Effect of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase in the plant growth-promotion abilities of bacterial endophytes and β-rhizobia

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“Tenho em mim todos os sonhos do Mundo”
Fernando Pessoa
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Life really has a way of surprising us and taking us where we belong.

My first years in Técnico were nothing but a struggle. A struggle in which I tried to catch up the lost time, burying myself in extra classes and extra work. All because the super-hard-on-herself person I once were thought that finishing the course one year later would be too much of a disappointment. Never was I so wrong. The constant struggle led to constant failure and loss of confidence. It was only when I gave myself permission to be free that truly amazing things started to happen. In only one year I finally had no subjects behind, I raised my grades, and suddenly found myself simultaneously involved in many different projects that made me feel successful, active, and even grateful for my previous failures. I learned so many new things and grew up so much that I finally built up the confidence to know that I am now (still) a super-hard-on-herself person, but with a better judgement, and able to overcome any rocky path ahead of me.

Despite this newly built-up confidence, I still did not know what my passion really was. I only knew I would be successful, no matter what. And I was really convinced that scientific investigation was not for me. Well… today I am convinced of the opposite. When I chose Brazil, and Floripa, to do my Master Thesis research, my only good reasons were, to be honest, the great weather and… the paradisiac island, of course. Nowadays, I am really glad I chose Brazil. Not only because of the good weather, but for the reason that I was able to experience realities so different from our own. I would never have gained such an experience if I had done my practical work in a European country. Besides, I found a line of investigation that I really care about. I was amazed every time I could see the wonderful bacterial effects on the plants I nourished as my own children. I believe in this line of investigation, and I believe it can make a difference in the world. I believe I can make a difference.

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Resumo

As práticas agrícolas, através do uso de fertilizantes químicos e outros poluentes, são responsáveis por danos ambientais, sendo necessárias alternativas a estes produtos. A solução pode residir na utilização de “bactérias promotoras do crescimento de plantas”, plant growth-promoting bacteria (PGPB), que se encontram naturalmente presentes nos solos e nos tecidos vegetais, sendo capazes de modular o desenvolvimento das plantas. A nodulação de legumes mediada por rizóbios, e a colonização endofítica são exemplos de importantes associações simbióticas entre plantas e bactérias. A presença da enzima 1-aminociclopropano-1-carboxilato (ACC) desaminase é um elemento chave nas capacidades de PGPB, uma vez que é responsável pela diminuição dos níveis de etileno, que sobretudo em condições de stress, limitam o desenvolvimento de plantas.

O objetivo principal desta tese é o estudo do papel da ACC desaminase nas capacidades de bactérias endófitas e de um β-rizóbio em promover o desenvolvimento da planta, e a sua nodulação. Neste trabalho, duas PGPB, uma endófita e um β-rizóbio foram transformadas para expressar ACC desaminase. Para além destas, uma estirpe endófita e o seu mutante acdS foram usados como referência. As bactérias que expressaram ACC desaminase foram capazes de aumentar o seu potencial simbiótico e promover eficientemente o crescimento das plantas testadas. Estas bactérias conferiram também um aumento da resistência ao stress, e diminuíram a taxa de senescência de uma flor sensível ao etileno.

Este trabalho trouxe novas perspetivas relativamente ao papel e uso de bactérias produtoras de ACC desaminase, que podem ser fundamentais para uma vasta gama de aplicações agrícolas e biotecnológicas.

Palavras-Chave: Ambiente, Bactérias promotoras do crescimento de plantas, Etileno, ACC desaminase, Endófita, Rizóbio
Abstract

Eco-friendly alternatives to the use of chemical fertilizers and other pollutants are necessary to minimize the environmental damage caused by agricultural practices. One solution may reside on the use of plant growth-promoting bacteria (PGPB) that are naturally present in soils and plant tissues, producing a wide range of compounds and capable of modulating plant development. Rhizobia nodulation of legumes and endophytic colonization are examples of important symbiotic associations between plants and bacteria. The presence of bacterial enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase is a key feature in the PGP abilities of these bacterial strains, since it can decrease deleterious ethylene levels that are extremely harmful to the plant host, especially under stress conditions.

The main objective of this thesis is the study of the role of ACC deaminase in the PGP abilities of bacterial endophytes and a β-rhizobium, particularly in their ability to promote plant development and nodulation. In this work, one endophyte and one β-rhizobium were transformed to express ACC deaminase. Also, an endophytic strain and its ACC deaminase knockout were used as reference. Bacteria expressing ACC deaminase were able to increase their PGP abilities and effectively promote the growth and development of the tested plants, as well as to enhance the nodulation process. ACC deaminase-producing bacteria were also able to increase plant resistance to stress, and decrease the senescence rate of an ethylene-sensitive flower.

This work brought new insights into the role and use of ACC deaminase-producing bacteria, which may be the key to a wide range of agricultural and biotechnological applications.

Keywords: Environment, Plant growth-promoting bacteria, Ethylene, ACC deaminase, Endophyte, Rhizobia
Contents

Acknowledgments ............................................................................................................. vii
Resumo ............................................................................................................................. ix
Abstract ............................................................................................................................. xi
List of Tables ..................................................................................................................... xvi
List of Figures ................................................................................................................... xix
List of Acronyms .............................................................................................................. xxi

General Introduction ........................................................................................................... 1
State of the Art ..................................................................................................................... 3
1 World population and food demand .............................................................................. 3
  1.1 Plant development and plant-growth-promoting bacteria ...................................... 3
  1.2 Ethylene, a powerful modulator of plant growth and development ...................... 5
    1.2.1 Ethylene biosynthesis ....................................................................................... 5
    1.2.2 Plants response to stress. The stress ethylene model ...................................... 6
    1.2.3 Ethylene and flowering .................................................................................... 7
2 ACC deaminase – a key to modulate ethylene levels ................................................... 8
  2.1 ACC deaminase biochemical properties ................................................................... 8
  2.2 ACC deaminase origin and distribution .................................................................. 9
  2.3 Transcriptional regulation of the acdS gene .......................................................... 10
  2.4 Bacterial modulation of ethylene levels by ACC deaminase ................................... 11
  2.5 ACC deaminase expression levels .......................................................................... 13
3. Rhizobia and endophytes: distinct categories of ACC deaminase-producing bacteria…… 13
  3.1 Rhizobia nodulation, ethylene and ACC deaminase effect .................................... 14
    3.1.1 Rhizobia and nodulation process ..................................................................... 14
    3.1.2 Ethylene effects in the nodulation process ..................................................... 16
    3.1.3 ACC deaminase in rhizobia and nodulation process ...................................... 17
  3.2 Endophytic colonization, ethylene and ACC deaminase effects ............................. 18
    3.2.1 Endophytic colonization ................................................................................ 18
    3.2.2 Ethylene role in bacterial endophytic colonization ......................................... 20
    3.2.3 ACC deaminase in endophytic colonization .................................................. 21
  3.3 Co-inoculation of legumes with rhizobia and other ACC deaminase-producing PGPB 21
4. Bacterial transformation with the acdS gene ............................................................... 22
Objectives and Motivation ................................................................................................. 23
Materials and Methods ..................................................................................................... 25
1. Transformation of endophytic and β-rhizobia strains to express ACC deaminase ....... 25
   1.1 Selection of strains ............................................................................................... 25
   1.2 Triparental conjugation method ......................................................................... 25
2. Determination of ACC deaminase enzymatic activity ........................................... 26
   2.1 Preparation of standard α-ketobutyrate solutions ......................................... 26
   2.2 Induction of ACC deaminase activity ............................................................. 27
   2.3 Sample preparation ......................................................................................... 27
   2.4 Quantitative measuring of produced α-ketobutyrate ....................................... 27
   2.5 Total protein quantification ............................................................................ 28

3. Studies on the role of ACC deaminase in plant-growth promotion abilities of endophytes. 28
   3.1 Cucumber plant-growth promotion assay ....................................................... 28
      3.1.1 Bacterial cell culture preparation .............................................................. 28
      3.1.2 Seed disinfection, germination and inoculation .......................................... 29
      3.1.3 Assay conditions ...................................................................................... 29
   3.2 Common bean nodulation and plant-growth promotion assay ......................... 29
      3.2.1 Bacterial cell culture preparation .............................................................. 30
      3.2.2 Seed disinfection, germination and inoculation .......................................... 30
      3.2.3 Assay conditions ...................................................................................... 30
      3.2.4 Root and nodule imaging ......................................................................... 31

4. Evaluation of the ACC deaminase effect in Cupriavidus taiwanensis STM894 nodulating abilities in Mimosa pudica ................................................................. 31
   4.1 Bacterial cell culture preparation .................................................................... 31
   4.2 Seed disinfection, germination and inoculation ................................................ 31
   4.3 Assay conditions ............................................................................................ 32

5. Evaluation of the ACC deaminase effect in the strain BXF1 ability to decrease the senescence of carnation flowers ................................................................. 32
   5.1 Bacterial cell culture preparation .................................................................... 32
   5.2 Assay conditions ............................................................................................ 32
   5.3 Evaluation of BXF1 and BXF1 pRKACC flower colonization by PCR Reaction ...... 33
      5.3.1 DNA extraction ....................................................................................... 34
      5.3.2 Primer design and PCR conditions ........................................................... 34
   5.4 Statistical analysis ......................................................................................... 34

Results and discussion .......................................................................................... 35
1. Obtaining and measuring of ACC deaminase activity in transformed strains ................ 35
2. Effect of ACC deaminase in bacterial endophyte’s ability to promote cucumber growth ................................................................................................................. 36
3. Effect of endophytic bacterial ACC deaminase production in common bean nodulation, growth and development ................................................................. 41
   3.1 ....... ACC deaminase effect in S. quinivorans BXF1 ability to promote bean growth and nodulation ............................................................. 41
   3.2 ....... ACC deaminase effect in P. fluorescens YsS6 ability to promote bean growth and nodulation ................................................................................... 45
4. The effect of ACC deaminase in the plant-growth promotion and nodulation process mediated by C. taiwanensis STM894 ................................................. 48
5. Evaluation of the ACC deaminase effect in the strain BXF1 ability to decrease the senescence of carnation flowers .................................................... 50
List of Tables

Table 1 - Characteristics of strains used in Triparental Conjugation. ........................................26
Table 2 - ACC deaminase activity values obtained in the transformed strains. Values refer to quantity of α-ketobutyrate (µmol), total protein (mg/mL) and total enzymatic activity (µmol α-ketobutyrate/mg protein/h). ........................................................................................................35

Appendix A - Bacterial Strains used in this Work
Table 3 - Bacterial Strains used in this work.................................................................63

Appendix C - Bacterial Characterization
Table 4 – Methods of bacterial characterization.........................................................67
Table 5 – Results of bacterial characterization.........................................................69
List of Figures

Figure 1 – U.S Census Bureau 2016 forecast of the world population by 2050. ........................................ 3
Figure 2 – Plant growth as a function of its age. .......................................................................................... 4
Figure 3 - Ethylene biosynthesis pathway. ................................................................................................. 6
Figure 4 - Plant ethylene production as a response to stress. ................................................................. 7
Figure 5 - Enzymatic reaction catalyzed by ACC deaminase. ................................................................. 9
Figure 6 - Model for the transcriptional regulation of ACC deaminase expression in Pseudomonas sp. UW4. ........................................................................................................................................ 11
Figure 7 – Schematic model representing how bacteria that produce both IAA and ACC deaminase can facilitate plant growth. ........................................................................................................ 12
Figure 8 - Lowering of stress ethylene by ACC deaminase-producing bacteria. .................................... 13
Figure 9 - Nodule organogenesis and infection process. ........................................................................ 16
Figure 10 - Endophytic modes of access into different plant tissues. ..................................................... 20
Figure 11 - Scale of senescence symptoms of carnation flower .............................................................. 33
Figure 12 – Schematic representation of the flower sections used for DNA extraction. ...................... 33
Figure 13 - Results obtained in the cucumber plate assay in plants inoculated with strain BXF1 or BXF1pRKACC, under normal and stress conditions (ISB). ......................................................... 37
Figure 14 – Results obtained in the cucumber pot assay in plants inoculated with strain BXF1 or BXF1pRKACC, under normal and stress conditions (ISB). ......................................................... 38
Figure 15 - Results obtained in the cucumber plate assay in plants inoculated with strain YsS6 or YsS6 acdS-, under normal and stress conditions (ISB). ......................................................... 39
Figure 16 - Results obtained in the cucumber pot assay in plants inoculated with strain YsS6 or YsS6 acdS-, under normal and stress conditions (ISB). ......................................................... 40
Figure 17 – Mean dry weight per plant of the co-inoculation assay of R. tropici CIAT 899 and S. quinivorans BXF1 wild-type and pRKACC in common bean. .......................................................... 42
Figure 18 – Presence of C. flaccumfaciens isolated in tissues of common bean. .................................. 42
Figure 19 - Common bean plants (A) and disease symptoms (B) obtained in the co-inoculation assay of R. tropici CIAT 899 and S. quinivorans BXF1 wild-type and pRKACC, 20 days after inoculation... ................................................................................................................. 43
Figure 20 – Nodulation assay in common bean with co-inoculation of R. tropici CIAT899 and S. quinivorans BXF1, 20 days after inoculation.................................................................................. 44
Figure 21 - Confocal Microscope Imaging of common bean tissues ....................................................... 44
Figure 22 – Mean dry weight per plant of the co-inoculation assay of R. tropici CIAT 899 and P. fluorescens YsS6 wild-type and acdS- in common bean. ........................................................................ 45
Figure 23 - Common bean plants (A) and disease symptoms (B) obtained in the co-inoculation assay of R. tropici CIAT 899 and P. fluorescens YsS6 wild-type and acdS-, 20 days after inoculation. ................................................................................................................. 46
Figure 24 – Nodulation assay in common bean with co-inoculation of R. tropici CIAT899 and P. fluorescens YsS6, 20 days after inoculation.................................................................................. 47
Figure 25 - *Mimosa pudica* assay inoculated with *C. taiwanensis* STM894. .......................... 49

Figure 26 - Differences in the number of nodules obtained in *Mimosa pudica* inoculated with STM894 and STM894 pRKACC.................................................. 50

Figure 27 - Variation of the senescence symptoms presented by carnation flowers inoculated with BXF1 and BXF1 pRKACC, during 8 days........................................... 51

Figure 28 – Carnation flowers after 8 days of incubation................................................. 51

Figure 29 – Results obtained after gel electrophoresis of the amplified DNA products......... 52

