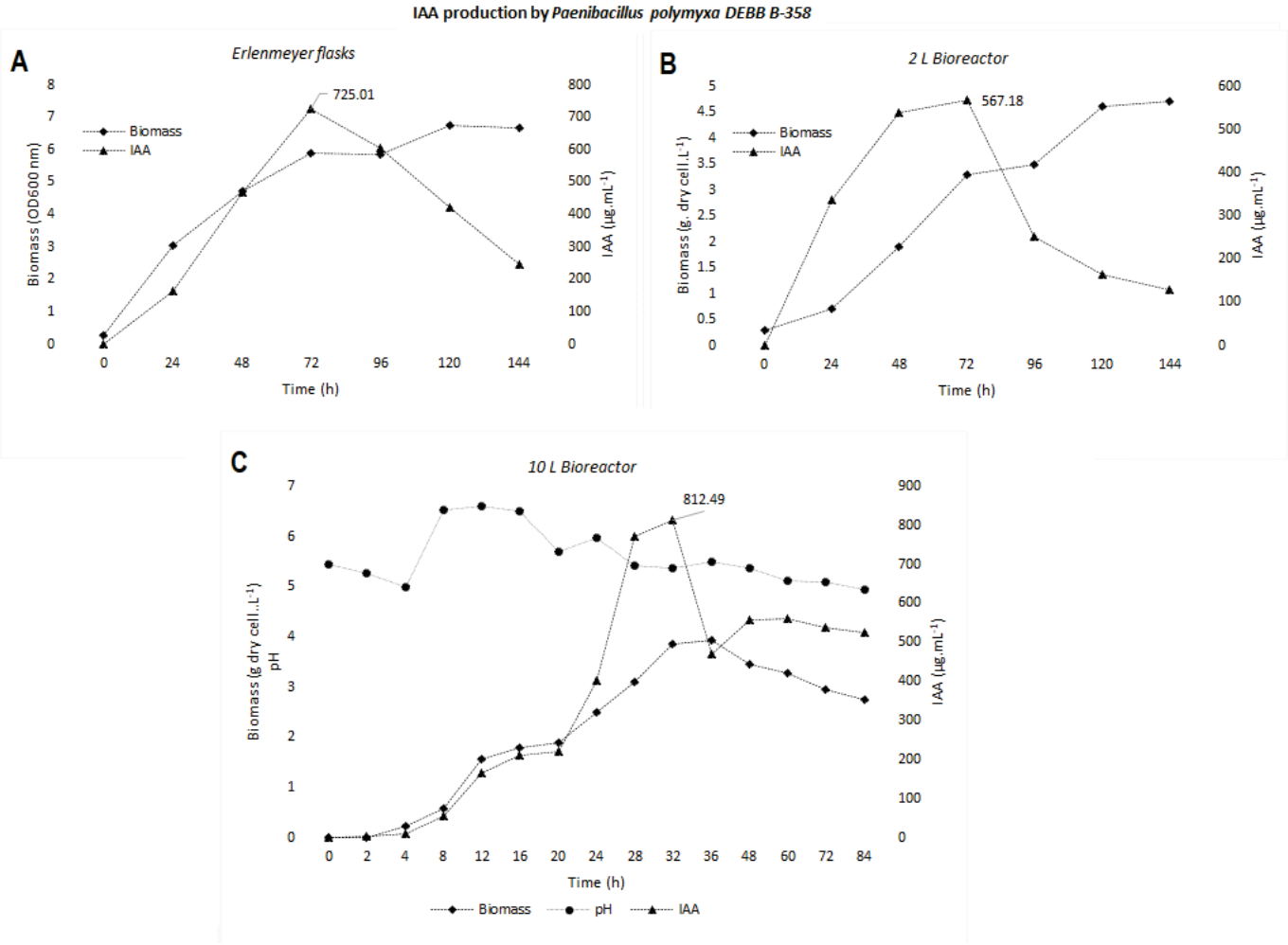

Figure 20. IAA production with the optimized parameters on Erlenmeyer flasks (A), 2 L Bioreator (B) and 10 L Bioreactor (C) Bioreactor by *P. polymyxa DEBB B-358.* The graphic indicates the values for IAA yield (µg.mL^{-1}) production and bacteria growth (OD) or Biomass weight (g of dry cell per litter) over the fermentation time. pH was also controlled in 10 L fermenter. Culture conditions: temperature: 30 °C, pH = 5±0.2, 120 rpm on flask, 400 rpm and 1VVM in bioreactor.

To confirm that the isolates were producing indol-3-acetic acid (IAA) we extracted the auxin-like compounds produced by the endophytes by liquid extraction and identified it by HPLC and FTIR as showed on fig. 22. The produced auxin compounds by the isolates showed the same retention time (6.4 min) for IAA peak on HPLC. The FTIR absorption spectra of the standard IAA and the isolates extract were similar. The graph confirmed a strong band due to N-H stretching in 3494 cm^{-1}, C-C peaks on 1639.4 and 1396.3 cm^{-1}, stretching frequencies of carboxylic acids.

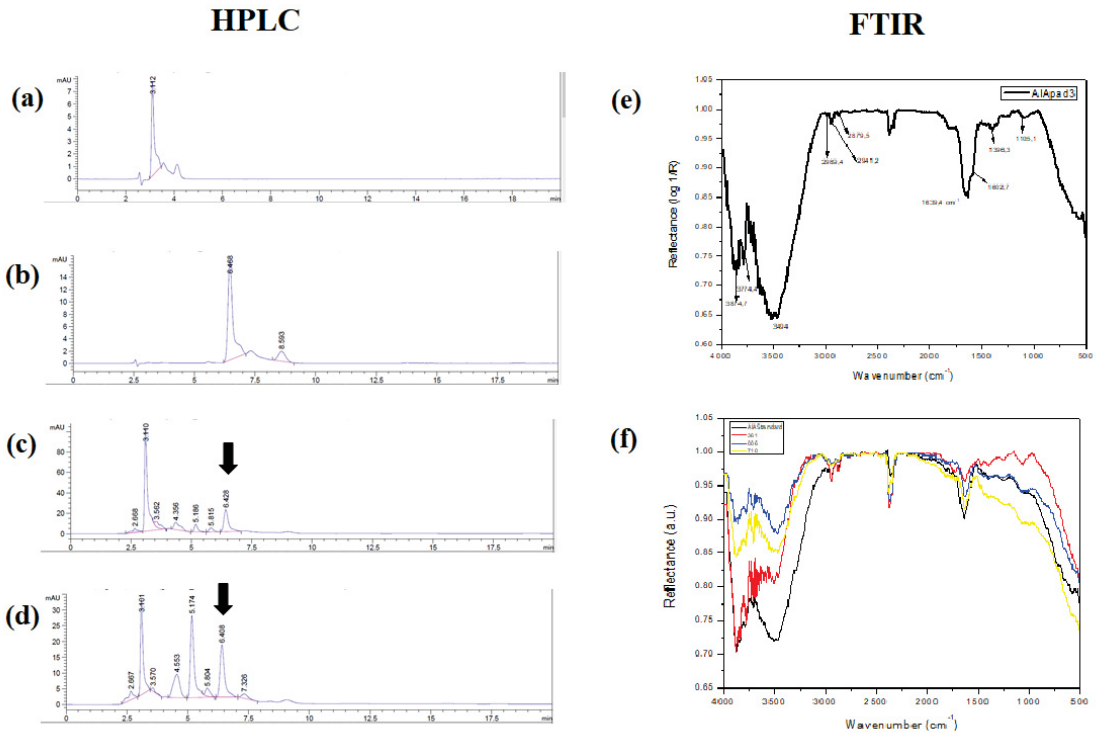

Figure 21. HPLC graphic of indol-3-acetic acid compounds and tryptophan. (a): Tryptophan standard peak at 3.112 min; (b): IAA standard peak at 6.468 min; confirmation of IAA production and tryptophan compounds on *B. megaterium* DEBB B-353 (c) and *P. polymyxa* DEBB B-358 (d). (e): standard IAA FTIR profile; (f): IAA FTIR produced by the isolates *B. megaterium* DEBB B-353 (blue line) and *P. polymyxa* DEBB B-358 (yellow line).

We evaluated the bacterial fermented culture on soy seed germination and plant growth. The treatments were with the endophytes sole or in combination as consortia, or as an additive in combination with a currently organic fertilizer TITAN ADVANCEFS soja (Baic, Brazil). No statistical difference was observed in plant experiments among the treatments. Both bacterial cultures showed better results on root growth, when applied

alone with 46.00±3.89 and 47.50±6.65 cm, respectively. As consortia, they increased the plant chlorophyll content, similar when both were mixed with TITAN, with chlorophyll total content of 7.69±1.52 and 8.20±0.02 $mg.g^{-1}$. Plant fresh and dry weight was higher on seeds treated with *P. polymyxa* DEBB B-358 (63.16±16.2 and 12.59±3.97 g) and when both bacteria were mixed with TITAN (61.14±19.11 and 14.28±5.29 g). At the first observation after 8 weeks, all treatments with bacteria fermented increased plant height (Fig. 23), and the highest plant height at the final was observed on plants treated with TITAN (63.20±11.62 cm) and the mixture of TITAN with *B. megaterium* DEBB B-353 (64.60±10.38 cm).