Appendix B - Scale of Disease Symptoms in common bean

Figure 30 – Scale of disease symptoms on common bean, caused by the pathogen *Curtobacterium flaccumfaciens*................................................................. 65
### List of Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ACC</td>
<td>1-aminocyclopropane-1-carboxylic acid</td>
</tr>
<tr>
<td>acdR</td>
<td>gene coding for Leucine Responsive Protein</td>
</tr>
<tr>
<td>acdS</td>
<td>gene coding for ACC deaminase</td>
</tr>
<tr>
<td>ACS</td>
<td>enzyme ACC synthase</td>
</tr>
<tr>
<td>AOA</td>
<td>Aminoxyacetic acid</td>
</tr>
<tr>
<td>AON</td>
<td>Autoregulation of nodulation</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>AVG</td>
<td>Aminoethoxyvinylglycine</td>
</tr>
<tr>
<td>CRP</td>
<td>Cyclic AMP receptor protein</td>
</tr>
<tr>
<td>FAO</td>
<td>Food and Agriculture Organization</td>
</tr>
<tr>
<td>FNR</td>
<td>Fumarate and nitrate reductase</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>HGT</td>
<td>Horizontal Gene Transfer</td>
</tr>
<tr>
<td>IAA</td>
<td>Indole-3-acetic acid</td>
</tr>
<tr>
<td>ISB</td>
<td>Isoxaben</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani medium</td>
</tr>
<tr>
<td>LRP</td>
<td>Leucine-responsive regulatory protein</td>
</tr>
<tr>
<td>MTA</td>
<td>5′-Methylthioadenosine</td>
</tr>
<tr>
<td>NBD</td>
<td>2,5-norbornadiene</td>
</tr>
<tr>
<td>nod</td>
<td>nodulation gene</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PGPB</td>
<td>Plant growth-promoting bacteria</td>
</tr>
<tr>
<td>PLP</td>
<td>Pyridoxal-5-Phosphate</td>
</tr>
<tr>
<td>r.p.m</td>
<td>Rotations per minute</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosylmethionine</td>
</tr>
<tr>
<td>STS</td>
<td>Silver Thiosulphate</td>
</tr>
<tr>
<td>TSB</td>
<td>Tryptic Soy Broth medium</td>
</tr>
<tr>
<td>YMB</td>
<td>Yeast Mannitol Broth medium</td>
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</table>
General Introduction

With the continuous and expected growth of the world’s population, greater levels of industrialization and agricultural practices are a direct consequence, leading to inevitable environmental damage. Feeding this growing population in a sustainable and environmentally friendly manner is, thus, a challenge. The application of traditional fertilizers, pesticides and herbicides is known to cause extreme environmental damage, due to, among other harnesses, their ability to migrate into our drinking water supplies, as well as infiltrate into water bodies, consequently changing natural ecosystems [1].

An alternative for the use of these chemicals may arise with the application of plant growth-promoting bacteria (PGPB). By being naturally present in soils and plant tissues and producing a wide range of compounds, these bacteria positively modulate plant development, and therefore, can be used as an environmental friendly alternative to the use of pollutant fertilizers. The symbiosis found between rhizobia and legumes is one of the most studied examples regarding the importance of bacteria when sustainable agricultural practices are concerned [2]. These bacteria, when associated with legumes, are able to form root nodules and fix atmospheric nitrogen (N₂), solubilizing it into ammonia (NH₄⁺), which can then be further used by the plant [2]. Besides rhizobia, which inhabit roots and the rhizosphere (immediate portion of soil surrounding the roots), there are other PGPB, known as endophytes, that are able to colonize plant internal tissues such as roots, shoots, leaves, flowers and fruits [3][4]. This ability may present an advantage when compared to rhizospheric bacteria, as endophytes are protected from the competitive, high-stress environment of the soil [4].

When facing stress conditions, such as flooding, drought, high salt concentrations, organic contamination, or bacterial and fungal pathogens, the plant is subjected to a series of events that cause hormonal alterations, which may lead to plant senescence, and ultimately, death. The phytohormone ethylene, found in all higher plants, is an important modulator of normal plant growth and development, and has the leading role when stress response is concerned [5]. In leguminous plants, this phytohormone is also known for its negative effects in the nodulation process, initiated by rhizobia, by inhibiting the formation and functioning of the nodules [6][7].

Some PGPB possess what can be called a key feature in contributing to plant growth, which is the production of the 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme. This enzyme is responsible for the breakdown of ACC, the direct precursor of ethylene, into ammonia and α-ketobutyrate, hence, decreasing the deleterious ethylene effects [8]. Bacteria expressing ACC deaminase have been proven to lower the impact of various stresses on plants. Furthermore, transformation of strains with ACC deaminase (acdS) genes and their regulatory regions, has been proven to increase symbiosis potential in many PGPB, by enhancing plant tolerance to several types of stresses [9],[10].

To understand the main mechanisms employed by these bacteria and the importance of the enzyme ACC deaminase in promoting plant growth is, therefore, an essential task and an important step towards their acceptance as suitable and effective adjuncts to agricultural practice.
State of the Art

1. World population and food demand

According to the U.S Census Bureau, by 2050 the world population will have reached the 9 billion mark, moving from to the actual 7 billion in only a few decades. To feed this growing population, Food and Agricultural Organization of the United Nations (FAO) consider that world food production must increase in 70%, and that food production in the developing world must double [11].

The projected growth of food production comes with an increase of industrial and agricultural practices, which historically have been known as major contributors to most of the environmental problems we face today. From highly polluted water, with heavy metals and hydrocarbons, used for crops irrigation, to the massive carbon emissions originated by livestock, an increase in food production accounts for many environmental issues. Furthermore, chemical pesticides and fertilizers have been extensively used throughout the years to deal with local pests, and some of these chemical residues infiltrate into water bodies, contaminating ground water and also the animals that eat those crops and plants.

![World Population: 1950-2050](image)

Figure 1 – U.S Census Bureau 2016 forecast of the world population by 2050.

Moreover, climate changes are already having an impact on ecosystems, as the weather patterns are gradually being altered. Thus, sustainable measures to use and recover natural resources as the population and food production increase is a crucial step towards the maintenance of the ecosystems.

1.1 Plant development and plant-growth-promoting bacteria

Plants represent a major asset in developing sustainable environmental measures, as they are fundamental in several areas, such as, agriculture, being at the very base of the food chain, air renewal, water cycle regulation and subterranean waters maintenance, soil recovery
and forestry, production of pharmaceuticals, and also consist of habitat for many different species. However, during their lifetime, plants are subjected to a number of non-lethal stresses that can have both biotic (e.g. herbivore or pathogen attacks) or abiotic (e.g. flooding, extreme temperatures or drought) origins. These stresses inevitably lead to a decrease of plants optimal growth, causing their life cycle to be characterized by periods of maximal growth interleaved with periods of growth inhibition (Figure 2) [12].

![Plant Growth Graph](image)

**Figure 2** – Plant growth as a function of its age. Maximum growth is represented by the slope of the maximum yield line, while the actual yield is represented by the slopes. Each arrow indicates a non-lethal stress, limiting of plant growth and development [12].

To remedy the effects of stress, plants are able to modify their physiology and metabolism, synthesizing various defensive compounds [12]. However, the presence of certain bacteria (attached or inside plant tissues) is also of great relevance, enhancing plants abilities to deal with stress responses. Soil bacteria are present in a concentration of around $10^8$ to $10^9$ cells per gram of soil [13], and can grow rapidly, using a very wide range of different substances as nutrient sources, yet, their distribution in the soil is not even. Due to high levels of nutrients, such as sugars, amino acids, organic acids, and other small molecules (e.g. flavonoids) near plant roots, the concentration of bacteria in the rhizosphere is much higher than in the rest of the soil. These molecules are exuded from the roots of plants, and used by rhizospheric bacteria to support their growth and metabolism. Also, bacteria can enter and thrive inside different plant tissues and organs and directly modulate some stress responses. The beneficial bacteria that interact with plants, stimulating their growth, are commonly denominated plant growth-promoting bacteria (PGPB) and that includes free-living bacteria (mostly in the rhizosphere); bacteria that form specific symbiotic relationships with plants, such as rhizobia (that form nodules and fix atmospheric nitrogen); bacterial endophytes that can colonize the interior of plant tissues; and cyanobacteria (that can do photosynthesis and also fix nitrogen). These bacteria can stimulate plant growth directly or indirectly, being the direct action related to bacteria’s ability to promote the plant acquisition of nutrients, such as nitrogen (e.g. nodule formation by rhizobia),
phosphorous or iron (phosphate solubilization and siderophore production), or through the modulation of plant hormones, such as auxin (e.g. indole-3-acetic acid), cytokinin or ethylene. The indirect action is related to PGPB’s biocontrol activity, decreasing the inhibitory effects of various pathogenic agents, via antibiotic production, for example [14].

Taking into consideration the complexity of plants physiology and their importance in maintaining a healthy ecosystem, to understand the main mechanisms applied by plants in order to support their growth and development is of extreme relevance. It is only by fully comprehending the intricate interactions between signaling molecules, phytohormones and microorganisms that it is possible to adapt those to environmental needs, and to create new sustainable biotechnological agricultural solutions.

1.2 Ethylene, a powerful modulator of plant growth and development

Phytohormones and signaling molecules play a crucial role in managing plant growth, and their discovery represented a significant progress towards a more complete understanding of plant physiology. Ethylene (C_2H_4) is one of the most important phytohormones, being a powerful modulator of plant growth and development. It is found in all higher plants [13] and occurs in all plant organs – roots, stems, leaves, bulbs, tubers, fruits or seeds [15], being responsible for many phases of the plant life cycle, including germination of seeds, root development, senescence, abscission, and fruit ripening [16]. Other aspects of plant life cycle, namely rhizobia nodulation of legumes, rooting of cuttings, endophytic colonization, and plant interaction with beneficial mycorrhizal fungi are also regulated by ethylene [17]–[21].

Plant ethylene production is dependent on many different factors, which include temperature, light, gravity, nutrition, the presence of other hormones, and also the presence of several types of stresses [5]. When facing stress conditions, such as the presence of metals, organic and inorganic chemicals, extreme temperatures, drought or flooding, ultraviolet light or pathogenic attacks, plants produce exaggerated levels of this phytohormone, which can ultimately lead to their death [15].

1.2.1 Ethylene biosynthesis

Ethylene biosynthetic pathway is described by the cycle proposed by Yang and Hoffman in 1984, named the Yang Cycle (Figure 3), in which the amino acid methionine is presented as fundamental to ethylene formation, and both S-adenosylmethionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC) are established as ethylene precursors [22]. In the presence of adenosine, approximately 80% of cellular methionine is converted to SAM by the enzyme SAM synthetase, at the expense of ATP utilization. Following SAM synthesis, the first major (and rate-limiting) step of ethylene biosynthesis takes place, and consists of the conversion of SAM into ACC, by ACC synthase (ACS). This reaction also produces 5-methylthioadenosine (MTA), a by-product that is further converted into methionine, ensuring the cycle continuity and guaranteeing that the pool of methionine is always replenished at the end of each round. As the
levels of this amino acid in plant tissue are usually very low, this step enables a continuous ethylene synthesis without demanding an increasing pool of methionine. Produced ACC is finally oxidized by ACC oxidase to form ethylene, CO$_2$ and cyanide that is further detoxified into β-cyanoalanine by β-cyanoalanine synthase to prevent toxicity through accumulated cyanide [16], [22].

Figure 3 - Ethylene biosynthesis pathway. Methionine is converted to S-adenosyl-methionine (S-AdoMet, SAM) by the enzyme SAM synthetase at the expense of one molecule of ATP. Through the action of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, SAM turns into ACC, with release of 5'-methylthioadenosine (MTA). MTA is recycled to methionine by several enzymatic reactions (transformation into 5-methylthioribose (MTR), which turns into 2-keto-4-methylthiobutyrate (KMB). SAM is also the precursor of the polyamines Spermidine/Spermine biosynthesis pathway. Ethylene is finally produced by the transformation of ACC by the enzyme ACC oxidase, which also generates carbon dioxide and hydrogen cyanide. ACC can also be transformed into Malonyl-ACC (MACC) [23].

1.2.2 Plants response to stress. The stress ethylene model.

Once exposed to stress, plants respond by producing ethylene. The response may though vary from plant to plant, and different plants may present different sensitivities to this phytohormone. Nevertheless, when under extreme stressful conditions, plants produce exaggerated ethylene levels, termed “stress ethylene” which can be harmful to their development and survival [12].

Ethylene has been observed to both lighten and aggravate some of the effects caused by stress [5], and this apparent paradox can be explained by ethylene being synthesized in two different peaks, following a model proposed by Glick et al. [24] (Figure 4). According to this model, the first peak is typically very small and occurs close in time, usually a few hours after stress is applied, whilst the second peak is much larger, and ensues one to three days later. These two moments of ethylene production suggest that the first small wave of ethylene consumes the existing pool of ACC within plant tissues, triggering protective responses by the plant, such as the transcription of pathogenesis-related genes and acquired resistance, while the second “stress ethylene” peak follows after further increased ACC production, developing more aggressive processes such as senescence, chlorosis and abscission [12].
Figure 4 - Plant ethylene production as a response to stress. Initially, plant responds by producing a small peak of ethylene, which is thought to trigger the transcription of some defense genes. Later in time, a second and much larger ethylene peak arises, causing adverse responses in the plant [12].

The overall effect of the second ethylene peak is inhibitory for plant growth, hence, following the application of a determined type of stress, a considerable portion of the damage is on account of stress ethylene, rather than the stress itself [21],[24]. Also, in the presence of stress ethylene, plants tend to produce even more ethylene, being this process termed autocatalytic ethylene synthesis. Taking this extremely deleterious and self-harmful effects into consideration, it is logical to assume that modulation of the second ethylene peak poses an exceptionally relevant strategy to promote plant growth and improve plant resistance to stress.

1.2.3 Ethylene and flowering

Flower senescence can be defined as a series of events that culminate with the death of a flower [25], being described by several characteristics, including petals enrolling, loss of color, and wilting, and also by the shedding of flower parts and gradual fading of the blossom.

Taking into account ethylene’s effect in senescence of flowers, these can be identified as ethylene sensitive or insensitive. Usually, dicotyledonous plants fall in the first category, while monocotyledonous plants belong to the latter. Furthermore, ethylene-sensitive flowers have been classified from not sensitive to highly sensitive, in five different categories [25]. Flowers such as zinnia, carnation, rose and geranium are regarded as highly sensitive.

The rise of endogenous ethylene levels in stressful conditions causes damaging effects in plant growth and development, namely in flower senescence, where it is thought to have a major role [5]. The application of some chemical inhibitors of plant ethylene production has come to prove this phytohormone’s part in flower decay and senescence. Silver thiosulphate (STS) [26], cyclic olefin norbornadiene (NBD) [27], and Aminoethoxyvinylglycine (AVG) [28], established ethylene inhibitors, have been reported as capable of extending the shelf-life of ethylene-sensitive flowers. However, despite their successful action in lowering ethylene levels and its harmful effects, these chemicals have serious disadvantages, making their recurrent use very unlikely. For instance, besides presenting a strong and unpleasant odor, NBD is also carcinogenic [27]. In addition, AVG and STS treatments are very costly, as well as environmentally hazardous and
potentially phytotoxic [5]. Therefore, alternatives to chemical treatments must be employed in order to decrease ethylene levels, which may include the use of PGPB producers of ACC deaminase.