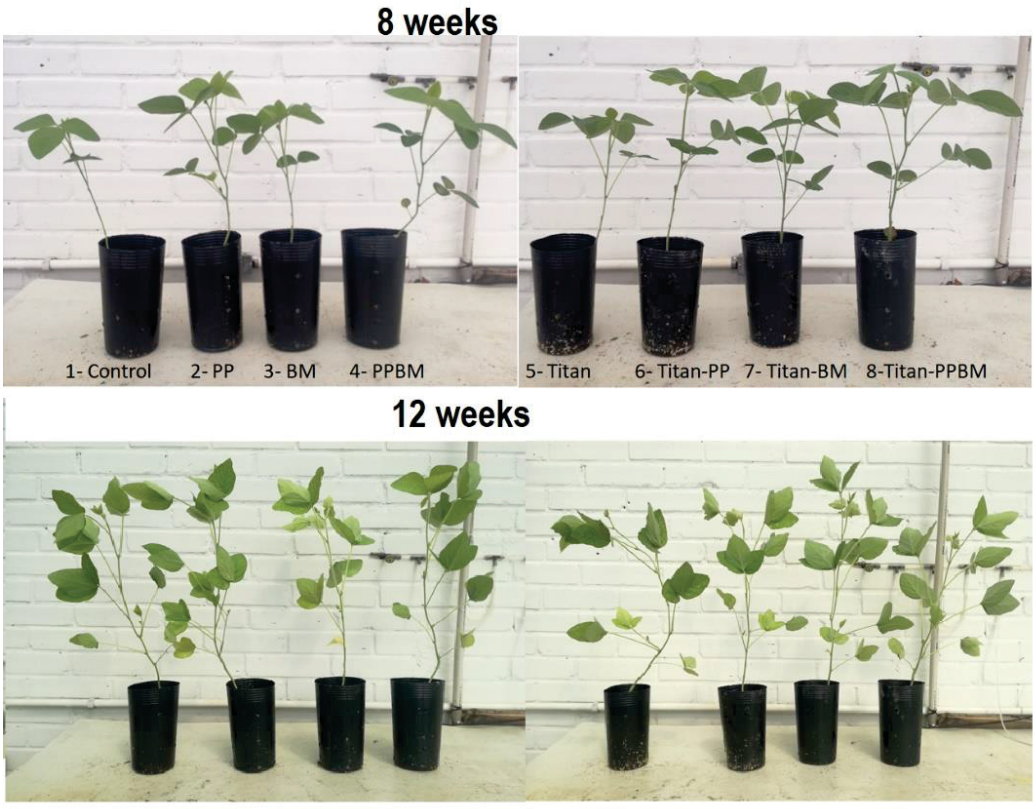

Figure 22. Evaluation of fermented culture, containing bacteria biomass and auxin contents. Treatments: 1) negative control with water; 2) 10% *Paenibacillus polymyxa* DEBB B-358 (PP); 3) 10% *B. megaterium* DEBB B-353 (BM); 4) bacteria consortia with 5% *Paenibacillus polymyxa* DEBB B-358 and 5% *B. megaterium* DEBB B-353; 5) 10% TITAN ADVANCEFS; 6) 5% TITAN + 5% *Paenibacillus polymyxa* DEBB B-358; 7) 5% TITAN + 5% *B. megaterium* DEBB B-353; 8) 5% TITAN + 5% *Paenibacillus polymyxa* DEBB B-358 + 5% *B. megaterium* DEBB B-353.

Table 11. Plant bio stimulation by *B. megaterium* DEBB B-353 and *P. polymyxa* DEBB B-358 broth on soy seeds after 85 days of harvest. Seeds treated with the strains isolated and as a consortium and mixed with natural fertilizer Titan Advance FS.

	Treatment	Germination (%)	Height (cm)	Root height (cm)	Leaves	Fresh weight (g)	Dry weight (g)	Chlorophyll a ($mg.g^{-1}$)	Chlorophyll b ($mg.g^{-1}$)	Chlorophyll total ($mg.g^{-1}$)
T1	Control	70.0	44.05±9.36	42.00±5.31	**33.00±10.45**	48.49±16.76	8.52±3.75	2.84±0.14	4.04±0.089	5.21±0.01
T2	*P. p*	80.0	53.40±9.95	**46.00±3.89**	31.50±3.77	**63.16±16.2**	**12.59±3.97**	4.71±2.28	4.87±0.68	7.10±2.29
T3	*B. m*	80.0	57.80±15.37	**47.50±6.65**	27.00±1.5	34.17±8.9	6.84±1.81	4.26±2.14	2.02±2.63	4.38±0.24
T4	*P. p* + *B. m*	80.0	51.60±13.68	43.00±10.96	27.00±5.30	38.63±18.44	7.94±4.04	**5.38±0.04**	**5.68±2.97**	**8.20±0.02**
T5	Titan	**90.0**	**63.20±11.62**	33.00±13.25	**33.00±5.0**	39.11±11.31	7.69±2.78	3.04±0.95	3.32±1.27	4.73±1.35
T6	Titan + *P. p*	**90.0**	58.55±13.06	43.50±17.11	30.00±2.77	47.15±15.65	11.06±4.47	3.38±1.19	3.68±1.95	5.24±1.97
T7	Titan + *B. m*	80.0	**64.60±10.38**	41.50±9.13	31.50±3.9	51.89±14.61	11.27±3.61	5.11±3.56	5.14±0.0	7.57±2.4
T8	Titan + *P.p* + *B. m*	80.0	48.00±9.94	34.50±4.56	27.00±4.53	**61.14±19.11**	**14.28±5.29**	**5.25±1.13**	**5.18±2.11**	**7.69±1.52**

Data represent the mean ± SD from the repetitions. Legend: *B.m* – *Bacillus megaterium* DEBB B-353; *P.p* – *Paenibacillus polymyxa* DEBB B-358;

Table 12. Cost for raw materials to produce 1L of product in USD (observed in April 2020, using Real and the USD quotation of 5.327 from 4/3/2020, https://br.advfn.com/moeda/dolar/2019).

Components	[g.L^{-1}]	Price USD $	Unit (Kg)	Price/ kg	Cost g material/ medium vol (USD)
Glycerol	48.23	33.79	25.0	1.35	0.0652
Corn steep liquor	9.09	56.31	1000	0.06	0.0005
K2HPO4	0.5	31.16	1.0	31.16	0.0156
KH2PO4	0.3	30.03	1.0	30.04	0.0090
MgSO4.7H2O	0.1	4.50	1.0	4.51	0.0005
NaCl	0.75	3.00	10	0.30	0.0002
CaCl2.6H2O	0.03	4.69	10	0.47	0.0000
KI	0.083	14.16	0.1	141.68	0.0118
Na2MoO4.2H2O	0.124	14.45	0.25	57.82	0.0072
H3BO3	0.03	4.69	1.0	4.69	0.0001
FeCl3	0.118	24.96	1.0	24.97	0.0029
ZnSo4.7H2O	0.287	3.56	1.0	3.57	0.0010
MnSO4.4H2O	0.223	33.99	5.0	6.80	0.0015
CuSO4.5H2O	0.249	52.18	25.0	2.09	0.0005
Total ($)	**-**	**-**	**-**	**-**	**USD 0.1167**

By the author.

The medium cost of a bioprocess is around 30-40% of the total production cost. According to the developed medium, the cost of the medium components to produce 1L of bioinoculant product will be around USD 0.12 cents. This estimated cost only considered medium components and did not consider other operational units as down and upstream steps, formulation, equipment, utility, and energy consumption.

DISCUSSION

Plant-microorganism symbiosis assures protection to the microorganism from abiotic stresses such as extreme variations in temperature, pH, nutrient, and water availability as well as biotic stresses such as microorganisms competition (Puri et al., 2018; Rosenblueth and Martínez-Romero, 2006) and on the other way, these microorganisms produce growth stimulating bioactive substances, positively promoting plant growth and development and enhancing plant tolerance to stress and pathogens (Pratap and Bahadur, 2016; Ramanuj and Shelat, 2018; Rosenblueth and Martínez-

Romero, 2006), bestowing a double way of advantage for both involved organisms, with direct or indirect benefits (Bhattacharyya and Jha, 2012; Yadav and Yadav, 2017).

Although rhizosphere microorganism's community exists in major concentration surrounding plant roots, studies with endophytic bacteria have been receiving more attention towards their benefits for agricultural use (Ahmed and Hasnain, 2014; Brader et al., 2014; Puri et al., 2018; Venugopalan and Srivastava, 2015; Zinniel et al., 2002). Their ability to spread systemically through the plant is an important factor as they can directly promote plant growth (Erdelyi et al., 2014) and decreases the need for continuous inoculations (Buragohain et al., 2018; Ribeiro et al., 2018). Therefore, screening for their potential to produce auxin can provide an effective PGB bacteria with an exceptional value in agricultural biotechnology (Erdelyi et al., 2014) in a short period of time (Etesami et al., 2015).

Bacillus and *Paenibacillus* genre members have been well documented as plant growth promoters bacteria and biocontrol agents by multiple mechanisms (McSpadden Gardener, 2004). Both are facultative, changing between the free-living and endophytic stages, and aerobic endospore-forming bacteria which are essentially ubiquitous in agricultural systems to their survival traits (McSpadden Gardener, 2004), allowing their adaptation to extreme abiotic conditions, such as extreme temperatures, pH, or pesticide (Dodd et al., 2010; Ribeiro et al., 2018; Sözer Bahadir et al., 2018). However, most research has focused on *Bacillus* species giving relatively less attention to *Paenibacillus* sp. (Jeong et al., 2019). And, so far, most studies related to *P. polymyxa* species are reported focusing on biocontrol mechanisms and with different antibiotic production (Timmusk et al., 2005).

P. paenibacillus isolates were described as having outstanding growth-promoting effects on several plant species (Grady et al., 2016; Ryu and Park, 1997), with the ability to produce phytohormones (Bhore et al., 2010; Da Mota et al., 2008; Lebuhn et al., 1997; Park et al., 2008; Timmusk et al., 1999; Xin et al., 2017), nitrogen fixation (Lindberg and Granhall., 1984; Timmusk et al., 2005), soil phosphorus solubilization (Singh and Singh, 1993), iron acquisition (Grady et al., 2016) and pathogen biocontrol ability (Lai et al., 2012) by antimicrobial and enzymes production (He et al., 2007; Raza et al., 2008), extracellular polysaccharide with antioxidant activity (Haggag, 2007; Raza et al., 2011), production of volatile compounds that elicit priming plant immunity (Jeong et al., 2019), probiotic for fish disease (Gupta et al.,

2014), biofuel production (Adlakha et al., 2015). All these wide ranges of properties with ecological and biotechnological importance, have turned this species for this two last decades more attractive for industrial processes and sustainable agriculture (Daud et al., 2019; Lal and Tabacchioni, 2009).

Auxin influence on plant development, especially modifying the plant root morphology and development (Ahmed and Hasnain, 2014), which consequently allows a better uptake of water and minerals (Gilbert et al., 2018; Goswami et al., 2015; Spaepen and Vanderleyden, 2011). Also linked to regulate some genes expression which are indirectly related to many plant processes (Ahmed and Hasnain, 2014) as the activation of the transcription of ACC synthase, and subsequently inhibits ethylene level (Glick, 1995). They are also pointed to mediate response on abiotic stress responses in plants (Daud et al., 2019; Figueiredo et al., 2008).

Several factors proved to interfere on auxin production by bacteria: pH, temperature, carbon and nitrogen sources, days of incubation, tryptophan, the main precursor for the synthesis of IAA in plants, bacteria strain and light (Arshad and Frankenberger, 1991; Spaepen et al., 2007). We evaluated the best pH for auxin production, and the results showed that acidic values (pH=5) stood to increase auxin concentration for both isolates. In the literature, pH range for auxin production varies according to the bacteria species and process. Similar to our report, *Paenibacillus spp*. SPT−03 revealed highest IAA production by pH 5.0 and at pH 7.0 42% decrease was observed (Acuña et al., 2011), while a *B. megaterium* isolate revealed acidic pH (below 6) to decrease IAA production, and the maximum amount of IAA was produced when pH of the culture medium was 8 (Mohite, 2013).

Corn steel liquor was appropriate for auxin production for both bacteria isolates, although with no statistical difference when compared to yeast extract for *B. megaterium* DEBB B-353. CSL is a by-product of the corn wet-milling industry, in bulk quantities, and at a low price, rich in nitrogen and growth factors (Madigan et al., 2011). It is normally used to ruminants and bird feed and as a nitrogen source for some industrial fermentation processes (Coelho et al., 2016). Its composition varies according to the raw material origin and processing, and according to our analysis, the used CSL has 3.8% total proteins in its composition. CSL has been reported to be acceptable as a nitrogen source for *Rhizobium* strains growth (Nutaratat et al., 2017; Stephens and Rask, 2000) and other biomolecules of interest (Madigan et al., 2011).

Therefore, functional microbial with positive results on bioconversion of agricultural waste into valuable and high demand products can take full advantage to emerge their applications in biotechnological processes, turning the process strategically more robust and economical for production of biomolecules (Madigan et al., 2011; Venugopalan and Srivastava, 2015) and to maximize ecological benefits (Daud et al., 2019).

CSL showed to stimulate the IAA production (400 $\mu g.mL^{-1}$) using a PGP bacterium *Enterobacter* sp. DMKU-RP206, but contrary to our results did not stand as better as yeast extract for IAA production (754.4 $\mu g.mL^{-1}$) (Nutaratat et al., 2017). In this experiment auxin production decreased in the presence of ammonium sulphate, although another different inorganic nitrogen sources as $NaNO_3$ was found suitable for IAA production with a *B. megaterium* isolate (~24.0 µg.ml-1) (Mohite, 2013) and $(NH4)_2SO_4$ for culturing *B . subtilis DR2* (KP455653) isolate with maximum IAA production (162.93 $\mu g.ml^{-1}$) (Kumari et al., 2018). These reports reinforce that the nitrogen source present in the medium affects auxin production among the different isolates.

In the same line, glycerol stands as a low-cost carbon source to provide energy and improves cell co-factor recycling and contributes to the overall efficiency of secondary metabolites biosynthesis (Kumari et al., 2018). Glycerol is a by-product of biodiesel production, with a high nutritional value at low-cost, being a viable way to contribute to a sustainable agro-industry (Díaz-Fernández et al., 2019; Scheidt et al., 2019). And interestingly, glycerol can be employed for biomass production and also can act as a protective agent of microbial cells, following for bioformulation processes (Lobo et al., 2019).

For our endophytic strains, glycerol increased auxin production in comparison with glucose and sucrose. Similar to our results, Balaji et al. (2012) reported higher IAA production (190 $\mu g.mL^{-1}$) using 2% of glycerol and observed total inhibition of auxin production with glucose, lactose, starch, and cellulose using a *Pseudomonas spp.* isolate. Different studies have screened and optimized auxin production with a range of different carbon sources as well as their combinations: glucose, sucrose, mannitol, glycerol, starch, cellulose, galactose and L-glutamic acid (Balaji et al., 2012; Dat et al., 2015; Kumari et al., 2018). The nature of carbon source has remarkable effect on microorganism growth and biomolecules production (Mutturi et al., 2016). And, during

a fermentation process, pH digression or upward is expected, and the source of carbon is directly related with this factor. Glycerol seems to cause relatively small pH digressions when compared to sugars like glucose and sucrose (Mutturi et al., 2016; Saharan et al., 2010). This can be an explanation for our best results with glycerol auxin production.

Optimizing multiple variables for a process of interest of bio-molecule production can be time consuming and laborious, especially when it is done by the classical single factor method. Wherefore, we used a central composite design (CCD) and response surface methodology (RSM) to optimize multiple variables with a minimum of experiments. Using a two factor CCD we found the best C:N ratio which directly influences auxin production and then we searched for the interaction of days, inoculum percentage, and tryptophan concentration on a three factor CCD.

Our peak of maximum production was observed after 72h (3 days of incubation) and 96h using bioreactor for DEBB B-353 isolate, which can be related to the attainment of the stationary growth phase of bacteria (Ahmed and Hasnain, 2014). Similar to our results, Kumari et al., (2018) found maximum auxin production at 96 h incubation and observed the decline after that period and Hussein et al., (2016) reported a decrease on IAA concentration after 72h, using a *B. subtilis* isolate with an auxin production of 106.38 $\mu g.ml^{-1}$. Auxin drop after certain culture time may be related to the release of enzymes that degrade auxins such as peroxidase and IAA oxidase (Hussein et al., 2016).

Tryptophan is an important precursor involved in two bacteria IAA biosynthesis, the indole-3-acetamide (IAM) and indole-3- pyruvic acid (IPyA) pathways (Arshad and Frankenberger, 1991). Lebuhn et al., (1997) have confirmed Trp-dependent conversion for auxin production by *P. polymyxa* strains. They have detected indole-3-latic acid (ILA) and indole-3-ethanol (tryptonophol) (TOL) metabolites which indicates the occurrence of IPyA and IAM pathways on auxin biosynthesis. Later on, Park et al., (2008) analyzing the genome sequence database on *P. polymyxa* E681 isolate reported the existence of only one IPA pathway for IAA biosynthesis. An earlier study showed that a *B. megaterium* br1 isolate could not produce auxin in medium without Trp (Mohite, 2013), indicating a Trp-dependent IAA biosynthesis pathway.

Besides Trp presence, IAA production is also dependent on culture conditions such as time, oxygenation, pH, and inoculum growth phase, as well as the species and

strains (Arshad and Frankenberger, 1991). Our CCD and RMS analysis revealed that days of incubation and Trp, primarily, had a more significant effect on auxin production for both strains. Using concentrations of Trp around 1030 and 1100.00 μg.mL^{-1} we could reach up to 640.11 and 745.63 μg.mL^{-1} of IAA in flasks after 72h of incubation for *B. megaterium DEBB B-353* and *P. polymyxa DEBB B-358*, 49.77 and 42.45 times higher than we first tested for auxin using LB media, respectively. Time of culture also affected auxin concentration, which showed a rise at the stationary phase, from 24-72h, and started to decline after 72-96h. We could maintain and observed a slight increase in auxin production rate on bioreactor and flask production, which is a good point when seeking a scale-up process. Another good aspect was the decrease of fermentation time frmo 72 h to 32 h in STR. The optimized results here reported to the endophytes are the highest reported until this study was conducted. Reports of IAA production for *P. polymyxa* varies from 11.84 μg.ml-1 (Park et al., 2008), 17 μg.ml^{-1} (Da Mota et al., 2008), 25.9 μg.ml^{-1} (Phi et al., 2010), 62.8±3.4 μg.ml^{-1} (Weselowski et al., 2016) and 91.7 μg.ml^{-1} (Lebuhn et al., 1997). And for *B. megaterium*: 8.86±2.18 μg.ml^{-1} (Đorđević et al., 2017), 48.1 μg.ml^{-1} (Baig et al., 2014), 50.3 μg.ml^{-1} (Lenin and Jayanthi, 2012) and 100.0 μg.ml^{-1} (Mohite, 2013) were reported.

However, the cost of the raw materials is a key factor that influences the price of any bioprocess. The use of low-cost substrates as material sources will decrease production costs, in particular those generated from industrial or agricultural by-products, e.g. cassava fibrous residue, chickpea and sweet whey have been used for this purpose (Srisuk et al., 2018). The medium cost of a bioprocess is around 30-40 % of the total production cost. Here, we developed a low-cost medium using primary glycerol and CS as carbon and nitrogen sources. To produce one liter of product was estimate to cost around R$ 0.62 of medium material, around USD 0.12 cents.

As mentioned above, species from *Paenibacillus* and *Bacillus* genre are known to have plant growth promotion abilities as nitrogen fixation, Ca-P solubilization, phytohormone production (Figueiredo et al., 2008; Jeong et al., 2019; Luo et al., 2011; Ribeiro et al., 2018) and biocontrol mechanisms of plant pathogens (Daud et al., 2019). Therefore, the combination of two or more of these mechanisms with the capability to produce phytohormones on one spore-forming Gram-positive bacterium turns out as a successful approach to be used as bioinoculant (Jeong et al., 2019) as they can resist a range of environmental stress conditions (Da Mota et al., 2008). When evaluating

the isolates inoculation on soy seeds, we observed higher germination and significative enhancement on plant growth on seeds inoculated with the bacterial culture when compared to the control treatment. Root growth may be related to the presence of IAA in the bacterial culture. When combined with the organic fertilizer, the bacterial culture showed to promote plant height, fresh and dry weight, and total chlorophyll content. This may be the first insight to use PGP bacterial as additives for other organic fertilizer products.

Enhanced production of IAA by isolated *P. polymyxa* improved plant tolerance to abiotic stress through lowering host ethylene levels by 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity (Weselowski et al., 2016). Moreover, *P. polymyxa* E681 has reported to colonize *A. thaliana* and incremented plant growth as foliar fresh weight and total leaf area (Jeong et al., 2019, 2006). As endophytic bacteria, they have the capacity to colonize various plant compartments such as roots, stem, leaves, flowers, fruits, and seeds and improve plant growth, improve plant development and protection (Da Mota et al., 2008; Puri et al., 2018). *P. polymyxa* was reported to efficiently colonize some important agricultural crops (Puri et al., 2018) and wood plant seedlings (Anand and Chanway, 2012). Co-inoculation of bean with *Rhizobium* and both *Paenibacillus* strains resulted in increased plant growth, nitrogen content, and nodulation (Figueiredo et al., 2008). *B. megaterium* strains extract isolated from *Ilex paraguariensis* showed a significant effect on growth also on *P. vulgaris* under *in vitro* conditions, increasing shoot and root length, shoot and root fresh weight, increase on root number and length of secondary roots (Grunennvaldt et al., 2018). Wahyudi et al. (2011) reported a positive effect on soybean seed germination, root and shoot length enhancement, and number of lateral roots to the production of phytohormone and siderophore and the capability to solubilize phosphate by *Bacillus* species.

In conclusion, in this study, we could develop a low-cost culture medium by using corn steep liquor and glycerol as nitrogen and carbon sources and optimized the auxin (IAA) production over 49.77 and 42.45 times higher, reaching up to 672.15 $\mu g.mL^{-1}$ and 725.01 $\mu g.mL^{-1}$ of IAA in flasks, for *B. megaterium DEBB B-353* and *P. polymyxa* DEBB B-358, respectively. The process was scaled-up to 2L and 10 L STR bioreactors and maintained the production and reduced the fermentation time about 40 h, with 638.45 $\mu g.mL^{-1}$ and 812.49 $\mu g.mL^{-1}$ for both endophytes. Both bacteria

fermented broth and its produced auxin showed to promote soy seed germination and plant and root development.