2. ACC deaminase – a key to modulate ethylene levels

Some bacteria and fungi express ACC deaminase, an enzyme which catalyzes the cleavage of ACC into ammonia and α-ketobutyrate. By lowering plant ACC levels, these microorganisms are able to modulate ethylene levels and aid plant in overcoming the effects of stress ethylene.

2.1 ACC deaminase biochemical properties

Discovered in 1978, the enzyme ACC deaminase was initially identified in the yeast *Hansenula saturnus* and in the bacterium *Pseudomonas* sp. ACP [8]. ACC deaminase has been defined as a multimeric enzyme (homodimer or homotrimer) with a subunit molecular mass of approximately 35-42 kDa [12], belonging to a large group of enzymes that require the co-factor pyridoxal 5'-phosphate (PLP) for enzymatic activity [26]. This group has been categorized, based on the three dimensional structure of enzymes, into four folding types: (i) tryptophan synthase, (ii) aspartate aminotransferase, (iii) D-amino acid aminotransferase and (iv) alanine racemase [27], and ACC deaminase falls into the category of tryptophan synthase beta superfamily, binding tightly to PLP in the amount of approximately one molecule of pyridoxal phosphate per trimeric subunit [28].

Plant-produced ACC is the main substrate of ACC deaminase, thus, being a cytoplasmic enzyme, the enzymatic reaction can only occur after ACC is taken up by the ACC deaminase-producing microbe [29]. Other substrates can also be used by this enzyme, such as several D-amino acids, especially D-serine and D-cysteine, yet the binding is less efficient. This enzyme also have strong inhibitors, including L-aminoacids, such as L-alanine and L-serine [28].

The reaction catalyzed by ACC deaminase is characterized by two main steps, which are the fragmentation of the cyclopropane ring, and the deamination of ACC, originating ammonia and α-ketobutyrate (Figure 5). The temperature and pH optima at which these two steps occur are in a range between 25-30°C and 8.0-8.5, respectively, and the K_m values for ACC, at a pH of 8.5, were estimated between 1.5 and 17.4 mM, thus, indicating that the enzyme does not have a particularly high affinity for this substrate [8],[12],[29]. Contrarily, plant ACC oxidase, the enzyme that catalyzes ethylene formation from ACC, has a much greater affinity for ACC than microbial ACC deaminase, hence, ACC deaminase can only compete with ACC oxidase for the substrate if present in a much greater concentration (100 to 1000 fold) [31].

As for ACC deaminase molecular structure, it comprises several key amino acid residues, which are a reactive thiol group at cysteine 162 that is located in the internal gap between the two domains of the enzyme, a pyridoxal phosphate binding site, found at lysine 51, and also a serine
tyrosine 295, glutamine 296, and leucine 322 which may be responsible for substrate docking and consequent degradation [26], [28].

\[
\begin{align*}
\text{ACC} & \xrightarrow{\text{deaminase}} \text{CO}_2^- + \text{NH}_4^+ + \alpha\text{-Ketobutyrate} \\
\end{align*}
\]

Figure 5 - Enzymatic reaction catalyzed by ACC deaminase. The reaction consists of the opening of the aminocyclopropane ring and in the deamination of ACC, resulting in ammonia and \( \alpha \)-ketobutyrate (adapted from [30]).

### 2.2 ACC deaminase origin and distribution

Several studies regarding the isolation and manipulation of \( acdS \) genes (structural gene encoding ACC deaminase) from various bacteria and fungi have been conducted [26]. Yet, the origin of the genes encoding ACC deaminase is relatively unknown. Nevertheless, phylogenetic studies have shown that both bacterial and fungal ACC deaminase share a common origin, an ancestral enzyme, belonging to a large group of deaminases dependent on the PLP co-factor and related to the tryptophan synthase beta subunit [26]. ACC deaminase is thought to have been originated as a result of specific mutations in this ancestor enzyme. The conserved characteristics of this type of enzymes allows them to use a wide range of related substrates, which is the case of ACC deaminase being able to metabolize, not only ACC, but also D-cysteine in many tested species. However, evolutionary forces, such as small amino acid mutations, led these enzymes to present some level of substrate specialization [26].

Nascimento et al. [26] proposed a model regarding ACC deaminase evolution and phylogenetic distribution, based on bacterial ACC deaminase protein (AcdS) phylogenetic analysis, AcdS protein sequence analysis, \( acdS \) gene location, organism habitat and origin. According to this model, the evolution of the \( acdS \) gene is very likely to have occurred via continuous vertical transmission, taking into account the similarity found in \( acdS \) gene sequences between closely related strains. Another fact sustaining this claim lies in the close similarity in \( acdS \) gene sequences between strains with different origins and isolated from different habitats. However, it is probable that several environmental events, including a change in habitats, caused bacteria to adapt, leading to \( acdS \) divergence rates and, sometimes, gene loss or acquisition. For instance, some bacterial strains may possess ACC deaminase activity without the \( acdS \) gene being identified in the chromosome. However, these bacteria may contain \( acdS \) genes in plasmids which can be readily transmitted between bacteria. This is a direct result of horizontal gene transfer (HGT) in which the gene is acquired through, for example, co-existence with other ACC deaminase - producing bacteria in an environment where ACC deaminase production provides the bacteria with advantages. The same model suggests that gene location in bacteria may have an impact in its loss or prevalence. Intragenomic transfers of \( acdS \) genes from primary chromosomes to plasmids may have led to HGT events and to divergence of \( acdS \) genes, due to
the higher evolutionary rates presented by plasmids when compared to primary chromosomes. In addition, it has been reported the existence of HGT between distantly related organisms, which is the case of yeast acdS acquisition from bacteria [26].

2.3 Transcriptional regulation of the acdS gene

The induction of the enzyme ACC deaminase, as well as its mode of regulation is a very complex process, depending on factors such as presence or absence of oxygen, concentration of substrate, and accumulation of products. Thus, due to such complexity, there is a great lack of knowledge regarding the mechanism of regulation of the gene acdS [32].

Considering an existing model for the acdS gene from Pseudomonas sp. UW4, several regulatory elements have been found in the DNA segment containing the acdS gene, including a CRP (cyclic AMP receptor protein) binding site, a FNR (fumarate-nitrate reduction regulatory protein) binding site, a LRP (leucine-responsive regulatory protein) binding site, an open reading frame encoding a LRP protein (termed acdR), one putative promoter sequence controlling the acdR, and two other putative promoter sequences (Figure 6). Furthermore, there is also an AcdB protein, which encodes glycerophosphoryl diester phosphodiesterase and forms a complex with ACC [12], [32]. According to this model, in the presence of ACC, LRP forms an octamer that will bind to a complex comprised by ACC and the protein AcdB. This trimeric complex binds to the promoter region of acdS, activating it, which results in ACC cleavage into ammonia and α-ketobutyrate. ACC deaminase is negatively regulated by the presence of leucine, which is synthesized from α-ketobutyrate. Concluding, increasing leucine concentrations favors the formation of an inactive LRP dimer, shutting down acdS transcription mechanism [12], [32].

Transcriptional regulation of acdS differs between species of bacteria. However, the presence of acdR, has been found in the majority of the studied cases, suggesting that this is the most common regulatory mechanism for ACC deaminase [26]. In some cases, as in Burkholderia sp. CCGE 1002 and B. phymatum STM 815, there are two copies of acdS, one in a megaplasmid, and the other in a second chromosome, however, there is only one regulatory acdR gene [32]. There are also some reports that do not include most of the regulatory elements found in the acdS gene of Pseudomonas sp. UW4, as is the example of Variovorax paradoxus 5C2 and Achromobacter xylosoxidans A551 [32]. Furthermore, in the case of Mesorhizobium sp. MAFF303099, acdS is only expressed inside the nodules, being under regulation of the nifA2 gene, which is a regulatory N₂ fixing unit. In this particular case, acdS is thought to be only expressed after the binding of nifA2 to a σ₅₄ RNA polymerase sigma recognition site. The expression of acdS in root nodules enables the decrease of ethylene levels, lowering their senescence [32].
Figure 6 - Model for the transcriptional regulation of ACC deaminase expression in *Pseudomonas* sp. UW4. The *acdR* gene encodes a LRP protein (octamer), which can either bind to a LRP box, preventing further transcription of *acdR*, or to a complex formed by ACC and AcdB. This trimeric complex in anaerobic conditions binds to FNR, or, when in aerobic conditions, to a CRP box, on the DNA. This binding enables the transcription of *acdS* by RNA polymerase, synthesizing ACC deaminase, which will cleave ACC into ammonia and α-ketobutyrate. This latter compound is a precursor of leucine. In the presence of this aminoacid, the LRP is dissociated from its octamer form into an inactive dimeric form, preventing further gene transcription [12].

Regarding the phylogeny of *acdR*, it was found that the phylogeny of *acdS* and *acdR* is related, which suggests that both genes have evolved in a similar and dependent fashion. However, in some cases, it appears that *acdS* and *acdR* are not inherited together or have experienced genomic rearrangements, which can be explained by the presence of other *acdS* regulatory genes in some bacteria. Furthermore, due to coding a regulatory protein, *acdR* is more prone to modifications, in order to perfectly adjust *acdS* transcription and expression, which causes differences between both genes in evolutionary rates. Despite the stated dissimilarities, it is accepted that evolution of *acdS* and *acdR* is coupled, restating the essential role of *acdR* in ACC deaminase optimum expression [26].

### 2.4 Bacterial modulation of ethylene levels by ACC deaminase

Plant growth-promoting bacteria can modulate ethylene levels through the production of the enzyme ACC deaminase. Glick et al. [29] (Figure 7) proposed a model for the interaction between ACC deaminase-producing bacteria and plants. In this model, the bacterium starts by binding to the plant surface, usually to roots or seeds and, once attached, begins to respond to tryptophan and to other small molecules from plant exudates. This response leads to bacterial production of indole-3-acetic acid (IAA), which is taken up by the plant and, together with endogenous plant IAA, stimulates plant cell proliferation, root elongation and consequently exudation. This auxin concentration in the plant cell may induce the production of ACC synthase, the enzyme responsible for ACC formation. Some of the synthesized ACC is exuded by the seeds, roots or leaves and can be further metabolized by ACC deaminase-producing PGPB, cleaving it into ammonia and α-ketobutyrate. In this particular model, bacteria act as sinks for plant ACC, whether endogenous or IAA-stimulated, thus, reducing ethylene levels. By decreasing ethylene levels, PGPB that produce ACC deaminase are able to lower the damaging effects caused by...
this phytohormone in several stress conditions. Thus, it is expected that plants growing in association with these bacteria present longer roots and shoots, as well as an increased resistance to growth inhibition provoked by ethylene [11],[12],[28].

Figure 7 – Schematic model representing how bacteria that produce both IAA and ACC deaminase can facilitate plant growth. According to this model, both bacteria and plant produced IAA induce the formation of ACC that is further exuded by plant roots. Some of this ACC is taken up by bacteria and cleaved into ammonia and α-ketobutyrate, reducing the amount of formed ethylene.

ACC deaminase-producing bacteria modulate ethylene levels, however, the first ethylene peak that occurs as a response to stress conditions (Figure 8), and, is essential to activate some plant defense responses, remains unaltered. This occurs because the first ethylene peak consumes the pool of ACC that is readily available inside the plant cell, by the rapid action of ACC oxidase, which has greater affinity for ACC. In this way, ACC deaminase-producing bacteria only decrease the second ethylene peak, which is a result of the increased ACC de novo production [12],[28]. In spite of radically decreasing this harmful peak, PGPB producers of ACC deaminase can never completely abolish it, due to the much higher affinity that ACC oxidase has for ACC, than that of ACC deaminase. Thus, the ratio ACC oxidase to ACC deaminase is determinant in the modulation of ethylene levels by ACC deaminase-producing bacteria [29].
Figure 8 - Lowering of stress ethylene by ACC deaminase-producing bacteria. A) Plant ethylene levels without the application of ACC deaminase-producing bacteria. In both cases, there is an initial small peak of ethylene, which is beneficial, since it induces the transcription of defensive genes. Afterwards, a second much larger ethylene peak appears, causing adverse responses in the plant. According to this model, ACC deaminase-producing PGPB can diminish this second deleterious peak [12].

2.5 ACC deaminase expression levels

Expression levels of ACC deaminase differ amongst microorganisms. In fact, ACC deaminase activity is strongly dependent of the producer organism, and of factors such as *acdS* location in the replicon, *acdS* copy number, and *acdS* transcriptional regulation [33]. Thus, ACC deaminase-producing organisms can be divided in two distinct categories, as they present high or low enzyme activity [12][34]. Organisms with high ACC deaminase activity are those that usually bind non-specifically to several plant surfaces, including the majority of organisms from the rhizosphere, phyllosphere, as well as some endophytes. These organisms act like a sink for plant-produced ACC following a response to some stress, thus, lowering overall ethylene levels [27],[32]. Usually, these bacteria possess ACC deaminase genes in their chromosomes. In contrast, organisms with low ACC deaminase activity seem to be host specific, binding only to a particular plant or tissue. This group comprises most rhizobia that, instead of lowering global ethylene levels, merely prevent a rise in this phytohormone levels. Their action occurs following infection and nodulation, which leads to a small ethylene production by the plant, demanding only a low level of ACC deaminase production [28]. Interestingly, most rhizobia possess ACC deaminase genes in plasmids, nearby the symbiotic gene clusters [26].

3. Rhizobia and endophytes: distinct categories of ACC deaminase-producing bacteria

Plant-microbe associations are a vastly-studied subject, being the symbiotic associations between nitrogen-fixing microorganisms, such as rhizobia, the most understood mechanism of PGPB in promoting plant growth. Interactions between plants and beneficial bacteria can have a profound effect on crop health, yield and also soil quality, and as bacteria can positively impact
plant growth and development, plants can somehow “select” their microbiome, in order to have beneficial bacterial colonizers[4][35].

While rhizobia are defined as a type of bacteria that colonizes plant roots, being able to fix atmospheric nitrogen and provide it to plants inside specialized structures, called nodules, endophytes are bacteria able to colonize the internal plant tissues. Endophytes were first discovered in Germany in 1903, and have been defined as “fungi or bacteria, which for all part of their life cycle, invade plant tissues, but cause no symptoms or disease” [36]. Bacterial endophytes are present in every plant tissue, such as roots, stems, leaves, seeds, fruits, tubers, ovules, and also inside legume nodules [37], being characterized as neither organ nor host specific.

Root colonization has been considered the main bacterial trait determining inoculum efficacy, in terms of crop yield enhancement and disease control. This fact has led to the selection of bacterial strains that were able to effectively colonize the root system, i.e. rhizosphere competent. Due to the high-stress and extremely competitive environment of the soil, endophytes thrive as advantageous when compared to rhizospheric bacteria, since when inside plant tissues, these bacteria are relatively protected from such stresses. Furthermore, plant growth promotion is often greater when it is induced by endophytes, rather than by bacteria restricted to the rhizosphere and root surface, since by colonizing the inside of plant tissues, endophytes can serve their host promptly and efficiently [4],[34].