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SUPPLEMENTARY DATA

Table 12. Analysis of variance (ANOVA) of Central composition design for IAA production, evaluating the interaction of corn steep liquor concentrations, as nitrogen source, and carbon:nitrogen ratio, using glycerol as carbon source, by *Bacillus megaterium* DEBB B-353 and *Paenibacillus polymyxa* DEBB B-358.

	Bacillus megaterium DEBB B-353				*Paenibacillus polymyxa* DEBB B-358			
Factors	Effect	Std.Err.	*p*	-95.%	Effect	Std.Err.	P	-95.%
Mean/Interc.	257.719	15.92311	0.000000	223.964	287.909	15.62895	0.000000	254.777
(1)CSL (L)	76.573	18.69927	0.000845	36.932	9.825	18.35383	0.599788	-29.083
CSL (Q)	-120.953	18.62347	0.000007	-160.433	-141.372	18.27943	0.000001	-180.123
(2)CN (L)	-57.005	17.83673	0.005625	-94.817	-27.384	17.50722	0.137336	-64.498
CN (Q)	-78.230	15.99178	0.000163	-112.131	-53.432	15.69635	0.003629	-86.707
1L by 2L	-28.169	29.07004	0.346972	-89.794	-52.417	28.53301	0.084846	-112.904
Mean/Interc.	257.719	15.92311	0.000000	223.964	287.909	15.62895	0.000000	254.777
(1)CSL (L)	76.573	18.69927	0.000845	36.932	9.825	18.35383	0.599788	-29.083

Bacillus megaterium DEBB B-353 R^2= 0.82; *Paenibacillus polymyxa* DEBB B-358 R^2= 0.80

Table 13. Coefficient and p-value of regression analysis for auxin production by *B. megaterium* DEBB B-353, R2=0.85, evaluating days of incubation, inoculum percentage and tryptophan concentration.

	Regression coef.	Std. Error	t(24)	P	-95% Coef. Limt
Mean	-1614.21	224.7082	-7.1836	0.000000	-2077.99
(1) Days (L)	740.81	70.7028	10.4777	0.000000	594.88
Days (Q)	-78.57	7.4906	-10.4897	0.000000	-94.03
(2) Inoculum (L)	67.57	37.6849	1.7929	0.085603	-10.21
Inoculum (Q)	-6.06	2.9940	-2.0247	0.054164	-12.24
(3) Tryptophan (L)	0.87	0.1846	4.7249	0.000084	0.49
Tryptophan (Q)	-0.00	0.0001	-4.7095	0.000087	-0.00
1L by 2L	-8.20	6.0670	-1.3517	0.189066	-20.72
1L by 3L	-0.07	0.0303	-2.1770	0.039545	-0.13
2L by 3L	0.01	0.0152	0.9306	0.361349	-0.02

Table 14. Coefficient and p-value of regression analysis for auxin production by *P. polymyxa* DEBB B-358, R2 = 0.65, evaluating days of incubation, inoculum percentage and tryptophan concentration.

	Regression coef.	Std. Error	t(24)	P	-95% Coef. Limt
Mean	-2501.20	629.8074	-3.97138	0.000566	-3801.06
(1) Days (L)	733.87	198.1643	3.70335	0.001111	324.88
Days (Q)	-77.47	20.9944	-3.69020	0.001148	-120.80
(2) Inoculum (L)	294.77	105.6224	2.79082	0.010143	76.78
Inoculum (Q)	-28.47	8.3914	-3.39260	0.002401	-45.79
(3) Tryptophan (L)	2.35	0.5174	4.54958	0.000131	1.29
Tryptophan (Q)	-0.00	0.0002	-3.90029	0.000678	-0.00
1L by 2L	-6.79	17.0045	-0.39959	0.692990	-41.89
1L by 3L	-0.16	0.0850	-1.84542	0.077350	-0.33
2L by 3L	-0.04	0.0425	-0.97630	0.338655	-0.13

PART V - GREEN BIOSYNTHESIS OF SINGLE AND BIMETALLIC NANOPARTICLES OF IRON AND MANGANESE USING BACTERIAL AUXIN COMPLEX TO ACT AS PLANT BIO-FERTILIZER

ABSTRACT

Iron and manganese nanoparticles were synthesized through a simple and rapid method using bacteria supernatant containing auxin complex (IAA), and evaluated as plant nano-fertilizer. Successful biosynthesis of singular FeO_x-NPs and MnO_x-NPs, and bimetallic MnO_x/FeO_x-NPs were obtained. UV–visible spectroscopy confirmed the formation of FeO_x-NPs with an absorbance peak 250-300 nm, and MnO_x-NPs around 280-300 nm. Transmission electron microscopy (TEM) indicated spherical nanoparticles agglomerated, and Fourier Transform Infrared spectrometer (FTIR) revealed correspondent bands of auxin at the synthesized NPs, confirming that the auxin complex acted as reductive/capping agent. In general, bimetallic MnO_x/FeO_x-NPs from bacterial supernatant showed best result on plant growth, especially in germination rates, root growth and fresh weight of maize plantlets, and showed to be suitable to be used as micronutrient nano-fertilizer.

Keywords: nano-fertilizer, indol-3-acetic acid, plant biostimulant, manganese nanoparticles, iron nanoparticles

GRAPHICAL ABSTRACT

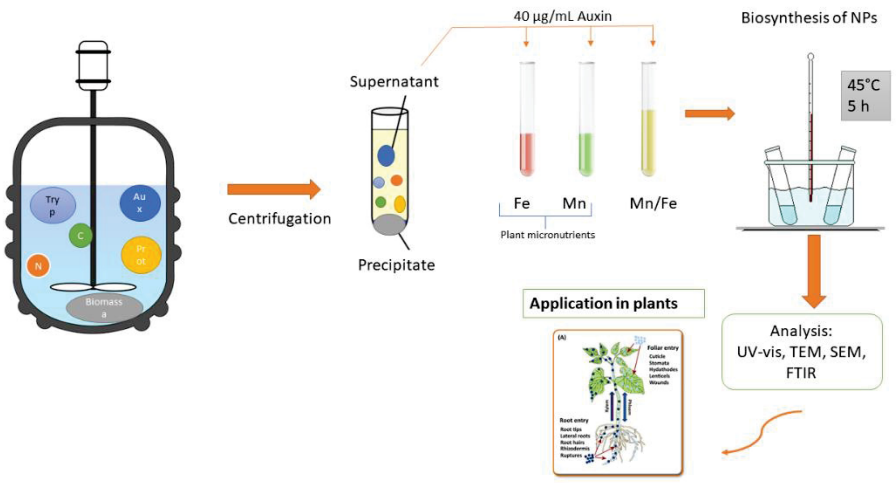

Figure 23. Graphical abstract representing mono and bimetallic nanoparticles of Iron and Manganese green biosynthesis.

INTRODUCTION

According to the United Nations reports, the agricultural sector needs to increase up to 50 % of the food production until 2050, to feed the expected 9.7 billion of the world population (Wiens, 2016). However, the adopted methodologies for food production must in somehow guarantee more safety, faster and precise rate of yield and environmentally friendly farming (Pandey, 2018; Shivani et al., 2018), avoiding the conventional agricultural methods with the indiscriminate use of chemical fertilizers and pesticides, which has led to many problems as environmental damage with water and air pollution, the loss of soil quality and biodiversity, food contamination, and intensification on pest resistance with the decreased effectiveness of the chemicals (Campos et al., 2018; Duhan et al., 2017; Kim et al., 2018).

Today, nanotechnology is one of the fastest growing fields in science and technology (Dasgupta et al., 2015), and has been receiving much attention to be used as a new technology to supersede the conventional farming practices and provide the state-of-the-art solution for current challenges faced in agriculture and future society (Shankramma et al., 2016; Feregrino-Perez et al., 2018; Shivani et al., 2018). In agriculture, application of nanotechnology is focused to improve results with more precise agricultural systems in the fertilization process, nutrient optimization to enhance plant growth and minimized the requirements of plant chemical fertilizers and pesticides (Nair et al., 2010; Pandey, 2018; Thakur et al., 2018). Nano-based products

have the advantage of large surface area that favors the specifically targeted and slow release of nutrients that regulate plant growth, with less eco-toxicity (Pandey, 2018; Shankramma et al., 2016). With this relative slow release of nutrient, the extended effective duration of nutrient supply to plant growth its guaranteed, ensuring that the nutrients are utilized optimally, increasing the fertilizer efficiency and subsequently reducing leaching (Shivani et al., 2018; Singh et al., 2016; Thakur et al., 2018).

In general, nanoparticles (NPs) can be synthesized by various physical and chemical methods. And recently the alternatively new green synthesis of NPs methods arise as an idea of developing eco-friendly methods (Thamilarasan et al., 2018). Green synthesis uses biological sources like plant extracts, microorganisms or its fermented extracts (Chandran et al., 2016; Mazumdar and Haloi, 2011), which are natural biogenic factories with an entire arsenal of biotransformation molecules such as enzymes/proteins, amino acids, organic acids, polysaccharides and vitamins that have the antioxidant properties and are capable to reduce or oxidize chemical compounds (Griffin et al., 2017; Hassan et al., 2018; Iravani, 2011; Singh et al., 2016), and in some cases as organizers for facilitating nucleation (Kumar et al., 2013), and lead to the formation of their elemental particles. This green synthesis has the advantage to be natural, easy and rapid to manipulate, fast, safe, with low cost, energy efficient and easy to scale up, and reduces waste (Chandran et al., 2016; Gottimukkala et al., 2017; Thakur et al., 2018). The biological methods withdraw the necessity of reactive and toxic reducing agents that have a negative impact on organism health and environment (Hoseinpour and Ghaemi, 2018) and offer better manipulation and control over crystal shape and size and their stabilization (Gottimukkala et al., 2017).

Synthesis of different nanoparticles such as Fe, Au, Ag, Zn nanoparticles, has been reported to be produced by plant extracts and microorganisms such as fungus, yeast, bacteria, algae etc (Alsoudi et al., 2018; Malik et al., 2014; Moon et al., 2010; Singh et al., 2016). Using bacteria cell-free supernatant directly to synthesized NPs in the medium has become an interesting green synthesis technique for NPs as it eliminates the downstream processing steps for the recovery of NPs in intracellular methodologies, including sonication to break down the cell wall, numerous centrifugation for biomass separation and washing steps for nanoparticle purification, and others (Singh et al., 2016). Thus, it is easier to apply and future scale-up (Malik et al., 2014).