The mechanisms applied by both type of bacteria in promoting plant growth are similar, and the production of the enzyme ACC deaminase poses as a main instrument in regulating plant growth, as some of ethylene effects are related to inhibiting primary root elongation and lateral root formation, being exacerbated under stress conditions. Furthermore, this phytohormone negatively affects some physiological processes regarding the symbiotic association of these bacteria with plants, namely, the nodule formation mediated by rhizobia, and the colonization of endophytic bacteria. Thus, the production of ACC deaminase, which converts ACC into ammonia and α-ketobutyrate, is able to decrease ACC levels, consequently reducing ethylene levels, and enabling a better symbiosis with PGPB and also increasing plant stress resistance.

This chapter focuses on the symbiotic activities of both rhizobia and endophytes, being present the challenges posed by ethylene in bacterial normal activities, and the effects of ACC deaminase in overcoming those challenges. It also approaches a relatively novel technique regarding co-inoculation of rhizobia and endophytes as a successful strategy of plant growth promotion.

3.1 Rhizobia nodulation, ethylene and ACC deaminase effect

3.1.1 Rhizobia and nodulation process

The leguminous plant-rhizobia is the most popular beneficial plant-microbe interaction studied [33], being traditionally used in agricultural practices to provide nitrogen to plants in an efficient and sustainable manner and, thereby, enhance plant growth. Although typically
performed by rhizobia, other bacteria, such as *Azospirillum* spp., are also able to fix nitrogen (without forming nodules) and provide it to plants [14]. Biological nitrogen fixation produces ammonia from molecular nitrogen. The enzyme responsible for this catalytic reactions is the nitrogenase enzyme complex, which is encoded by rhizobial *nif* genes, and consists of two proteins - the iron protein (encoded by nifH) and the molybdenum - iron protein (encoded by nifD and nifK) [15]. After catalyzing the reaction, rhizobia release ammonia, which in turn needs to be immediately converted into organic forms in the root nodules before being transported to the shoot via the xylem, in order to avoid toxicity [15].

The symbiosis between rhizobia and legumes is not obligatory, and the majority of these nitrogen-fixing prokaryotes lives in the soil, generally independent of other organisms [33]. However, when under nitrogen-limited conditions, symbionts seek each other through an elaborate set of signaling processes, which involve specific genes in both the host and the symbionts - *nodulin* (*Nod*) genes and *nodulation* (*nod*) genes, respectively [15].

The success of the symbiotic process lies in two fundamental steps, bacterial infection and nodule organogenesis, which occur simultaneously [33]. Nodules are special organs of the plant host that enclose nitrogen-fixing bacteria wherein rhizobia reduce atmospheric nitrogen into ammonia [15]. The process is initiated with bacterial migration towards the roots of the plant, as a chemotactic response to chemical attractants, namely flavonoids, exuded by the roots. Flavonoids and other chemical attractants activate and bind tightly to the rhizobial *NodD* protein, which, besides being the major determinant of rhizobial host specificity (each strain of rhizobia identifies only a limited number of flavonoids structures, and each species of legumes produces its own set of flavonoids [33]) induce the transcription of other *nod* genes [15], [33]. These will in turn induce an extreme curling of the root hair cells, where rhizobia become enclosed and produce a lipochitooligosaccharide *nod* factor which, after binding to a legume root receptor, will activate mitotic cell division in root cortical cells, leading to the formation of the nodule primordium [15], [38], [39]. Upon the formation of the nodule primordium, an infection thread (an internal tubular extension of the plasma membrane produced by the fusion of Golgi-derived membrane vesicles at the site of the infection [15]) filled with proliferating rhizobia, arises and continues to grow until it reaches the nodule primordium, which develops to the ultimate nodule form, after rhizobia release and differentiation into a specialized symbiotic organelle-like form, termed bacteroid [38].
Figure 9 - Nodule organogenesis and infection process. A) Rhizobia binding to the root hair, as a response to chemical attractants (e.g. flavonoids). B) Extreme curling of the root hair cells in response to nod factors and rhizobia proliferation. C) Formation of the infection thread, derived from secretory vesicles of Golgi from root cells. D) Membrane of the infection thread fuses with the plasma membrane of the root hair cell. E) Release of rhizobia and formation of the nodule primordium. F) Infection thread extends and branches until it reaches specialized cells within the nodule. [15]

Although widely used, the process of nitrogen fixation has a few downsides, since it involves a large expenditure of energy in the form of ATP [14][40], and processes such as molecular signaling, rhizobial attachment, root hair curling, infection thread formation, nodule formation, and nitrogen fixation are severely affected by various stresses [41]–[43]. The main stress comes from the plant immunity response, since these processes cause plants to locally produce ethylene, which may characterize rhizobial infection as a self-limiting process [44], [45]. Nevertheless, rhizobia can produce either rhizobitoxine, an inhibitor of the enzyme ACC synthase, or ACC deaminase, enabling these strains to lower ethylene levels [12].

### 3.1.2 Ethylene effects in the nodulation process

In leguminous plants, ethylene is identified for its inhibitory action towards the nodulation process, acting in the formation and functioning of nodules [6], [7]. As several studies reported, the presence of ethylene reduced the frequency of nodule primordia formation in most nodulating plants, such as *Phaseolus vulgaris*, *Pisum sativum*, *Trifolium repens*, *Medicago sativa* or *Melilotus alba* [17], [46], [47]. Moreover, the application of ethephon, a compound that releases ethylene upon biological conversion by the plant cells, has been observed to reduce the number of nodules, as well as their nitrogen fixation abilities, in *Pisum sativum*, [48]. Furthermore, treatment of roots with ethylene biosynthesis inhibitors, including AVG (aminooxyacetic acid) and AOA (aminooxyacetic acid), or with ethylene perception blockers, such as Ag+, has been reported to increase the number as well as the developmental state of nodules, in *Phaseolus vulgaris* and in *P. sativum* [47], [49].
In addition to experiments with exogenous application of ethylene and its biosynthesis inhibitors or perception blockers, experiments with transgenic plants were crucial in understanding the role of this phytohormone in nodulation. For instance, several mutant plants displaying insensitivity to ethylene, such as *Medicago truncatula* [86], or *Lotus japonicus* [51], formed an increased number of nodules compared to the wild-type form, as well as higher numbers of infection threads and nodule primordia, suggesting that ethylene perception not only assists the negative feedback regulation of nodule initiation, but also has a fundamental part in the development of infection threads, especially in their initiation and elongation[33]. Furthermore, ethylene has also been exposed as an inhibitor of various steps of the nodulation process and symbiosis, including the initial response to bacterial Nod factors, nodule development, senescence and abscission [52], [53]. Due to its role in controlling the epidermal responses during nodulation, ethylene is responsible for preventing rhizobial infection, blocking it as soon as the infection thread reaches the basal epidermal cell or the outer cortical cells of the plant [47]. This findings are supported by the observation that endogenous ethylene production significantly increases in roots infected by some nodulating species, such as *Rhizobium* or *Bradyrhizobium*, preventing nodule formation [54], [55].

Ethylene has also been reported to act as a major participant in the autoregulation of nodulation (AON), a systemic process through which legumes control the number of nodules [44], [56], [57]. Therefore, rhizobial strategies of modulating ethylene levels, such as the production of ACC deaminase, are essential to overcome the limiting effects of this phytohormone.

### 3.1.3 ACC deaminase in rhizobia and nodulation process

The *acdS* gene is found mainly in α-rhizobia (rhizobia belonging to the α-Proteobacteria class), in bacteria such as *Azhorhizobium*, *Bradyrhizobium*, *Methylbacterium*, *Mesorhizobium*, *Rhizobium*, and *Ensifer* (*Sinorhizobium*), *Devosia*, *Microvirga*, and *Bosea*. However, *acdS* can also be found in β-rhizobia (rhizobia belonging to the β-Proteobacteria class), like *Burkholderia* and *Cupriavidus* [26].

Despite belonging to the group of bacteria with low ACC deaminase, different rhizobia present different enzymatic activities [33]. As previously mentioned, *acdS* location in the replicon is a factor influencing the activity of ACC deaminase and there is a great disparity regarding gene location in several rhizobia [33]. For instance, in *Rhizobium* and *Sinorhizobium*, *acdS* are located in symbiotic plasmids, whereas in *Azhorhizobium*, *Bradyrhizobium*, the gene is found mainly in the chromosome, far from symbiotic islands. However, in many *Mesorhizobium* spp. *acdS* genes can be found in symbiotic islands next to the symbiotic genes [58]. Regarding β-rhizobia, *Burkholderia* and *Cupriavidus* strains possess two copies of *acdS*, one in a second chromosome, and the other in a symbiotic plasmid [26].

Several studies performed with rhizobial *acdS* deletion mutants, as well as rhizobial strains expressing exogenous *acdS* genes have proven the important role of ACC deaminase in the nodulation process. In these assays, conclusions were drawn based on the observations that the great majority of *acdS* deletion mutants had a reduction of their ability to nodulate their host,
as well as a decrease in their symbiotic capacities, when compared to the wild-type form [33]. Furthermore, most of these ACC deaminase deletion mutants decreased their nodule occupancy abilities, and had a reduction in plant growth promotion abilities, as it is the example of the acdS minus mutant of *R. leguminosarum* bv. *viciae* 128C53K, that showed a 25% reduction of its nodulation abilities as well as a 23% decrease in its ability to increase shoot dry weight in *P. sativum* [59]. In addition, experiments with rhizobial strains expressing exogenous acdS genes, showed that, compared with the wild-type form, these strains had an increased nodulation efficiency and rhizobial competitiveness. When transformed to express an exogenous acdS gene, *S. meliloti* Rm1021 was able to produce 35-40% more nodules in *M. sativa* plants when compared to its wild-type form [60], and also *Mesorhizobium* sp. MAFF303099 that, with an extra copy of the acdS gene in its chromosome, was able to form more nodules in *L. japonicus* and in *L. tenuis*, and be more competitive than the wild-type strain [61]. Moreover, a strain of *Mesorhizobium ciceri* LMS-1 transformed with an exogenous acdS gene, through the plasmid pRKACC containing acdS and acdR genes of *Pseudomonas* sp. UW4, presented enhanced nodulatory abilities in *Cicer arietinum* (127%), and also formed more developed nodules in earlier stages of nodulation [62]. The increased symbiotic capacity and nodulation abilities displayed by these transformants, possessing exogenous acdS genes, may be explained by their ability to use ACC as a nutrient source, giving them the capacity to better proliferate in the infection threads and to reduce ethylene levels that inhibit the nodulation process. Taking into consideration these observations, it is reasonable to state that ACC deaminase is an effective strategy for modulating ethylene levels in root tissues, thus, enhancing nodulation and the competitiveness of the rhizobia [33].

Despite many studies have been performed in α-rhizobia, not much is understood about the role of ACC deaminase in the nodulation process of β-rhizobia. However, ACC deaminase activity has been detected in root nodulating *Burkholderia* [63]. These bacteria form nodules in leguminous trees, such as *Mimosa* spp., being mostly endemic to Brazil and South America [64]. Further studies are necessary to understand the role of ethylene and ACC deaminase in the nodulation process of leguminous trees.

### 3.2 Endophytic colonization, ethylene and ACC deaminase effects

#### 3.2.1 Endophytic colonization

The rhizosphere is a highly competitive environment for microorganisms, in terms of space and nutrients availability. Regarding plant colonization, endophytes, that are bacteria able to establish a mutualistic association within plant tissues without causing harm [65], are believed to have different mechanisms to gain entry into plant tissues, when comparing to specialized rhizosphere living bacteria, due to their very efficient interaction with plant hosts. A true endophytic colonization is characterized by two main endophytic abilities: proper spread among different organs of the plant, and maintenance of endophytic state for bacterial generations within the plant environment [66]. These characteristics distinguish true endophytes from transient
microorganisms which sometimes enter plant tissues by accident, thus, not being able to survive for long periods of time.

Apart from the already established seed-endophytes, the most usual way of entry into a plant tissue is through the roots. Hence, when colonizing a plant, before entering plant tissues, endophytes begin to inhabit the rhizosphere (soil near the roots), or the phyllosphere (aboveground surfaces of a plant that are used as microbial habitat) [35], [66]. To gain access into plants, endophytes employ different mechanisms, being able to enter through different points. The most recognized entry point for bacterial colonization are root cracks and wounds, since at these sites there is some leakage of plant metabolites, which attract bacteria [35]. However, other entrance points have been documented, such as, tissue wounds, stomata, lenticels, root hair cells and germinating radicles (Figure 10). In addition, production of cell wall degradative enzymes was also observed as a suitable mechanism for bacterial colonization [35].

Once inside the plant, endophytes may occupy different locations within its tissues. Hence, they can either remain at the point of entry, or spread themselves throughout the plant, whether in intracellular spaces, in the vascular system, and/or within the cells [67]–[70]. Habitat selection occurs accordingly to endophytes tendency to become established in those environments. For instance, the xylem has been proven as a selective environment for nitrogen-fixing endophytic bacteria, such as Acetobacter diazotrophicus, Bacillus pumilus, Gluconacetobacter diazotrophicus, Herbaspirillum seropedicae, Klebsiella pneumoniae and Serratia marcescens [66].

Regarding hosts, a great variety of plants has been identified as suitable host for endophytic bacteria, going from herbaceous plants to woody plants, monocotyledonous genera to dicotyledonous [71][71]. Despite not having any record of a plant species completely free of endophytes, [65], densities of endophytic population are lower than those of rhizospheric bacteria or bacterial pathogens [57]. However, endophytic niche is believed to protect bacteria from numerous environmental stresses, and a large number of bacterial endophytes have been isolated from almost every part or tissue of the plant [72].
Figure 10 - Endophytic modes of access into different plant tissues. The most common point of entry are root cracks. In the aerial part, endophytes can enter through lenticels, stomata (on leaves and young cells). Vertical seed transmission is another way to inherit endophytes through plant host generations, as well as through hydrolysis of root cells. Rhizobia can also thrive as endophytes, colonizing the internal plant tissues and form root nodules [35].

3.2.2 Ethylene role in bacterial endophytic colonization

Similar to what is verified with nodulation and rhizobial infection, ethylene also modulates endophytic colonization of plants [73]. As stated before, the mechanisms employed in endophytic colonization are likely similar, at least in the initial phases, to colonization of plant roots by rhizobacteria [67]. In fact, many soil bacteria could turn into efficacious endosphere (internal plant tissues) colonizers once they can overcome the challenges imposed [73].

Infection of plants by bacteria, especially in the early stages, is a phenomenon widely monitored by the many molecular mechanisms available to plant, due to the impact such invasion could pose. These mechanisms can either improve or decrease bacterial colonization and, among them, genes of the ethylene-signaling pathway are present and differentially expressed, even in the presence of beneficial endophytes [74]. Ethylene, as a potent modulator of plant growth and development, is involved in microbe-plant interactions and its role in endophytic colonization has been studied and reported in the literature. For instance, the endophyte Klebsiella pneumoniae 342 hypercolonized an ethylene-insensitive mutant of Medicago trunculata but not the wild-type plant [73]. Moreover, studies with the same bacteria and host showed that the addition of an exogenous ethylene inhibitor, 1-methylcyclopropene (1-MCP) resulted in an enhancement of the K. pneumoniae 342 colonization [73]. Furthermore, the addition of ethylene precursor, ACC, to a parental Medicago sativa resulted in a diminished invasion of bacteria [73].

Altogether, these findings suggest that ethylene is a key regulator of the colonization of plant tissues by bacteria, and that its effect on the plant signaling pathways is in all likelihood responsible for mediating this response [75].
3.2.3 ACC deaminase in endophytic colonization

Taking into account the major ethylene effect in regulating the occupation of plant tissues by bacteria, affecting plant signaling pathways [75] and imposing great restrictions to endophytic colonization, modulation of ethylene levels by bacterial endophytes becomes extremely important. Also, since bacterial endophytes are closer to plant cells in which ethylene biosynthesis occur, ACC deaminase-producing endophytes can be considered more competent PGPB [75].

Some endophytes express the enzyme ACC deaminase which increases their plant growth promotion potential, due to its efficacy in lowering ethylene levels [12]. Thus, the acdS gene is central to the plant-growth promoting abilities of many endophytic strains. The endophyte Burkholderia phytofirmans PsJN lost its canola root elongation abilities when its acdS gene was deleted [76]. Onofre-Lemus et al. [63] demonstrated that an acdS mutant of the tomato endophyte B. unamae MTI-641 lost its tomato-growth promotion capability. Recently, Ali et al. [77] demonstrated that endophytes, P. fluorescens YsS6 and P. migulae 8R6, promoted tomato plant growth under control and salinity stress conditions, while its ACC deaminase deficient mutants did not.

Interestingly, plants may be able to select ACC deaminase-producing endophytes, in order to decrease this phytohormone levels [75]. Plant selection of beneficial endophytes can occur in an early stage of development, such as, seed germination. In fact, ethylene is needed in relatively high concentrations to break seed dormancy, however, after germination the presence of this phytohormone is extremely inhibitory of root elongation [75]. Thus, bacterial endophytes producing ACC deaminase are an extremely important asset to the plant, due to their ability of lowering stress imposed by extreme ethylene levels. For example, Truyens et al. [12] demonstrated that ACC deaminase-producing bacterial endophytes were predominantly found in Arabidopsis thaliana plants (seeds) which had been exposed to 2 mM cadmium for several generations, but not so predominant in plant seeds not exposed to stress. ACC deaminase activity can, thus, be considered a beneficial evolutionary characteristic in plant endophytes.

3.3 Co-inoculation of legumes with rhizobia and other ACC deaminase-producing PGPB

As previously stated, most rhizobia are included in the low activity ACC deaminase-producing bacteria, using this enzyme locally to promote nodulation. Despite good results in the process of nodulation, these bacteria are not able to decrease the high levels of stress ethylene occurring throughout the plant [13]. Nevertheless, when growing in inhospitable soils, legumes are subjected to high levels of environmental stresses, which in turn will favor ethylene formation and inhibit the legume-rhizobia symbiosis.

A novel strategy to promote nodulation and legume plant-growth, especially under stress conditions, may rely on the combination of rhizobial strains and other ACC deaminase-producing bacteria (possessing high ACC deaminase activity) [33]. This technique has been shown to be effective, as is the example of the co-inoculation of a PGPB possessing ACC deaminase activity.
and B. japonicum which resulted in an increase of 48% in mung bean nodulation [78]. In addition, further studies with a consortium of PGPB and rhizobia also showed enhanced nodulation in common beans, lentils and chickpea, proving the positive effects of co-inoculation in lowering ethylene concentrations in infected roots, thus, enhancing nodulation [79]–[81]. Moreover, additional studies regarding co-inoculation of several rhizobia with other ACC deaminase-producing PGPB in numerous plants, have shown that this approach not only contributed to a higher nodulation ability, but also improved plant growth and yield, even when grown in stressful environments, such as salinity or drought. Furthermore, co-inoculation has also been proven as a powerful tool in enhancing the uptake of essential nutrients, including among others nitrogen, phosphorous and sodium [33].

Overall, a collection of data suggests that rhizobia when co-inoculated with PGPB containing high ACC deaminase activity, increase their symbiotic potential and nodulation abilities, while, at the same time, provides plants with an enhanced resistance to environmental stresses, by lowering stress-induced ethylene levels [33]. Thus, future commercial inoculants may consist on the application of both type of bacteria, instead of selecting only rhizobia.

4. Bacterial transformation with the acdS gene

In order to study the effect of the enzyme ACC deaminase in plant-microbe interactions, some bacterial strains with no ACC deaminase activity have been transformed with acdS genes and their regulatory regions. The results of these transformations confirmed that this enzyme is a great asset in terms of promoting nodulation (as previously described), plant growth and development. For instance, after transforming Escherichia coli and Pseudomonas strains that lacked ACC deaminase activity with an exogenous acdS gene (in the pRKACC plasmid), these bacteria were able to promote the elongation of canola roots [82]. In addition, Pseudomonas strains expressing Pseudomonas sp. UW4 acdS gene also improved their biocontrol effectiveness [83].

Nonetheless, the complex regulatory system of the acdS gene is sometimes host specific, as it is the efficiency of the expression of the introduced acdS genes. This can be illustrated by the case of Azospirillum brasilense Cd, which did not show ACC deaminase activity after transformation with the acdS gene of Pseudomonas sp. UW4, and its acdR regulatory gene [83]. After replacing this native regulatory region by an E. coli lac promoter, acdS gene was expressed at a high level, with consequent enhancement of the growth-promoting activity of the transformed Azospirillum strain. The same was verified by a further study in which lac promoter was substituted by a tetracycline promoter, TetR, a constitutive promoter weaker than lac, and therefore with less metabolic load [84]. Despite some constrains, bacterial transformation with exogenous acdS genes contributes to a better understanding of the role of ACC deaminase in promoting plant growth, and therefore, to find efficient and sustainable solutions to improve plant growth, yield and development.
Objectives and Motivation

Environmental problems, mainly caused by human action, have been leading to a decrease in the quality of life of many people and animal species from around the world. Climate changes and ozone depletion are currently changing ecosystems and endangering the world as we know it. Moreover, the forecasted growth of the world population is an evidence that this situation is only likely to worsen, with the increase in agricultural practices, which entail several wrongful practices regarding environmental health. The substitution of chemical fertilizers and pesticides by environmental-friendly PGPB inoculants is, thus, a very important alternative and deserves further studies in order to maximize its efficiency and application. Also, controlling stress ethylene levels is key to a wide range of agricultural and biotechnological applications.

Taking this into consideration, the main objective of this work is to better understand the mechanisms employed by PGPB, more precisely, the ACC deaminase effect in plant-microbe interactions, with special emphasis in endophyte and rhizobial plant-growth promoting abilities. Hence, several objectives are proposed in this work:

- Transformation of an endophyte and a β-rhizobium with an exogenous ACC deaminase gene.
- Understand the role of ACC deaminase in plant-growth promotion abilities of endophytes and the nodulation process of β-rhizobia.
- Evaluate the effect of ACC deaminase-producing endophytes in the nodulation abilities of rhizobia.
- Study the effect of ACC deaminase in the endophyte ability to delay the senescence of carnation flowers.
Materials and Methods

1. Transformation of endophytic and β-rhizobia strains to express ACC deaminase

1.1 Selection of strains

Two bacterial strains, *Serratia quinivorans* BXF1 and *Cupriavidus taiwanensis* STM894, were selected to be transformed to express ACC deaminase in order to assess the effect of this enzyme in the plant growth promotion abilities of an endophyte and β-rhizobia, respectively. The endophyte *S. quinivorans* BXF1 was previously isolated from the nematode *Bursaphelenchus xylophilus* Portuguese isolate Bx7-D, which was originally acquired from a diseased *Pinus pinaster* tree in Oliveira do Hospital, Coimbra (Portugal) in 2009 [85]. This bacterium was found to be a generalist endophyte (pine, tomato, cucumber) with plant-growth promoting abilities and its genome was recently sequenced. It contains a single chromosome of approximately 5.4 Mbp and does not carry any plasmids (Nascimento et al., unpublished results). *Cupriavidus taiwanensis* STM894 was isolated from the root nodules of *Mimosa pudica* in Taiwan [86] and it is able to form an efficient symbiotic relationship with several leguminous plants. Its genome has been sequenced and it contains two chromosomes (chromosome 1, aprox. 3.4 Mbp; chromosome 2, aprox. 2.5 Mbp), and a plasmid (pRalta, 557,200 bp) containing the symbiotic genes [87]. The ACC deaminase gene was not found in these strains.

1.2 Triparental conjugation method

The triparental conjugation method was applied in order to transform both strains with the pRKACC plasmid [82] containing the *acdS* and *acdR* gene of *Pseudomonas sp. UW4* and its flanking regions. In this methodology, the recipient (strain to be transformed) and two *E. coli* strains were used: the helper, *E. coli* MT616, containing the pRK600 plasmid encoding the genes responsible for transferring genetic material between strains (conjugation), and the donor, *E. coli* DH5α, containing the pRKACC plasmid.

*E. coli* strains were grown overnight at 37°C in Luria Bertani (LB) medium (composition per liter: 10.0 g tryptone, 5 g yeast extract, 10 g NaCl) supplemented with the respective antibiotics (Table 1). The recipient strains (BXF1 or STM894) were grown in Tryptic Soy Broth (TSB) medium (composition per liter: 17.0 g Tryptone, 3.0 g Soy Peptone, 5.0 g NaCl, 2.5 g K₂HPO₄, 2.5 g glucose) at 28°C. After overnight growth, 50 µL of the bacterial cultures were individually suspended in a sterile 1.5 mL Eppendorf tube and centrifuged at 10000g for 1 minute, in order to remove the supernatant. The pellet was suspended in 50 µL of TSB medium without antibiotics. The strains (recipient, donor and helper) were mixed (1:1:1 v/v) in a sterile 1.5 mL Eppendorf tube and vortexed for 30 seconds. Posteriorly, 100 µL of this mixture was plated in the center of a TSA plate without antibiotics and incubated overnight at 28°C. Transformants were
selected by its ability to grow in TSA containing 15 µg/mL tetracycline and its colony morphology. Bacterial identity was further confirmed by 16S rDNA sequencing. Bacterial DNA was extracted using Sigma GenElute™ Bacterial Genomic DNA extraction kit following the manufacturer’s protocol. The obtained DNA was quantified in a NanoDrop (Thermofisher) according to the manufacturer’s protocol, and was posteriorly sent to Macrogen Inc. (Korea) in order to perform the 16S rDNA sequencing by in-house protocols using the universal primers 24F and 1492R [88].

Furthermore, a *S. quinivorans* BXF1 strain carrying the p519ngfp plasmid containing the Green Flourescence Protein (GFP) gene (Nascimento, unpublished results) was also transformed with the pRKACC plasmid as described above. This strain was selected on TSA plates containing 15 µg/mL tetracycline (Tet) and 150 µg/mL kanamycin (Kan).

Table 1 - Characteristics of strains used in Triparental Conjugation.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid and characteristics</th>
<th>Antibiotics Resistance</th>
<th>Growth Conditions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> MT616</td>
<td>pRK600</td>
<td>25 µg/ml Cloramphenicol</td>
<td>LB, 37°C</td>
<td>Finan et al. 1986 [89]</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>pRKACC: pRK415 containing <em>Pseudomonas</em> sp. UW4 acdS gene and its flanking regions</td>
<td>15 µg/ml Tetracyclin</td>
<td>LB, 37°C</td>
<td>Shah et al. 1998 [82]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>acdS (GeneBank) ID: 14055601</td>
</tr>
<tr>
<td><em>S. quinivorans</em> BXF1</td>
<td>No plasmids</td>
<td>-</td>
<td>TSB, 28°C</td>
<td>Vicente et al. 2011 [85]</td>
</tr>
<tr>
<td><em>S. quinivorans</em> BXF1 p519ngfp</td>
<td>p519ngfp containing the gfp gene</td>
<td>150 µg/ml Kanamycin</td>
<td>TSB 28°C supplemented with 150 µg/mL Kan</td>
<td>Nascimento et al. (Unpublished results)</td>
</tr>
<tr>
<td><em>C. taiwanensis</em> STM 894</td>
<td>pRalta containing symbiotic genes</td>
<td>-</td>
<td>TSB, 28°C</td>
<td>Amadou et al. 2008 [87]</td>
</tr>
</tbody>
</table>

2. Determination of ACC deaminase enzymatic activity

ACC deaminase activity of the transformed strains was evaluated as described by Honma and Shimomura [8] and Penrose and Glick [90] a method which quantifies the produced α-ketobutyrate, a resulting product from the cleavage of ACC by ACC deaminase.

In order to determine the produced quantity (µmol) of α-ketobutyrate in the sample, its absorbance at 540 nm was compared to that of known standards. For each measurement, a negative control (without ACC) and two duplicates were (containing ACC) were performed. Final ACC deaminase activity was expressed in µmol of produced α-ketobutyrate/ mg total protein/ incubation time. The strain *Pseudomonas* sp. UW4 was used as a positive control strain.

2.1 Preparation of standard α-ketobutyrate solutions

Standards of concentrations 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 M were prepared from a stock solution of α-ketobutyrate (100 mM) in 0.1M Tris-HCl, at pH 8.5. To 500 µL of each standard, 400
µL of a 0.56 N HCl solution were added, as well as 150 µL of 2,4-Dinitrophenylhydrazine. This solution was vortexed at maximum speed for 5 seconds and incubated at 30ºC, for 30 minutes. In acidic conditions, 2,4-Dinitrophenylhydrazine binds to the formed α-ketobutyrate, resulting in 2,4-Dinitrophenylhydrazone, a reaction that occurs during the thirty-minute period of incubation. Subsequently, 1 mL of a 2N NaOH solution was added, enabling the change of color of 2,4-Dinitrophenylhydrazone, due to a turn of pH from acidic to alkaline. Finally, absorbance was measured in a spectrophotometer at a 540 nm wavelength, in which negative control was prepared with all the reagents except for α-ketobutyrate, and a standard-curve was generated.