In the meantime, less attention has been paid to iron and manganese to be used for sustainable nanotechnology, especially for manganese (Hoseinpour and Ghaemi, 2018). Many research and applications of NPs as fertilizers, pesticides, and herbicides are concentrated on carbon nanotubes, silver, gold, zinc, copper, and titanium NPs (Antonoglou et al., 2018; Duhan et al., 2017; Xiong et al., 2018). For this reason, we choose to evaluate singular and bimetallic green biosynthesis of iron and manganese NPs, and apply them as nano-fertilizers. This approach is in convergence with the necessity to offer these micronutrients to plant uptake in response to the challenge left with the extensive use of chemical fertilizers, which have contributed to macro and micronutrient concentration decrease in soil, and turned necessary the input of these nutrients for plant development.

Micronutrients like Fe and Mn are essential for diverse metabolic processes for plant growth and development, related to many physiological reactions and are important components of chlorophyll (Shankramma et al., 2016). They are required by the plants in low quantities (less than 100 ppm) (Feregrino-Perez et al., 2018).

Iron is the fourth most abundant element on the Earth, but exists at low concentration or is absent in the available form to plants and microorganisms uptake because of the low solubility of minerals containing it (Askary et al., 2017). Therefore, iron deficiency is one of the main limiting factors affecting crop yields, food quality and human nutrition (Zuo and Zhang, 2011). Iron has a primary role in plant productivity, high required for proper photosynthetic reactions, and complex protein machinery located in mitochondria and plastids (Briat et al., 2007). Many enzymes including cytochromes, which are involved in the electron transport chain, chlorophyll synthesis, and the structure of chloroplast maintenance are iron-dependent (Briat et al., 2007; Mamathaand and Ramesh, 2015). Manganese occurs in small amounts in practically all soil (Kelley, 1912). It is an essential micronutrient for several plant metabolic processes, like in photosynthesis involvement in the water-splitting system of photosystem II (PSII), which provides the necessary electrons for photosynthetic electron transport, as an enzyme activator and cofactor, in chlorophyll biosynthesis, synthesis of amino acids and hormone activation (indol-3-acetic acid, IAA) throughout the IAA-oxidases (Burnell, 1988; Dučić and Polle, 2005; Millaleo et al., 2010). Manganese is also important in the plant's tolerance against oxidative stress, promoting plant cell´s adaptive responses under environmental stresses such as salt

stress (Millaleo et al., 2010). Meanwhile, Mn toxicity is a world-wide problem in areas with acid soils, which turns the excessive Mn amounts toxic for the plants and affects physiological, biochemical and molecular processes at the cell level (Millaleo et al., 2010).

Herein, we studied the effect of the fermented broth of an endophytic bacterium, which contains auxins and other trace metabolites to be used as reducer/capping agent to synthetized singular and bimetal Fe and Mn NPs. By far, this is the first research involving phytohormones supernatant produced by endophytic bacteria to produce green NPs and to be tested as nano-fertilizers.

MATERIALS AND METHODS

Auxin production

IAA-producing endophytic bacterium *Paenibacillus polymyxa* DEBB B-358 isolated from micro propagated *Eucalyptus* leaves were chosen for the study. The strain was isolated and maintained in laboratory of Bioprocess Engineering and Biotechnology Department – UFPR (Curitiba-Brazil). The auxin production was done using as basal media composition ($g.L^{-1}$): K2HPO4:0.5; KH2PO4:0.3; MgSO4·7H2O:0.1; NaCl:0.75; CaCl2·6H2O:0.03; corn steep liquor:8.27 and glycerol:46.45, 1 mL of trace element solution (in gL^{-1}: $FeCl_3$:0.02, KI:0.83, $Na_2MoO_4.2H_2O$:0.124, H_3Bo_3:0.03, $ZnSO_4.7H_2O$:0.287, $MnSO_4.4H_2O$:0.223, $CuSO_4.5H_2O$:0.249) (Blinkov et al., 2014), pH= 5. The medium was autoclaved for 15 min at 120 °C. Bacteria fermentation was carried out in 250 mL Erlenmeyer with 50 mL of medium with 3.5% of bacterial inoculum and supplemented with 1000 $\mu g.mL^{-1}$ of Tryptophan (Trp). It was incubated for 4 days at 30°C, in a shaker with 120 rpm. For auxin quantitative colorimetric analysis, we used Salkowski's reagent (a mixture of 50 mL 35% perchloric acid and 1 mL $FeCl_3$ 0.5M). Bacterial culture was centrifuged at 958 g for 15 min and 1 mL of supernatant was collected and mixed with 2 mL of Salkowski's reagent and allowed to stand for 30 min on temperature room in dark and read in a spectrophotometer in 530 nm. A standard curve was developed with a pure indole-3-acetic acid solution (0 - 100 $\mu g.mL^{-1}$, R^2= 0.98).

Synthesis of singular and bimetal nanoparticles

Solutions preparations: the bacterial supernatant containing auxin was filtrated using a 0.22 µm membrane and diluted until reach a concentration of 200 $\mu g.mL^{-1}$ (solution I). An aqueous solution of 20 mM $FeCl_3.6H_2O$ was prepared by dissolving the necessary amount of the iron salt in ultrapure water (solution II). The same was done for $MnSO_4.H_2O$ to get a solution of 20 mM (solution III).

The synthesis of the NP was carried out as follows: (1) for singular NP synthesis, a certain volume of the solutions I, II and III were taken so that the final concentrations of auxin and the metal solution in the reaction mixture were finally 50 $\mu g.mL^{-1}$ and 2 mM (for solution II and III), respectively; (2) for bimetal NP synthesis, the final reaction mixture was 50 $\mu g.mL^{-1}$ of solution I, 1 mM of solution II and III, respectively. The reaction took place using a water bath at 45°C for 5 hours without mixing or shaking and covered from the light (Hu and Hsieh, 2016). The supernatant solution pH was maintained ± 4.8. As control, NPs were synthesized using the basal medium composition.

Characterization of the NP

After the reaction, the resulting solutions of nanoparticles (FeO-NP, MnO-NP and MnO/FeO-NP, as unreacted bulk reagents were not removed) were submitted to:

UV–vis absorbance spectroscopy analysis: The bioreduction of the Fe and Mn^{+} ions was monitored by absorbance measurement using a UV-vis spectrophotometer (Shimadzu, UV-1601 PC) in an interval of 5 hours, measured in 200-800 nm range with a resolution of 1 nm, and recorded using a quartz cuvette with water as reference.

Transmission Electron Microscopy (TEM) analysis: The nanoparticle size and morphology were analyzed by using transmission electron microscope (JEOL, JEM 1200EX-II) operating at 120 kV. TEM samples were prepared by placing a drop of the suspension of hybrid mono and bimetal auxin/NP on carbon-coated copper grids and allowing water to evaporate at an incubator at 30°C. The analysis of the selected area electron diffraction (SAED) patterns were captured to identify iron and manganese crystal planes.

Fourier-Transformed Infrared (FT-IR) spectroscopy analysis: The changes in the surface chemical bonds and surface composition due to the possible

participation of bioactive functional groups in capping and stabilization of nanoparticles after the bio-reduction of the metals were obtained using the FT-IR (Bruker, Vertex 70) with DRIFT (diffuse reflectance) accessory. The reflectance technique was used with 64 scans, resolution of 4 cm^{-1}, without elimination of atmospheric compensation in the region between 4000 to 400 cm^{-1}. The resulting solutions of nanoparticles were dried at 45°C and the dried powders were mixed with potassium bromide (KBr) in a ratio of 1:5 using an agate mortar and pestle (Anthony et al., 2014).

Evaluation on plant germination and growth

Maize seeds were selected to evaluate the NPs effect on seed germination and plant development. The seeds were submersed in solutions (1 % v/v) with the respective NPs for 30 seconds and sown in plastic vases containing land and incubated in green-house. The respective treatments were: (1) negative control (only water), (2) positive control with indol-3-acetic acid (IAA) solution 50 µg/mL, (3) Bacteria supernatant (BS) FeO-NPs solution (1%), (4) BS MnO-NPS solution (1%), (5) BS MnO/ FeO-NPs solution (1%), (6) Medium (Me) FeO-NPs solution (1%), (7) Me MnO-NPS solution (1%), (8) Me MnO/ FeO-NPs solution (1%). All solutions were prepared using distilled water. Each treatment consisted of 11 seeds. After 21 days, seed germination, root and shoot growth, number of leaves and fresh weight were evaluated.

RESULTS AND DISCUSSION

We successfully produced a singular and bimetallic iron and manganese NPs using the endophyte *Paenibacillus polymyxa* DEBB B-358 isolate supernatant solution containing auxin complex (IAA).

Auxin is a secondary metabolite, a class of phytohormone responsible in many developmental processes in plants, including plant cell division stimulation and cell elongation promotion, cell differentiation, pattern formation, apical dominance, and tropic responses (Kochar et al., 2013; Normanly et al., 1995) and responsible for bacteria-plant signaling (Spaepen and Vanderleyden, 2011a). Secondary metabolites, such as terpenoids and flavonoids, sugars and certain amino acids have also been employed successfully to generate metal nanoparticles (Makarov et al., 2014). Besides the auxins compounds, the supernatant may contain traces of other natural organic compounds like sugars, amino acids, peptides, proteins and enzymes, even at

millimolar concentrations therein which have significative roles acting as reducing agents and natural capping and stabilizing agents to NPs (Griffin et al., 2017; Singh et al., 2016). One of these compounds may be tryptophan, as we used it as the auxin precursor on fermentation. Tryptophan residues from peptides were pointed to also act to reduce metal ions and promote silver and gold nanoparticles synthesis (Griffin et al., 2017; Si and Mandal, 2007). These bio-molecules metabolites contain numerous functional groups such as C-C (Alkenyl), C-N (amide), O-H (phenolic and alcohol), NAH (amine), CAH and COOA (carboxylic group) that are constantly involved in the redox reaction of salts into their nanosized particles (Kuppusamy et al., 2016).

Most of the green biosynthesis of iron and manganese nanoparticles were reported using plant extracts as Seaweeds *Dictyota dicotoma* (Chandran et al., 2016), tea leaves (Kuang et al., 2013; Shahwan et al., 2011), *Citrus maxima* leaves (Hu et al., 2017), *Eucalyptus* leaves (Wang et al., 2014) and *Camellia sinensis* leaf extract (Gottimukkala et al., 2017), *Syzygium aromaticum* L.) flower buds (Kumar et al., 2017), *Adhatoda vasica Nees / Justicia adhatoda* (Prasad, 2017), lemon extract as reducing agent and turmeric curcumin as a stabilizing agent (Jayandran et al., 2015), *Azadirachta indica* (Neem) plant (Sharma et al., 2016), and few using microorganisms *Aspergillus oryzae* TFR9 (Tarafdar and Raliya, 2013) and *Acinetobacter* spp. (Bharde et al., 2005), *Bacillus sp*. cells (Sinha et al., 2011), *Streptomyces sp*. HBUM171191 (Waghmare et al., 2011), *Thermoanaerobacter sp*. TOR-39 (Moon et al., 2010).

Moreover, Fe an Mn NPs were observed as well as on the treatments using bacteria medium, showing that the composition mainly with glycerol and CSL could reduce the salts to its nano sizes. Figure 22 shows the UV-vis spectra obtained for the reducing agent of Fe and Mn to nanoparticles with different patterns. The confirmation of FeO_x-NPs formation was done by observing a strong absorption peak located between 250 to 300 nm, which increased until 3h of incubation (Huang et al., 2014; Kumar et al., 2013) with bacteria supernatant and basal medium. The same was observed by Herrera-Becerra et al., (2010) when produced iron oxide NPs using gallic acid and tannic acid as a reducer, with a maximum of absorption around 527 nm and 544 nm, respectively.

Furthermore, a broad shoulder in the 470–540 nm region was mentioned as a characteristic spectra that indicate the formation of chemical species composed of Fe^{III} and IAA in aquatic systems (Kovács et al., 2008). First, the interaction between IAA

and Fe^{III} results in dimeric Fe^{III}–IAA complex at acid pH, and with subsequent reactions, some Fe^{III} are reduced to Fe^{II} and compounds like wustite (FeO) and magnetite (Fe_3O_4), which produces oxidized products of IAA (Herrera-Becerra et al., 2010; Kovács et al., 2008). Since the time zero when the solutions were mixed, the FeO_x-NPs turned from colorless to yellowish and MnO_x-NPs stayed colorless. The resulted color in the solutions may come from the coordination of the IAA oxidized products to Fe^{II} (Kovács et al., 2008), as this might be a more stabilized form of iron in aqueous systems (Milić Komić et al., 2016). The detected change in the absorbance characteristics of the medium at 265 nm wavelength with FeO_x-NPs can be due to protein and amino acids electronic excitation, as tryptophan and tyrosine residue (Mazumdar and Haloi, 2011).

The surface plasmon absorbance of the MnO_x-NPs increased along the 5 hours, especially around 280-300 nm, showing an increased production of MnO_x-NPs, even without color change. By mixing both Fe and Mn salts to NP synthesis, the peaks were similar to both singular NPs reduction. The surface plasmon resonance bands are influenced by size, shape, morphology, composition, and the refractive index and dielectric environment of the surrounding media and the separation between the particles (Herrera-Becerra et al., 2010; Kelly et al., 2003).

Figure 23 shows TEM image showing spherical NPs morphology, with the characteristic of agglomerated NPs. The selected area electron diffraction (SAED) pattern revealed the polycrystalline nature of the nanoparticles. And yet, size is the most important parameter that should be considered for characterization, and it is critical for determining the interactions of nanoparticles with living systems (Dasgupta et al., 2015). Therefore, with TEM micrograph illustration we could estimate the NPs sizes average as for FeO_x-NPs = 26.65 (min: 13.71 and max: 48.88 nm), MnO_x-NPs= 22.32 (min: 7.20 and max: 35.68 nm) and MnO_x/FeO_x NPs = 23.42 nm (min: 11.28 and max: 53.59 nm). For the size average, 170 particles were measured. The heterogenic size distribution is probably due to the fact that the supernatant may contain naturally derived compounds with different reducing properties besides the auxins (Wang et al., 2014).

A similar average MnO nanoparticles size was found by (Kumar and Sangwan, 2013) with 25-30 nm. The figure displays spherical nanoparticles with an average

diameter of 25 nm, with sizes ranging from 10 to 35 nm of Mn_3O_4 nanoparticles (Karimi and Eshraghi, 2017)

However, chemical and physical factors such as pH, temperature, time of the process, reducing agent have been mentioned to interfere with NP synthesis. Acid pH values are pointed to induce NPs aggregation in the process of reduction. Larger number of functional groups that bind and nucleate metal ions become accessible at pH 3.0 and 4.0 compared with pH 2.0. And at pH 2.0, the most accessible metal ions are involved in a smaller number of nucleation events, which leads to the agglomeration of the metal (Singh et al., 2016). The pH value influenced the size of zero-valent iron nanoparticles, at pH= 3 the average size was 23±12 nm, whereas the particle distribution under pH of 9 was 45±4.8, 197.5±28.9 and 895.8±90.7 nm (Desalegn et al., 2019). Therefore, magnetic properties of the nanoparticles tend to agglomerate in water rapidly and form micron size or larger aggregates (Lu et al., 2007; Smuleac et al., 2011). This tendency was also observed by Smuleac et al. (2011) when studied bimetallic nanoparticles using Iron with another metallics system as Fe/Pt, Fe/Ag, Fe/Cu, Fe/Ni, and Fe/Pd. Besides size, pH also seems to influence the nanoparticle composition. Generally, Manganese forms structural and compositional variants oxides such as MnO, Mn_2O_4, Mn_3O_4, MnO_2 as well as the metastable Mn_5O_8 (Sanfélix, 2016). This diversity of Mn oxides depends primarily on temperature and pH. At pH of 4–6 release of MnO and Mn_2O_3 is dominated, while pH above 7 favors the formation of manganese hydroxides and oxyhydroxides. Also, a temperature below 90 °C induces most of the Mn+ ions oxidation to form Mn^{2+} and Mn^{3+}, resulting in a mixture of Mn oxides as MnO and Mn_2O_3 (Prasad, 2017).

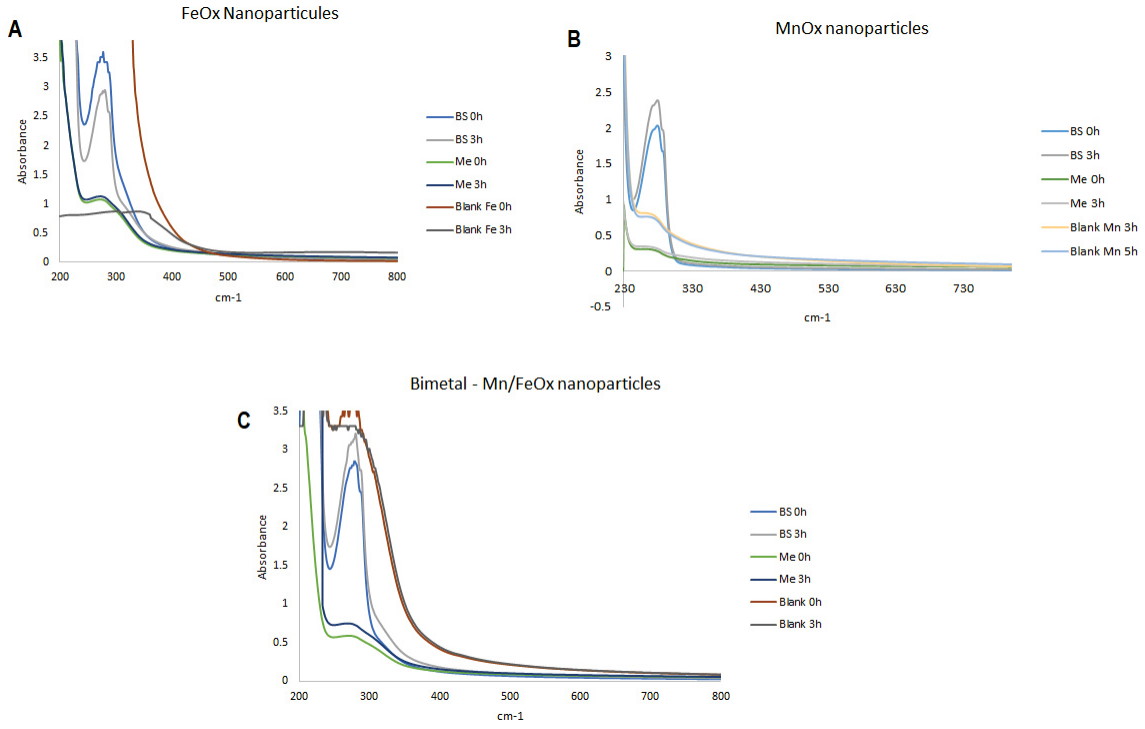

Figure 24. UV-vis spectra analysis of iron and manganese nanoparticles (200-800 nm) using bacterial supernatant (BS) with auxin compounds, the basal medium (Me) and its respective salts (Blank), from 0 and 3h of synthesis. (A) Iron nanoparticles (FeOx) using 1mM of $FeCl_3$; (B) Manganese nanoparticles (MnOx) using $Mn(NO_3)_2$ and; (C) Bimetallic manganese/iron nanoparticles (Mn/FeOx) mixing 1mM of FeCl3 + 1mM of $Mn(NO_3)_2$. Peaks around 250-300 nm confirm the NPs synthesis.

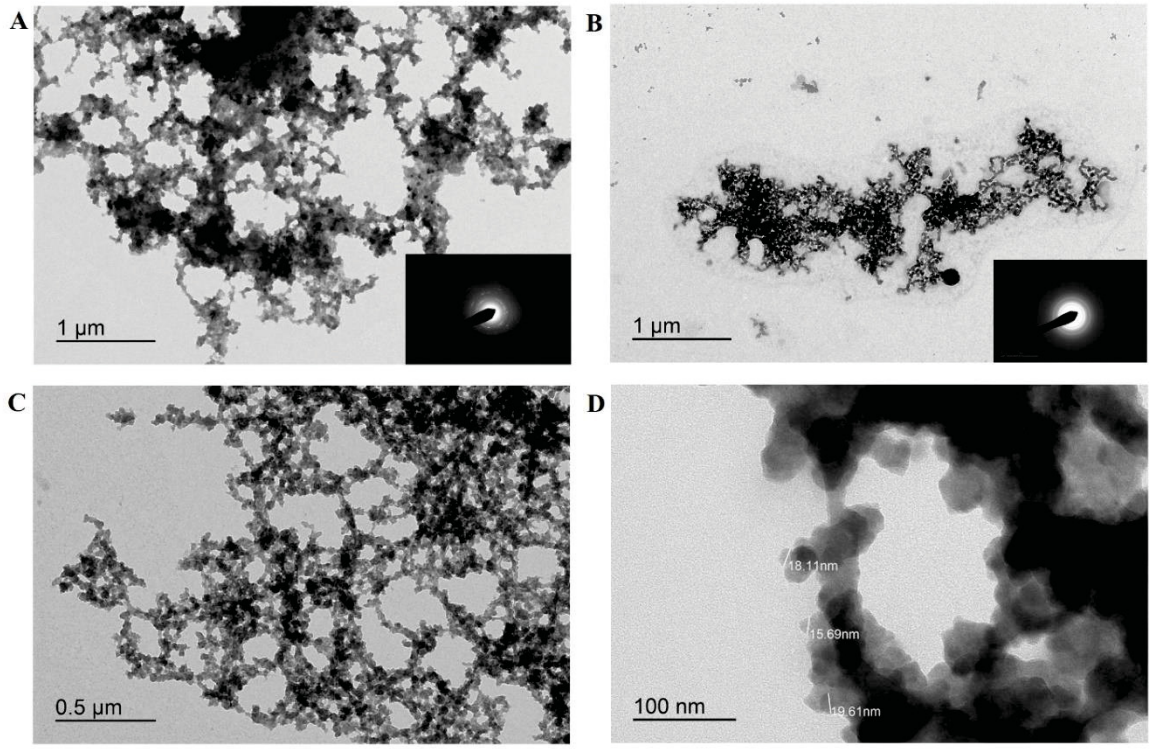

Figure 25. TEM Images of (A) iron NPs (B) manganese NPs with its respective SAED, and (C) and (D) Manganese/iron NPs.