2.2 Induction of ACC deaminase activity

To induce enzyme activity, putative transformed strains of *S. quinivorans* BXF1 and *C. taiwanensis* STM89 as well as their negative control (wild-type) were grown in TSB medium, and supplemented with the respective antibiotics when necessary. *Pseudomonas* sp. UW4 was also used in the assay as a positive control. After overnight growth, bacterial cultures were centrifuged at 10000g for 5 minutes, suspended in 5 mL of DF salts minimal medium (Composition per liter: 4.0 g KH₂PO₄, 6.0 g Na₂HPO₄, 0.2 g MgSO₄·7H₂O, 2.0 g glucose, 2.0 g gluconic acid and 2.0 g citric acid with trace elements: 1 mg FeSO₄·7H₂O, 10 mg H₃BO₃, 11.19 mg MnSO₄·H₂O, 124.6 mg ZnSO₄·7H₂O, 78.22 mg CuSO₄·5H₂O, 10 mg MoO₃, pH 7.2) containing 5 mM ACC as sole nitrogen source and incubated for 24 hours at 28ºC.

2.3 Sample preparation

After the induction of enzymatic activity, samples were prepared for measuring α-ketobutyrate quantity and total protein. Hence, DF grown cultures were centrifuged at 10000g for 5 minutes, the pellets were washed twice with a 0.1 M Tris-HCl pH 8 solution, and suspended in 400 µL of the same Tris-HCl solution. Posteriorly, 20 µL of toluene was added to the solution in order to lyse the cell wall and release ACC deaminase (cytoplasmatic enzyme). The solution was then vortexed at maximum speed for 30 seconds, and four aliquots were taken for each sample: three aliquots of 50 µL for measuring of ACC deaminase activity (two repetitions and one negative control), and one of 50 µL for total protein quantification.

2.4 Quantitative measuring of produced α-ketobutyrate

To 50 µL of the previous prepared aliquots, 5 µL of a 0.5 M ACC solution were added (when necessary, the negative control did not receive ACC), following a vortex at maximum speed for 5 seconds, and incubation at 30ºC (optimal temperature for ACC deaminase activity) for thirty minutes. After this period, 500 µL of a 0.56 M HCl solution was added (acidic pH stops enzymatic activity), and this mixture was centrifuged at 14000g for 2 minutes. To 500 µL of supernatant 400 µL of 0.56 M HCl and 150 µL of 2,4-Dinitrophenylhydrazine were added, following a vortex at maximum speed for 5 seconds, and incubation at 28ºC for thirty minutes. Subsequently, 1 mL 2M NaOH was added to the samples and the color change was observed. Afterwards, the
absorbance was measured at 540 nm using the spectrophotometer, against a blank consisting of all the cited reagents, except for the bacterial lysate.

The α-ketobutyrate production was quantified by a comparison between the results and the standard-curve.

2.5 Total protein quantification

Quantification of total protein was performed based on Bradford method. On that account, to 50 µL of prepared sample, 100 µL of a 0.1 M NaOH solution were applied and the resulting solution was boiled in a water bath for 10 minutes, to induce cellular lysis. Afterwards, the solution was cooled at room temperature.

Following lysis, 3 mL of Bradford reagent were mixed to 50 µL of the lysed solution with a pipette, and left to settle 5 minutes at room temperature. Following this period, the solution was once again mixed and read in a spectrophotometer at 595 nm against a blank consisting of 0.1 M Tris-HCl, NaOH, Bradford reagent, pH 8.0.

Results were obtained by comparison with a standard-curve of Bovine Serum Albumine (BSA), which was performed by adding 3 mL of Bradford reagent to 100 µL of a BSA solution of known concentration (0.25, 0.5, 1.0, 1.4 mg/mL).

3. Studies on the role of ACC deaminase in plant-growth promotion abilities of endophytes

3.1 Cucumber plant-growth promotion assay

In order to test the effect of ACC deaminase in endophytes plant-growth promotion abilities an assay was conducted using cucumber (Cucumis sativus). This plant was selected due to its high germination rate and fast growth. The assay was conducted under normal and stress conditions. Stress conditions were imposed by the use of isoxaben (ISB), a potent cellulose inhibitor. Isoxaben applications leads to an increase of ACC production [91]. Four strains were used in this assay: S. quinivorans BXF1 and S. quinivorans BXF1 pRKACC (obtained in this study); and P. fluorescens YsS6 and its acdS insertion mutant (tetracycline gene disrupted the ACC deaminase gene), P. fluorescens YsS6 acdS (gently ceded by Prof. Bernard Glick, Canada).

Two independent experiments were performed: one in agar plates and other in plastic pots containing vermiculite. Treatments consisted of a negative control (without added bacteria) and each bacterial strain inoculation (wild-type and mutants), under normal and stress conditions, in a total of ten independent treatments.

3.1.1 Bacterial cell culture preparation

Bacterial cultures were grown in TSB medium containing the appropriate antibiotics when necessary, for 48h at 28°C in a rotating shaker, with agitation set for 150 r.p.m. Once grown,
cultures were centrifuged at 6000g for 3 minutes and the pellets were suspended in 0.03 M MgSO₄. The bacterial cultures were adjusted to an optical density (OD) of 0.15 at a 600 nm wavelength using a spectrophotometer.

### 3.1.2 Seed disinfection, germination and inoculation

Cucumber seeds were surface sterilized, through a three-step disinfection process. This consisted of submerging the seeds for 1 minute in a 70% ethanol solution, followed by 3 minutes in a 1% commercial sodium hypochlorite solution, and ending with 5 washes with sterile distilled water to remove any traces of the former solutions.

### 3.1.3 Assay conditions

- **Agar plate assay**

  After disinfection, seeds were immersed in the respective bacterial strain solutions for 1 hour. After this period, four seedlings of each treatment were placed in half strength Murashige and Skoog (MS) [92] agar medium containing 150 nM isoxaben when necessary (stress conditions). Plates were then incubated for 3 days, at 25°C. After this time, the plants were harvested and main roots and lateral roots were counted and its length was measured.

- **Plastic pot assay**

  After the disinfection process, seedlings were placed in 1% agar plates and incubated for 3 days in the dark, at 25°C. Inoculation occurred after seedlings were germinated and placed in a plastic pot containing 100 g vermiculite and, 30 mL of distilled water. After sowing, 1 mL of bacterial solution with adjusted optical density (0.15) was inoculated. Stress with isoxaben (250 nM) was applied once by irrigation, three days after inoculation. The plants were watered every 2 days with 10 mL of 0.03 M MgSO₄ per pot. Eight plants per treatment were used. Ten days after inoculation, the plants were harvested and main roots and lateral root (first lateral root) length were measured. Afterwards, the plants were dried at 60°C for 2 days, and weighed in an analytical scale so that parameters such as root dry weight, shoot dry weight and leaf dry weight were assessed for each treatment.

### 3.2 Common bean nodulation and plant-growth promotion assay

Previous studies have shown that endophytes were able to increase the nodulation abilities of rhizobia, however, the role of ACC deaminase in this process was never investigated. In this sense, a nodulation and plant growth promotion assay was conducted using the endophytes *S. quinivorans* BXF1 and *P. fluorescens* YsS6 and its respective ACC deaminase overproducing (pRKACC) and minus (acdS gene disrupted) mutants. For this assay, the interaction between common bean (*Phaseolus vulgaris*) and *Rhizobium tropici* CIAT899 was used as the symbiotic model. Common bean is a fast growing legume and it forms nodules in a very
early stage of development. *R. tropici* CIAT899 is a fast growing rhizobia and it is used as a commercial inoculant [93]. Its genome was also sequenced [93].

Two independent co-inoculation experiments were conducted under greenhouse conditions: one using BXF1 or BXF1 pRKACC co-inoculated with *R. tropici* CIAT899 and other using YsS6 or YsS6 `acdS` co-inoculated with CIAT899. Treatments consisted of a negative control (without added bacteria), inoculation with CIAT899 only, and inoculation with each bacterial strain (wild-type or mutants) plus CIAT899, under normal (greenhouse) conditions.

3.2.1 Bacterial cell culture preparation

Bacterial cultures were grown in a rotary shaker, with an agitation of 150 rpm, for 2 days at 28°C. The *Serratia* and *Pseudomonas* were grown in TSB medium supplemented with the respective antibiotics, whenever necessary, and *R. tropici* CIAT899 was grown in Yeast Mannitol Broth (YMB) medium (Composition per liter: 1.0 g yeast extract, 10.0 g Mannitol, 0.5 g K$_2$HPO$_4$). Following a 2-day period of growth, cultures were centrifuged at 6000 g for 3 minutes, and suspended in a 0.03 M MgSO$_4$ solution. Cell optical density was, then, adjusted to 0.3 in a spectrophotometer at a 600 nm wavelength.

3.2.2 Seed disinfection, germination and inoculation

Common bean seeds were disinfected by the same method applied to cucumber seeds (see 3.1.2), and also germinated in 1% agar plates, in the dark, at 28°C for 3 days. Following the germination period, one seedling was planted per pot which was filled with a sterilized mixture of sand and vermiculite (1:1), and inoculated with 5 mL of the bacterial solution.

3.2.3 Assay conditions

The assays were conducted under greenhouse conditions in the period of June to August, 2016, in Florianópolis (average temperatures of 23°C maximum and 14°C minimum). Each treatment consisted of eight plant replicates. Plants were irrigated every 2 days with 15 mL of a nitrogen-free nutrient solution [94]. The plants were collected 20 days after inoculation, and parameters like root, shoot and leaf dry weight, as well as nodule number were evaluated. Roots, shoots and leaves were cut separately and dried at 60°C for 3 days, so that dry weights could be measured in an analytical scale.

Despite surface disinfection, after germination all seedlings presented some biotic disease symptoms, hence, suggesting that bean seeds carried the pathogen. In order to unveil the pathogen responsible for the disease symptoms, some portions of affected shoots and leaves were surface sterilized with 70% ethanol for 2 min, placed in 100 µL sterile 0.03 M MgSO$_4$ and grounded with a sterile mortar and pestle. The 100 µL containing the debris were inoculated in TSA plates and incubated at 28°C until bacterial growth was observed. Pink colonies were obtained in great numbers, and after Gram coloration, they could be identified as Gram-Positive. After some research, the pathogenic bacteria was thought to be *Curtobacterium flaccumfaciens*.
In Brazil, this bacterium is commonly found as a pathogenic agent of beans. Moreover, no fungal growth could be observed in the damaged plant tissues, even after incubation in humid chambers, further confirming the previous results.

3.2.4 Root and nodule imaging

In order to assess the colonization of roots and nodules (qualitative) by *S. quinivorans* BXF1 pRKACC expressing GFP, a small assay was conducted using similar conditions described above. Roots and nodules were collected from plants, 20 days after inoculation, using a sterile scalpel. The plant material was visualized in a Leica TCS SP5 confocal microscope, under different fluorescent wavelengths. Images were composed in the Leica LAS X interface program (Leica-microsystems).

4. Evaluation of the ACC deaminase effect in *Cupriavidus taiwanensis* STM894 nodulating abilities in *Mimosa pudica*

Despite the knowledge of the ACC deaminase effect in the nodulation abilities of α-rhizobia, not much is understood about its effect in the association between β-rhizobia and leguminous plants. Therefore, a plant growth promotion and nodulation assay, using *Mimosa pudica*, was conducted in order to evaluate the effect of ACC deaminase in the nodulation abilities of the β-rhizobia, *Cupriavidus taiwanensis* STM894 expressing an exogenous ACC deaminase gene.

4.1 Bacterial cell culture preparation

Both strains of *C. taiwanensis* STM 894 (wild-type and pRKACC) were cultured in YMB medium, supplemented with 15 µg/mL tetracycline in the case of the transformed strain. After incubation for 2 days, at 28°C in a rotary shaker with an agitation of 150 r.p.m, cell cultures were centrifuged at 6000g and suspended in 0.03 M MgSO₄. Bacterial cultures were adjusted to an OD₆₀₀ of 0.3 using a spectrophotometer.

4.2 Seed disinfection, germination and inoculation

Seeds of *Mimosa pudica* were surface sterilized with sulfuric acid in order to break seed dormancy. The applied method consisted of submerging cells in 98% H₂SO₄, for 10 minutes, followed by 10 minutes in 3% sodium hypochlorite solution, and ending with 5 washes with sterile distilled water to remove any trace of the previous solutions. After disinfection, seeds were placed in 1% agar plates and incubated in the dark, for 3 days at 28°C. Subsequently, 1 seedling was placed per pot which was filled with a sterile mixture of sand and vermiculite (1:1) and inoculated with 5 mL of bacterial cell culture.
4.3 Assay conditions

The assay was conducted under greenhouse conditions in June 2016 (average temperatures of 23ºC max. and 14ºC min.), in Florianópolis. In total, 4 treatments were applied, which consisted of a negative control (without bacteria inoculation and nitrogen supplementation), positive control (without bacteria inoculation and supplemented with nitrogen), inoculation of *C. taiwanensis* STM894 (without nitrogen), and inoculation with *C. taiwanensis* STM894 pRKACC (without nitrogen). Eight seedlings were used per treatment.

Solutions used for irrigation, performed each 2 days with 5 mL, consisted of Broughton and Dillworth [94] nutrient solution, supplemented with 0.05% KNO₃ in the case of the positive control.

The experiment had the duration of 30 days (after inoculation). Following this period, plants were harvested and parameters like root, shoot and leaf dry weight, as well as nodule number were evaluated. Roots, shoots and leaves were cut separately and dried at 60ºC for 3 days, so that dry weights could be measured in an analytical scale.

5. Evaluation of the ACC deaminase effect in the strain BXF1 ability to decrease the senescence of carnation flowers

Ethylene plays a major role in promoting flower senescence and death. Endophytic bacteria with ACC deaminase may help to decrease deleterious ACC and ethylene levels, and consequently, decrease the senescence rate of flowers. Therefore, an assay using the endophyte *Serratia quinivorans* BXF1 expressing an exogenous ACC deaminase gene was performed in order to assess the importance of the ACC deaminase enzyme in preventing the senescence of carnation (*Diantus caryophyllus*), a flower highly sensitive to ethylene.

5.1 Bacterial cell culture preparation

Both strains of *S. quinivorans* BXF1, wild type and pRKACC, were grown in TSB medium, supplemented with 15 µg/mL Tet, in the case of the transformed strain. Incubation occurred overnight at 28ºC. After this period, cells were centrifuged at 6000 g for 3 minutes, and the pellet suspended in 0.03 M MgSO₄. Bacterial cultures were adjusted to an OD₆₀₀ of 0.15 using a spectrophotometer.

5.2 Assay conditions

Carnation flowers were bought from a commercial flower store in Florianópolis, Brazil. The flowers were selected by its similar size and characteristics, in order to avoid high variability, and were cut to have 20 centimeters, from the shoot base to the receptacle. After being cut, the plants were immediately placed in test tubes containing 5 mL of the respective bacterial solution OD₆₀₀ of 0.15 or 5 mL 0.03 M MgSO₄ (control). Ten carnation flowers were used per treatment: control (without bacteria), inoculation with strain BXF1 and inoculation with strain BXF1 pRKACC.
The assay was conducted for 8 days, under laboratory conditions, with an average temperature of 24°C. All treatments were irrigated with 2 mL of sterile 0.03 M MgSO₄, whenever necessary, in order to maintain the shoot submerged at all times. Flower senescence rate was evaluated everyday according to Figure 11.