Analyzing the FT-IR spectrum helps in identifying specific functional groups present in the synthesized NPs which play the roles of capping agent and reducing agent. As shown in figure 27, there are various reflectance signals located at specific wavenumbers (cm^{-1}) for the auxin substance used and the corresponding synthesized iron and manganese NPs, reinforcing the hypothesis that the auxin molecules reduced the salts into their nano-size and capped the surface of the NPs (Kumar et al., 2013).

The strong and broad peak at ~3397.8 cm^{-1} indicates the presence of hydroxyl groups (O-H stretching) in aliphatic and aromatic structures (Boeriu et al., 2004), and 2944.4 cm^{-1} can be attributed to C-H aliphatic stretching (Singh and Dhepe, 2016), and 2890.5 cm^{-1}, 2085.7 cm^{-1} and 1407 cm^{-1} may be due to –CH2 stretching, =C-H stretching and –C-H stretching vibrations (Kumar and Sangwan, 2013). The peak at 1646.5 cm^{-1} can be assigned to C=C (Chandran et al., 2016). The peaks at 1042.9-1017.4 cm^{-1} can be attributed to various vibrations like C-O-C, C-H and C=O (Gosselink et al., 2004; Gottimukkala et al., 2017), 1330.5 cm^{-1} to -CN bending, 1106.6 cm^{-1} to C-O stretching (Chandran et al., 2016). Furthermore, adsorption bands at around 600-400 cm^{-1} may refer to metal-O absorption stretches of the produced FeOx-NPs and MnOx-NPs (Smuleac et al., 2011; Wang et al., 2014; Yang et al., 2010). Similarly, the confirmation of Fe NPS was observed in broad absorption at a wavelength around 500 nm-600 nm by Huang et al. (2014), Kumar et al. (2013) and Herrera-Becerra et al. (2010). Using gallic acid and tannic acid to iron NP synthesis, Herrera-Becerra et al. (2010) reported maximum absorption at 527 nm and 544 nm for iron oxide NPs, respectively.

In the case of Mn NPs, absorbances around at 515, 480 and 339.60 cm^{-1} were characteristics to manganese oxide metal nanoparticles bands (Kumar and Sangwan, 2013), and wavelengths 597 nm and 642 nm to the absorption by Mn_3O_4 (Prasad, 2017). Significant peaks were observed between 400-650 cm^{-1} spectra. Similarly, Ullah et al. (2017) described three significant peaks in the range of 400–650 cm-1 attributed to Mn_3O_4 NPs. The first absorbance at 624.8 cm-1 was referred to be Mn-O stretching modes in tetrahedral sites, the vibration frequency located at 526.5 cm-1 as characteristic to the Mn-O distortion vibration in an octahedral environment, and vibration at 415.6 cm^{-1} was ascribed to the Mn species (Mn^{3+}) vibration in the octahedral site of Mn_3O_4.

Table 15. Absorption band obtained through Fourier Transform Infrared Resonance (FTIR) spectrum of iron and manganese nanoparticles reduced by auxin (indol-3-acetic acid).

Wavenumber (cm-1)	Functional group
3397.8	O-H group
2944.4	C-H aliphatic hydrocarbon
2890.5	–CH2
2085.7	=C-H
1646.5	C=C aromatic ring
1407.7	–C-H
1330.5	-CN for aromatic amines
1113.7	C-O
600-400	Metal-O (Fe-O; Mn-O)

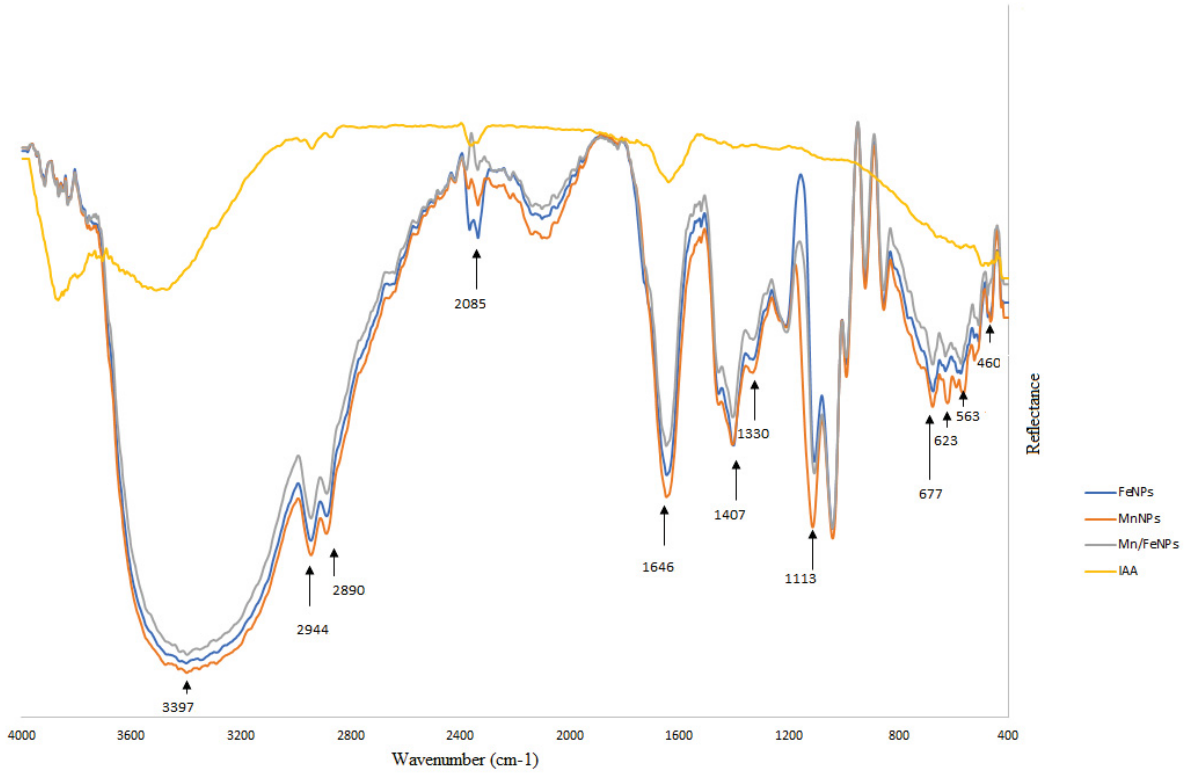

Figure 26. FTIR spectra of iron nanoparticles, manganese nanoparticles, bimetallic manganese/iron nanoparticles and indol-3-acetic acid pattern.

The NPs can be applied as seed treatments, by foliar spray, or directly in soil (Shivani et al., 2018). Plant-NPs communication occurs by chemical interaction involving membrane transport action, active oxygen species construction, lipid peroxidation, ion-cell disorder, and oxidative-breakage, leading to various physiological and structural changes (Pandey, 2018). Due to the small size, their large

surface area and essentially an extremely high surface-to-mass ratio, nanoparticles have a unique behavior and can modify the physicochemical properties of the materials or organisms (Kaur et al., 2018; Mazumdar and Haloi, 2011). This means the resultant nano-fertilizer can easily pass through the membranous structure in plant cells, and cover more parts of the plant for nutrition with the same volume, allowing the nutrients delivery at their site of action, with slow-release, therefore increasing the efficiency fertilization (Feregrino-Perez et al., 2018; Shankramma et al., 2016; Shivani et al., 2018).

Using from the beginning as seed treatments coat, NPs are likely to break-through into seeds, by its surface nanopores, facilitates more diffusion of water and oxygen penetration and boost the nutrient availability and accessibility to the growing seedling, improving seed germination, root and shoot length, and overall plant growth, quality and yield (Khodakovskaya et al., 2011; Pandey, 2018; Shivani et al., 2018). Thinking on the molecular and genetic levels, the effect of nano-materials on plant physiology, Khodakovskaya et al. (2011) demonstrated that multiwall carbon nanotubes (CNTs) could act as an external factor to induce/alter gene expression on tomato seedlings. They reported upregulation of genes involved in the regulation of transcription and hormone pathways (Les.17.1.S1—transcription factor; Les.85.1.S1—ethylene receptor; Les.3703.1.S1—IAA9 protein), expression of mitogen-activated protein kinase (Les.699. 1.S1), which is related in the control of plant cell division and growth and have important functions in stress signal transduction pathways in plants. It is also involved in the activation of many stress-related genes including the gene for tomato water-channel protein (LeAqp2), which have a significant impact on the observed phenomena of activation, enhancement of germination and growth of tomato seedlings on a medium supplemented with CNTs (Khodakovskaya et al., 2011).

In our experiments, the resulted NPs positively influenced the soy seed germination and plant growth, showing more vigor particularly on treatments with the basal medium (Fig. 28). Seed germination was 100 % for IAA, BS-FeO_x, Me-MnO_x and Me-FeO_x treatments, higher than control (90%), BS-MnO_x (90%) and Me-MnO_x (81%) treatments, although with no statistical difference. FeO_x-NPs increased root number, similar to IAA solution and bimetal MnO_x/FeO_x-NPs, although differing statistically only from Me-MnO_x-NPs. Medium FeO_x-NPs increased root growth, plant height and fresh weight with similar values from bacterial supernatant bimetallic MnO_x/FeO_x-NPs.

Furthermore, BS bimetallic showed best results at all evaluated parameters, including root number and germination, if we compare the overall experiment (Table 16). NP-s had improved root development similar or even higher than IAA solution, and higher than the negative control, especially when applied BS MnO_x/ FeO_x NPs and Me-FeO_x NPs. The positive effects on plant growth using medium NPs solution can also result from the high quantities of nitrogen provided from CSL. Meanwhile, this is a preliminary evaluation, a long-term experiment must still be conducted to observed the real effects on plant growth.

Yet, the differences in the physiological response may be related to the different size, shape, the level of aggregation, type of charge of the functional group in the surface of applied, crystallinity and other properties for bioeffects that each nano-materials are synthesized (Khodakovskaya et al., 2013). Shankramma et al. (2016) reported *Solanum lycopersicum* seed germination enhancement and plant growth when applied Fe_2O_3 NPs over to the control. FeNPs supplementation was linked with the increased amount of Fe^{total} (=Fe^{2+} and Fe^{3+}) in plants (Shankramma et al., 2016). Early-stage, during the initial heterotrophic phase, iron influx and efflux have been identified to be essential for young seedlings germinating and development (Briat et al., 2007; Kim et al., 2006; Lanquar et al., 2005). Later on, in plants, iron storage and metabolism are involved in the photosynthetic apparatus where is essential for the biosynthesis of cytochromes electron transport system and various heme- and [Fe–S] cluster-containing proteins (Askary et al., 2017; Briat et al., 2007).

Table 16. Evaluation on maize seed germination and plant growth using iron and manganese, mono and bimetallic nanoparticles synthesized using bacterial supernatant containing auxin and basal medium.

Treatment	Germination (%)	Leaves number	Root number	Root lenght (cm)	Shoot height (cm)	Fresh weight (g)
1 - CN	90^{ab}	3.27±0.79	**5.72±1.75^{ab}**	16.65±3.32^{c}	22.02±4.71	1.73±0.62^{ab}
2 – PC	100^{a}	3.45±0.49	**6.09±0.67^{a}**	19.75±1.70^{abc}	22.77±2.15	1.86±0.22^{ab}
3 - BS-FeOx	100^{a}	3.45±0.5	**6.27±0.66^{a}**	20.82±2.71^{abc}	23.58±3.24	1.73±0.21^{ab}
4- BS-MnOx	90^{ab}	3.45±0.79	5.09±1.37^{ab}	19.16±4.49^{bc}	22.68±4.66	1.62±0.36^{b}
5 - BS-MnOx/FeOx	100^{a}	**3.54±0.49**	**6.09±0.83^{ab}**	**21.85±1.93^{ab}**	**25.93±2.74**	**2.03±0.48^{ab}**
6- Me-FeOx	100^{a}	3.45±0.49	5.27±1.02^{ab}	**24.35±3.05^{a}**	**26.89±3.73**	**2.27±0.52^{a}**
7- Me-MnOx	100^{a}	3.27±0.79	4.81±1.17^{ab}	20.02±4.79^{abc}	22.47±5.39	1.92±0.59^{ab}
8- Me- MnOx/FeOx	81^{b}	3.09±1.15	4.54±1.75^{a}	18.36±6.67^{bc}	22.11±8.18	**2.01±0.88^{ab}**

(1) CN: negative control (water); (2) PC: positive control (auxin solution 50 µg.ml^{-1}); (3) BS-FeOx – 1% bacteria supernatant FeOx NP; (4) Bs-MnOx – 1% bacteria supernatant MnOx NP; (5) BS-MnOx/FeOx – 1% bacteria supernatant bimetallic MnOx/FeOx NP; (6) Me-FeOx – 1% Medium FeOx NP; (7) Me-MnOx – 1% Medium MnOx NP; (8) Me-MnOx/FeOx – 1% Medium bimetallic MnOx/FeOx NP.

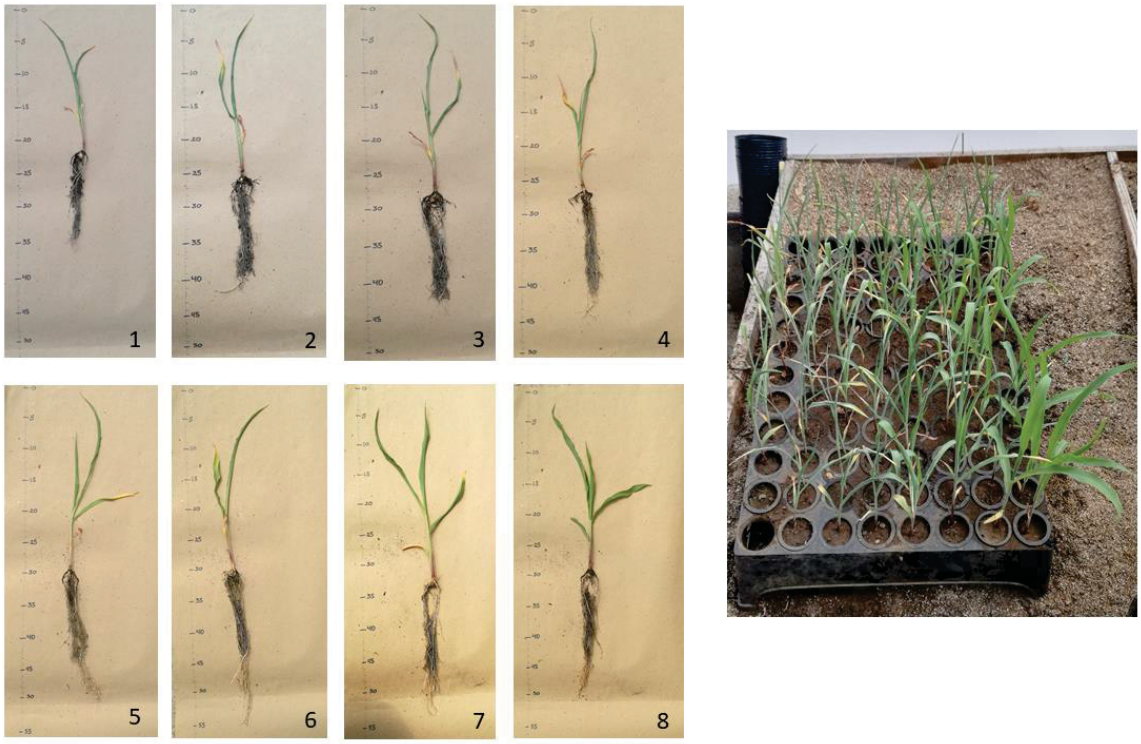

Figure 27. Maize plantlets after 21 days of germination in green house. The seeds were submitted to different treatments: (1) CN: negative control (water); (2) PC: positive control (auxin solution 50 µg.ml^{-1}); (3) BS-FeOx – 1% bacteria supernatant FeOx NP; (4) Bs-MnOx – 1% bacteria supernatant MnOx NP; (5) BS-MnOx/FeOx – 1% bacteria supernatant bimetallic MnOx/FeOx NP; (6) Me-FeOx – 1% Medium FeOx NP; (7) Me-MnOx – 1% Medium MnOx NP; (8) Me-MnOx/FeOx – 1% Medium bimetallic MnOx/FeOx NP.

Root application of Fe-NPs was reported to have a positive effect on plant growth in water-melon (*Citrullus lanatus*), it also increased seed germination, root growth, chlorophyll content (Li et al., 2013). In peanut (*Arachis hypogaea)* plants it increased root length, plant height, biomass, and chlorophyll levels (Rui et al., 2016). Fe2O3 NPs have enhanced the seed germination and plant growth of *S. lycopersicum* plant (Shankramma et al., 2016). And no visible signs of phytotoxicity are evident in *C. maxima* leaves under all treatments (Hu et al., 2017). This reports enforces that Fe-NPs can possibly replace traditional iron fertilizers and an effective fertilizer for the alleviation of Fe-deficiency in plants (Hu et al., 2017; Shankramma et al., 2016).

Few reports mention the use of manganese oxide nanoparticles as nano-fertilizer. They are most attractive in catalysis applications, ion exchange, molecular adsorption, biosensor, and particularly, energy storage (Kumar and Sangwan, 2013). In the meantime, a full study reported the importance to use MnNPs as plant micronutrient source nano-fertilizer without disturbing ROS-antioxidative equilibrium. Pradhan et al. (2013) demonstrated the photosynthetic efficacy of stable MnNPs on leguminous plant mung bean (*Vigna radiata*) when compared with commercially available manganese salts. Similar to our results, MnNPs was effective and increased root and shoot length, fresh and dry weight of MnNP-treated plants with respect to control treatment. Likewise, did not show any toxicity symptoms neither in leaf nor in root at higher concentration, no ultrastructural abnormalities or any disruption to the structural integrity, of chloroplasts system of plant tissues, no significant changes in protein and lipid contents in both treated leaf and root. Therefore, they also evaluated MnNP biosafety toward beneficial soil microorganism (*Trichoderma viride*) and observed no growth retardation on the microbial community, and no animal complications when acute oral toxicity tests in the mice model, with no death, recorded.

Furthermore, when compared to many other metallic NPs, iron and manganese oxide nanoparticles are constant, less expensive and appear to show no toxicity effects on plant physiologies, when used at lower concentrations (in the range of 50-200 $mg.L^{-1}$) (Askary et al., 2017; Shankramma et al., 2016). Assessing the effects of laboratory-prepared of micronutrients oxide NPs in low concentrations (< 50 ppm) on the germination of lettuce seeds in a water medium, Liu and Lal (2015) showed that MnO_x and FeO_x NPs were less toxic than Cu and Zn NPs and also significantly stimulated the growth of the seedlings by 12–54%.

Herein in this study, we proposed a green synthesis of iron and manganese nanoparticles using bacteria fermented supernatant containing auxin complex compounds as a redactor agent, to be a suitable alternative to be used as micronutrients fertilizers source for crop production. The NPs biosynthesis using auxin complex was successfully achieved, using a rapid and easy method. The NPs showed spherical shape and agglomerated. The NPs showed positive effects on maize seedling growth especially the bimetallic NPs, increasing seed germination, root development, and fresh weight.

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CONCLUSION

In general, we can conclude that the metagenomic analysis revealed a mix of endophytes community from the in vitro Eucalyptus explants. And, from the isolated endophytes, some had shown the ability to produce auxin. Two isolates with higher auxin production were chosen for further analysis and demonstrated ability of other plant growth promotion mechanisms such as nitrogen fixation, phosphate solubilization, and ammonium production.

Corn steep liquor and glycerol showed to be suitable as nitrogen and carbon sources for auxin production by the endophytic strains. This resulted in the development of a low-cost medium. The results obtained for auxin production by *Bacillus megaterium* DEBB B-353 and *Paenibacillus polymyxa* DEBB B-358 were the highest amount reported until now. When evaluated in seed treatments, all endophytic strains and their respective produced auxins, showed to promote plant growth and development, indicating that they can be used as bioinoculants for crop production. Furthermore, bacteria fermented supernatant containing auxin could reduce iron and manganese salts and produce mono and bimetallic nanoparticles, which later on showed to promote plant growth.

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