Two plants of each bacterial treatment were collected 48 hours after incubation in order to evaluate bacterial ability to migrate through the shoot of the flower, as described below.

5.3 Evaluation of BXF1 and BXF1 pRKACC flower colonization by PCR

After 48 hours of incubation, two plants of each bacterial treatment were removed and shoots were separated from the flower. The shoot of each plant was cut and two radial sections of 1 cm (length) were removed: the first section corresponded to the shoot base (0-1 cm) and other to the above shoot itself (10-11cm region), as shown in Figure 12. The sections were weighted to contain approximately 100 mg and total DNA was extracted (described below), which was further used as template for the PCR reaction.
5.3.1 DNA extraction

Each plant section weighting approximately 100 mg was flash frozen using liquid nitrogen and grounded with a sterile pestle in order to mechanically disrupt cells and increase the efficiency of cell lysis. After disruption, total DNA was extracted by using the Plant/Fungi DNA Isolation Kit MiniPrep (Norgen, Canada) according to the manufacturer’s protocol. The obtained DNA was quantified in a NanoDrop (Thermofisher) according to the manufacturer’s protocol, and adjusted to a concentration of 50 ng/μL.

5.3.2 Primer design and PCR conditions

The PCR method was employed in order to qualitatively confirm the presence of strains BXF1 wild-type and pRKACC inside the carnation flowers. For this assay specific primers able to identify strain BXF1 were constructed based on the available genome sequence. In this regard, the chiD gene (encoding a chitinase) of strain BXF1 was chosen and used as reference. This gene was found to be only present in Serratia strains and presents great variability between strains (Nascimento et al., unpublished results). Hence, the primer was constructed in the internal region of the gene, diverging from all other Serratia chitinase genes available in the NCBI database. Sequence comparisons were made using Blast analysis (http://blast.ncbi.nlm.nih.gov). The primers were designed using Primer3 (http://primer3.ut.ee) using default parameters, and were chosen based on the region of interest (variable region only found in strain BXF1). The selected primers were: chiDF 5’- CGTCTTACCAGCAGCTGA-3’; and chiDR 5’- CAGGCACCTTTACCACCATT-3’. These primers amplified a 225 bp fragment.

For the amplification reaction, it was used a reaction volume of 25 μL, containing 2.5 μL of 10X Taq —DNA Polymerase Buffer, 2 μL of 25mM MgCl2, 0.5 μL of 10 mM dNTP’s, 5 μL of each primer (5 pmol), 0.1 μl of DNA Taq Polymerase (1 U), 8.9 μl of MilliQ water, and 1 μl of DNA (50 ng/ μl). DNA was amplified using a termocycler with the following program: the initialization step occurred for 4 minutes, at 94ºC, followed by 35 cycles of denaturation at 94ºC, for 45 seconds, annealing for 1 minute, at 52ºC, and elongation for 1:30 minutes, at 72ºC. After the 35 repetitions, the last step consisted of 72ºC for 10 minutes. The PCR products were visualized by gel electrophoresis in agarose gel (1%).

5.4 Statistical analysis

Statistical analysis in all plant experiments was performed by T-student test by using the SPSS Statistics v.22 software (SPSS Inc., IBM Company).
Results and discussion

1. Obtaining and measuring of ACC deaminase activity in transformed strains

In order to perform studies regarding the role of ACC deaminase in plant growth, development and nodulation, two strains, *S. quinivorans* BXF1 and *C. taiwanensis* STM894, were selected and transformed with the plasmid pRKACC. After the transformation procedure both bacterial strains gained the ability to grow on TSA medium supplemented with tetracycline, thus suggesting the successful acquisition of the pRKACC plasmid. The strains were selected by colony morphology and its identity was further confirmed by the 16S rDNA sequencing results. The obtained 16S rDNA sequences were compared to other sequences in the NCBI database and this comparison showed that the selected colonies corresponded to the recipient bacteria and not *E. coli*. Also, *S. quinivorans* BXF1 p519ngfp was able to incorporate the pRKACC plasmid. This strain was selected on TSA plates containing 15 µg/mL tetracycline and 150 µg/mL kanamycin and presented fluorescence (gfp).

In order to evaluate the success of the transformations with the pRKACC plasmid and consequent ACC deaminase expression, the enzymatic activity of ACC deaminase was quantified in the bacterial strains. Values for the produced α-ketobutyrate, resulting from ACC deamination, and total protein are shown in Table 2. Both wild-type strains were unable to degrade ACC, which is consistent with the absence of *acdS* genes in its genomes. On the other hand, both transformed strains were able to produce ACC deaminase and consequently deaminate ACC into ammonia and α-ketobutyrate, thus indicating the presence and consequent use of the plasmid pRKACC by the transformed strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>α-ketobutyrate (µmol)</th>
<th>Total protein (mg/mL)</th>
<th>Incubation time (h)</th>
<th>µmol α-ketobutyrate/mg protein/h</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. quinivorans</em> BXF1</td>
<td>0</td>
<td>n.d</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td><em>S. quinivorans</em> BXF1</td>
<td>0.6</td>
<td>2.1</td>
<td>0.5</td>
<td>10.6</td>
</tr>
<tr>
<td><em>S. quinivorans</em> BXF1</td>
<td>0.5</td>
<td>2.1</td>
<td>0.5</td>
<td>9.9</td>
</tr>
<tr>
<td><em>C. taiwanensis</em></td>
<td>0</td>
<td>n.d</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td><em>C. taiwanensis</em></td>
<td>0.3</td>
<td>1.9</td>
<td>0.5</td>
<td>7.5</td>
</tr>
<tr>
<td><em>Pseudomonas</em> sp. UW4</td>
<td>0.5</td>
<td>1.8</td>
<td>0.5</td>
<td>12.2</td>
</tr>
</tbody>
</table>
Despite the fact that the strains were transformed with the \textit{acdS} gene of \textit{Pseudomonas} sp. UW4, their enzymatic activity was slightly different of that of \textit{Pseudomonas} sp. UW4 (12.2 µmol α-ketobutyrate/mg protein/h) (Table 2). This difference can be explained by the fact that \textit{Pseudomonas} sp. UW4 possesses the \textit{acdS} gene in its genome \cite{95}, in contrast to the transformed strains that contain the \textit{acdS} gene in the low copy plasmid, pRKACC. Being a low copy plasmid, pRKACC is present only in reduced quantity inside cells, which leads to a limited expression of ACC deaminase. In addition, some differences between enzymatic activities of the transformed strains were also observed, which might be due to the fact that the strains belong to distinct bacterial genera. \textit{Serratia} belongs to the Y-Proteobacteria class and Enterobacteriaceae family, and \textit{C. taiwanensis} belongs to the β-Proteobacteria class and Burkholderiaceae family. Bacteria from distinct genera present significant differences in parameters, such as, bacterial generation time, number of plasmids and efficiency of replication, energetic needs, and also in levels of gene expression \cite{26}. Therefore, such differences may have contributed to the disparity found between enzymatic activities of the transformed strains.

Interestingly, \textit{C. taiwanensis} possesses one plasmid, which contains the symbiotic genes \cite{87}. On the other hand, \textit{S. quinivorans} BXF1 does not carry any plasmids (Nascimento et al., unpublished results). This fact can also affect the success of pRKACC replication, maintenance and, consequently, gene expression. Nevertheless, \textit{S. quinivorans} BXF1 possessing both plasmid p519ngfp and pRKACC presented high ACC deaminase values.

Nevertheless, the obtained ACC deaminase activity values for the transformed strains can be considered high and within the range of the ACC deaminase activity values presented by free-living bacteria \cite{13}. This ACC deaminase activity can readily impact plant growth and development. Moreover, rhizobia only possess low activity values (0.076 to 0.274 µmol α-ketobutyrate/mg protein/h in \textit{Rhizobium} spp. \cite{96}) and can still have an effect in plant-growth promotion \cite{33}.

### 2. Effect of ACC deaminase in bacterial endophyte's ability to promote cucumber growth

To assess the effect of ACC deaminase in the bacterial endophytes ability to promote cucumber plant growth, two bacterial strains (\textit{S. quinivorans} BXF1 and \textit{P. fluorescens} YsS6) and their respective ACC deaminase overproducing (BXF1 pRKACC) or minus mutants (YsS6 \textit{acdS}-) were used.

When assessing the effect of ACC deaminase in cucumber root development, under normal conditions, results obtained in the plate showed that the expression of the exogenous \textit{acdS} gene enhanced \textit{S. quinivorans} BXF1 ability to elongate primary roots and to induce lateral root formation (Figure 13), however, not significantly. Under stress conditions, induced by high concentrations of isoxaben, primary root length and the number of lateral roots formed were the most affected parameters. Nevertheless, BXF1 pRKACC was able to significantly enhance (in about 30%) the lateral root number when compared to the wild-type strain (Figure 13).
Results obtained in the cucumber plate assay in plants inoculated with strain BXF1 or BXF1pRKACC, under normal and stress conditions (ISB). Plants obtained 3 days after inoculation. Statistically significant differences (P < 0.05) are marked with * Caption: PRL – Primary Root Length; LRL – Lateral Root Length; LRN – Lateral Root Number. Results based on 4 plants.

In the pot assay, the transformed strain, BXF1 pRKACC, significantly promoted cucumber lateral root formation and elongation in a higher extent (increase of approximately 50 and 10%, respectively) when compared to the BXF1 wild-type strain, under control conditions (Figure 14). Other parameters, including total biomass, shoot and leaf dry weight, root dry weight, and primary root length were also found to be enhanced in the BXF1 pRKACC treatment, however the differences were not statistically significant (Figure 14). Under stress conditions, the BXF1 pRKACC strain was able to significantly increase primary (30 %) and lateral root length (45 %), root, shoot and leaf dry weight when compared to the BXF1 wild-type strain (Figure 14). These results suggest that the presence of an exogenous ACC deaminase seems to be more important in strain BXF1 plant growth promotion abilities under stressful conditions.
Figure 14 – Results obtained in the cucumber pot assay in plants inoculated with strain BXF1 or BXF1pRKACC, under normal and stress conditions (ISB). Plants obtained 10 days after inoculation. Statistically significant differences (P < 0.05) are marked with *. Caption: PRL – Primary Root Length; LRL – Lateral Root Length; RDW – Root Dry Weight; SLDW – Shoot and Leaf dry weight; TB – Total Biomass. Results based on 8 plants.

Concerning the experiment with *P. fluorescens* YsS6, results obtained in the plate assay showed that the deletion of the *acdS* gene led to a significant decrease of *P. fluorescens* YsS6 cucumber growth-promoting abilities (Figure 15). For instance, under control conditions, plants inoculated with *P. fluorescens* YsS6 *acdS* showed a significant reduction of about 20% in primary root length when compared to plants inoculated with the wild-type. Also, lateral root length suffered a 40% decrease, and lateral root number was reduced in about 40% (Figure 15). Under stress conditions, plants inoculated with the mutant strain, *P. fluorescens* YsS6 *acdS*, presented significant reductions in all assessed parameters, when compared to plants inoculated with the
wild-type strain (Figure 15). The stressful effects of isoxaben were much more pronounced in plants inoculated with the mutant strain than with the wild-type (Figure 15).

Interestingly, under stress conditions, wild-type YsS6 inoculated plants presented an increased lateral root length when compared to the same treatment under normal conditions (Figure 15). However, plants inoculated with the acdS mutant strain presented a great reduction in lateral root length. Overall, plants treated with the mutant YsS6 acdS strain were less able to develop their primary and lateral roots, and to resist stress effects, when compared to the wild-type strain (Figure 15).

![Graph](image)

Figure 15 - Results obtained in the cucumber plate assay in plants inoculated with strain YsS6 or YsS6 acdS-, under normal and stress conditions (ISB). Plants obtained 3 days after inoculation. Statistically significant differences (P < 0.05) are marked with *. Caption: PRL – Primary Root Length; LRL – Lateral Root Length; LRN – Lateral Root Number. Results based on 4 plants.

As for the plastic pot assay, results showed that the deletion of the acdS gene generally resulted in a reduction of YsS6 cucumber plant-growth promotion abilities (Figure 16). Hence, under control conditions, plants inoculated with YsS6 acdS mutant strain presented a significant reduction in primary (15 %) and lateral root length (45 %), as well as, root dry weight (35 %) when compared to its wild-type counterpart (Figure 16). Total biomass, and shoot and leaf dry weight also decreased in plants inoculated with YsS6 acdS, however not significantly (Figure 16). Furthermore, as previously demonstrated, treatment with ISB led to a negative effect of the
majority of the evaluated parameters, when compared to control conditions (Figure 16). The negative effects of isoxaben were more pronounced in plants inoculated with YSS6 acdS when compared to plants receiving the Ys6 wild-type (Figure 16). In this sense, the Ys6 acdS mutant presented a decreased ability to promote primary and lateral root length (reductions of 12% and 57%, respectively), root dry weight (reduction of 40%) as well as to develop plant aerial part, when compared to Ys6 wild-type (Figure 16).

![Graph showing normalization of parameters in relation to YS56 (%)](image)

**Control** | **ISB**
---|---
Ys6 | Ys6 acdS-

Figure 16 - Results obtained in the cucumber pot assay in plants inoculated with strain YsS6 or YsS6 acdS-, under normal and stress conditions (ISB). Plants obtained 10 days after inoculation. Statistically significant differences (P < 0.05) are marked with *.

Altogether, these results indicate that ACC deaminase plays a significant role in bacterial endophytes ability to promote plant development, more significantly in root development. Previous studies have demonstrated that other ACC deaminase-producing bacteria were able to
promote and increase root development. For example, Glick et al. [97] demonstrated that bacteria expressing ACC deaminase were able to promote canola primary root elongation. Similarly, Compant et al. [4] indicated that the endophyte Burkholderia phytofirmans PsJN presenting ACC deaminase activity promoted root development in Vitis vinifera. These results are in accordance with literature reports indicating that ethylene inhibits primary and lateral root formation [98]. Therefore, bacteria that possess ACC deaminase are able to decrease ACC and consequently ethylene levels [13] that limit primary and lateral roots development.

Under stress conditions endophytes expressing ACC deaminase were able to decrease the negative effects of isoxaben. It is known that ISB is a potent inhibitor of root development and leads to the accumulation of ACC [91]. Thus, bacteria that express ACC deaminase are able to metabolize exuded ACC that is formed in response to stress, and lower the negative effects of stress ethylene. Therefore, it is only natural to assume that bacterial ACC deaminase has a main function in decreasing the harmful effects of abiotic stress, which has been reported several times in literature. For example, Sheng et al. [99] demonstrated that the ACC deaminase-producer endophyte P. fluorescens G16 increased plant resistance to heavy metals. Furthermore, as demonstrated by Ali et al. [77], high salinity showed less stressful effects on tomato plants treated with Pseudomonas fluorescens YsS6 and Pseudomonas migulae 8R6, both endophytes producers of ACC deaminase.

### 3. Effect of endophytic bacterial ACC deaminase production in common bean nodulation, growth and development

An assay with common bean (Phaseolus vulgaris) was conducted in order to evaluate the effect of endophytic bacterial ACC deaminase in bean growth and development, as well as in assisting in the nodulation process mediated by Rhizobium tropici CIAT 899.

#### 3.1 ACC deaminase effect in S. quinivorans BXF1 ability to promote bean growth and nodulation

Results showed that co-inoculation of R. tropici CIAT 899 and S. quinivorans BXF1 generally enhanced plant total biomass, when compared to sole rhizobial inoculation, though not significantly (Figure 17). However, when BXF1 expressed ACC deaminase, every estimated parameter suffered a drastic increase, in relation to the results obtained with the other treatments applied. For instance, root and shoot dry weight, and consequently total biomass increased significantly in 56%, 63%, and 62%, respectively, when compared to results obtained with the BXF1 wild-type strain (Figure 17).
Figure 17 - Mean dry weight per plant regarding the co-inoculation assay of *R. tropici* CIAT 899 and *S. quinivorans* BXF1 wild-type and pRKACC in common bean. Statistically significant differences (P < 0.05) are marked with *. Caption: RDW – Root Dry Weight; SLDW – Shoot and Leaf Dry Weight; TB – Total Biomass. Results based on 8 plants.

Interestingly, all common bean plants showed some disease symptoms even after the disinfection procedure. Chlorosis on leaves and shoots but not the roots, were observed in most plants, with greater extent in non-inoculated (control) plants. These symptoms were clearly similar to those induced by *Curtobacterium flaccumfaciens*, a common pathogen naturally found in bean seeds. The *C. flaccumfaciens* infection of bean is a seed-borne disease widely distributed in Brazil [100]. Pink Gram-positive bacteria (typical coloration of *Curtobacterium*) were isolated from the shoots of infected bean plants (Figure 18), suggesting its presence in the diseased plants.

Importantly, plants inoculated with the *S. quinivorans* BXF1 pRKACC presented less disease symptoms when compared to *R. tropici* CIAT899 alone or in combination with wild-type BXF1 (Figure 19), hence indicating the role of ACC deaminase in the enhanced plant resistance to the biotic stress.

Figure 18 – Presence of *C. flaccumfaciens* isolated in tissues of common bean.
In order to evaluate the role of endophytic ACC deaminase in assisting *R. tropici* CIAT 899 in the nodulation process of common bean, the number of root nodules formed was evaluated. Co-inoculation of *R. tropici* CIAT899 and *S. quinivorans* BXF1 generally enhanced root nodulation, when compared to results obtained with sole rhizobial inoculation (Figure 20). However, statistical significant results were only obtained in the presence of *S. quinivorans* BXF1 pRKACC, which increased the number of nodules formed by CIAT899 in 127%, when compared to the BXF1 wild-type strain (average of 11 nodules by *S. quinivorans* BXF1 vs. an average of 25 nodules by *S. quinivorans* BXF1 pRKACC) (Figure 20). Concerning nodule development, it was found that ACC deaminase production by the BXF1 endophyte not only induces the formation of a greater number of nodules by CIAT899, but also increased nodule development, with the formed nodules appearing larger and with a more pronounced pink tone (indicative of nitrogen fixation) than the ones obtained in the co-inoculation of *R. tropici* CIAT899 with *S. quinivorans* BXF1 wild-type (Figure 20).
In order to observe the endophytic colonization of roots and nodules, vegetal tissues were observed under a confocal microscope with *S. quinivorans* BXF1 marked with Green Fluorescent Protein (GFP) (Figure 21).

**Figure 20** – Nodulation assay in common bean with co-inoculation of *R. tropici* CIAT899 and *S. quinivorans* BXF1, 20 days after inoculation. A) Mean number of nodules per plant. B) co-inoculation of *R. tropici* CIAT 899 and *S. quinivorans* BXF1. C) co-inoculation of *R. tropici* CIAT 899 and *S. quinivorans* BXF1 pRKACC. Statistically significant differences (P < 0.05) are marked with *.

**Figure 21** - Confocal Microscope Imaging of common bean tissues. A) Endophytic *S. quinivorans* BXF1 pRKACC presence in the intercellular spaces of the root. B) Hypercolonization of *S. quinivorans* BXF1 pRKACC on the nodule surface.
Regarding root colonization, it can be observed the presence of *S. quinivorans* BXF1 in the intercellular spaces of the roots, effectively colonizing all the extension of the considered sample (Fig. 21A). *S. quinivorans* BXF1 pRKACC also hipercolonizes the surface of the root nodule (Fig. 21B), which may facilitate *R. tropici* CIAT 899 nodulation abilities.

### 3.2 ACC deaminase effect in *P. fluorescens* YsS6 ability to promote bean growth and nodulation

Plants inoculated with both *R. tropici* CIAT899 and *P. fluorescens* YsS6 presented significantly higher values for root dry weight, shoot and leaf dry weight, and total biomass (28%, 22% and 24%, respectively) when compared to the results obtained with the mutant strain (Figure 22). When compared to the single rhizobial inoculation (CIAT899), treatment with the wild-type strain of *P. fluorescens* YsS6 and CIAT899 significantly enhanced root dry weight, shoot and leaf dry weight, and total biomass in 39%, 111% and 43%, respectively (Figure 22).

![Figure 22 – Mean dry weight per plant of the co-inoculation assay of *R. tropici* CIAT 899 and *P. fluorescens* YsS6 wild-type and acdS- in common bean. Statistically significant differences (P < 0.05) are marked with *. Caption: RDW – Root Dry Weight; SLDW – Shoot and Leaf Dry Weight; TB – Total Biomass. Results based on 8 plants.](image)

When in comparison to plants treated with CIAT899 and *P. fluorescens* YsS6 acdS-, plants treated with CIAT899 and the wild-type of *P. fluorescens* YsS6 presented longer roots, taller shoots and more developed leaves (Figure 23). Furthermore, these plants also showed enhanced resistance to stress caused by the pathogen *C. flaccumfaciens*, observable especially on the leaves, which appear to be more developed and asymptomatic, when in comparison to the other applied treatments (Figure 23). The presence of ACC deaminase in *P. fluorescens* YsS6 revealed a crucial importance in bacterial induced plant growth and protection. Results show that the wild-type strain of *P. fluorescens* YsS6 yielded no symptoms of disease (Figure 23), suggesting that ACC deaminase may be a great asset to the plant, when facing stressful conditions. On the other hand, *P. fluorescens* YsS6 acdS- presented a decreased ability to protect plants against *Curtobacterium* infection.
Figure 23 - Common bean plants (A) and disease symptoms (B) obtained in the co-inoculation assay of *R. tropici* CIAT 899 (CIAT 899) and *P. fluorescens* YsS6 (YsS6) wild-type and *acdS*- (YsS6 *acdS*-), 20 days after inoculation. Disease symptoms scale can be found in Appendix B. Caption: NC – Negative Control

Regarding the number of nodules, as well as their developmental state, co-inoculation of *R. tropici* CIAT 899 and *P. fluorescens* YsS6, resulted in an increased rhizobial nodulatory activity (Figure 24). The endophyte *P. fluorescens* YsS6 induced a significant increase in the number of nodules formed by CIAT899, enhancing nodulation in over 200%, when compared to the results obtained with the YsS6 *acdS*- mutant (Figure 24). Regarding nodule development, plants co-inoculated with *P. fluorescens* YsS6 presented more developed nodules than the ones existing on the roots of plants treated with the YsS6 *acdS*- mutant, which once again indicates an active role of YsS6 ACC deaminase in assisting the nodulation process (Figure 24).
Figure 24 – Nodulation assay in common bean with co-inoculation of *R. tropici* CIAT899 and *P. fluorescens* YsS6, 20 days after inoculation. A) Mean number of nodules per plant. B) co-inoculation of *R. tropici* CIAT 899 and *P. fluorescens* YsS6. C) co-inoculation of *R. tropici* CIAT 899 and *P. fluorescens* YsS6 acdS-. Statistically significant differences (P < 0.05) are marked with *.

Overall, co-inoculation of *R. tropici* CIAT 899 with ACC deaminase producing endophytes presented positive results. In general, in the presence of ACC deaminase-producing bacteria, plants presented longer and thicker roots, with an increased number of lateral roots, and a greater biomass. The aerial part of the plant (shoot and leaves) was also positively affected in the presence of endophytes containing the ACC deaminase gene, presenting greater biomass and an enhanced development. This is consistent with the previous obtained results.

Nodulation abilities of CIAT899 were also found to be enhanced, as beans co-inoculated in the presence of endophytes containing the ACC deaminase gene grew roots with a greater number of nodules, and in a more advanced state of development. These results may suggest that co-inoculation with ACC deaminase-producing bacteria plays an important role in assisting rhizobial colonization of roots, as well as, in the formation of nodules. This suggestion is in agreement with some reports that indicate that the inoculation of legumes with a combination of both rhizobial strains and other ACC deaminase-producing bacteria, is an effective strategy to increase nodulation. In fact, Shaharoona et al. [78] demonstrated that *B. japonicum* co-inoculation with a PGPB possessing ACC deaminase activity enhanced by 48% the number of nodules on mung bean plants, when compared to the sole rhizobial inoculation. Equally, Remans et al., [79]
reported that co-inoculation of PGPB with a strain of \textit{R. etli} enhanced nodulation in common beans. In addition, co-inoculation of chickpea and lentil plants with rhizobia and PGPB expressing high ACC deaminase activities increased plant nodulation and growth, in comparison to single rhizobial inoculation \cite{80, 81}.

Furthermore, CIAT 899 co-inoculation with ACC deaminase producing endophytes enhanced plant resistance to stress caused by the natural bean pathogen \textit{C. flaccumfaciens}. Therefore, these results represent further evidence regarding the importance of the enzyme ACC deaminase in lowering the deleterious effects of biotic stress. Some reports have showed the impact of ACC deaminase in biotic stress control. Hao et al. \cite{101} demonstrated that \textit{Agrobacterium tumefaciens} C58 partially lost its ability to induce crown gall tumors on tomato plants and castor bean plants, after being transformed with an exogenous \textit{acdS} gene. Additionally, Toklikishvili et al. \cite{102} observed that the rhizobacterium \textit{Pseudomonas} sp. UW4, and the endophyte \textit{B. phytofirmans} PsJN, naturally possessing the \textit{acdS} gene, were able to reduce the development of tumors on tomato plants infected with \textit{Agrobacterium} strains. Furthermore, \textit{acdS} deletion mutants of \textit{Pseudomonas} sp. UW4 and \textit{B. phytofirmans} PsJN, were not able to significantly reduce \textit{Agrobacterium}-induced tumors. Thus, these data suggest that rhizobacteria and endophytes producing ACC deaminase are effective biocontrol agents, and the possibility of using these bacteria to protect plants from biotic stresses is potentially of great importance in improving crops productivity.

4. The effect of ACC deaminase in the plant-growth promotion and nodulation process mediated by \textit{C. taiwanensis} STM894

The effect of ACC deaminase on the nodulation process mediated by \textit{C. taiwanensis} STM894 was tested in \textit{M. pudica}. In order to do so, two strains of this β-rhizobium were used, the wild-type strain, and an overproducing ACC deaminase strain (\textit{C. taiwanensis} STM894 pRKACC).

Plants inoculated with the wild-type strain STM894 presented an increased growth when compared to non-inoculated plants (negative control), and a similar growth and development as plants supplemented with nitrogen (positive control), hence, indicating the beneficial plant-growth promoting abilities of the STM894 strain. However, the transformed strain \textit{C. taiwanensis} STM894 pRKACC significantly promoted plant growth (all studied parameters) in greater extent, when compared to the wild-type strain (Figure 25). Plants inoculated with STM894 pRKACC also presented more elongated primary and lateral roots, and a more developed shoot when compared to plants treated with the wild-type (Figure 25).

The role of ACC deaminase in affecting the nodulation abilities of \textit{C. taiwanensis} STM 894, was also studied (Figure 26). There was a significant increase in the number of nodules formed by strain \textit{C. taiwanensis} STM894 pRKACC when compared to the wild-type strain, indicating that the exogenous \textit{acdS} gene was able to improve nodulation abilities of \textit{C. taiwanensis} STM 894 by 125%. Moreover, these results consist of the first report regarding a β-
rhizobium transformed with the plasmid pRKACC, successfully increasing its nodulation abilities on a legume.

Figure 25 - *Mimosa pudica* assay inoculated with *C. taiwanensis* STM894. A) Representative plants of each treatment applied, 30 days after inoculation. B) Mean results per plant of Root dry weight (RDW), shoot dry weight (SDW), and total biomass (TB). Caption: NC – Negative Control, PC – Positive Control. Results based on 8 plants.
Several other studies using α-rhizobia expressing exogenous ACC deaminase genes have been performed. For instance, Ma et al. [60] described that *S. meliloti* Rm1021, transformed to express an exogenous ACC deaminase gene, increased its ability to nodulate *M. sativa* plants in 40% when compared to its wild-type form. Furthermore, Nascimento et al. [9] revealed that the strain *M. ciceri* LMS-1 expressing an exogenous ACC deaminase (through plasmid pRKACC) enhanced nodulation of *C. arietinum* by 127%, when compared to the wild-type form. Similarly, Brigido et al. [103], demonstrated that salt-sensitive *Mesorhizobium* strains increased their nodulation abilities by expressing an exogenous acdS gene. In addition, Kong et al. also showed that the expression of the plasmid pRKACC in *S. meliloti* CCNWSX0020 increased nodulation of *Medicago lupulina* plants [10]. Altogether, these reports indicate that ACC deaminase poses an important strategy in modulating ethylene levels in roots, which consequently leads to an increase in nodulation and to a better plant growth and development. By inducing a high enzymatic activity, the insertion of an exogenous acdS gene might be considered an important asset to the development of a rhizobial inocula, with increased nodulation abilities, however, its use is restricted by the regulation on the use of Genetically Modified Organisms (GMO’s). In this sense, co-inoculation of rhizobia and free-living ACC deaminase-producing bacteria poses an alternative.

![Figure 26 - Differences in the number of nodules obtained in *Mimosa pudica* inoculated with STM894 and STM894 pRKACC. Statistically significant differences (P < 0.05) are marked with *.

**5. Evaluation of the ACC deaminase effect in the strain BXF1 ability to decrease the senescence of carnation flowers**

To evaluate the effect of endophytic ACC deaminase on decreasing the senescence rate of carnation, a highly ethylene-sensitive flower, an assay featuring *S. quinivorans* BXF1 and its ACC deaminase-overproducing strain *S. quinivorans* BXF1 pRKACC was performed.

During 192 hours (8 days), symptoms for 8 plants were annotated, being the average values for each treatment presented in Figure 27.
The inoculation of flowers with the wild-type BXF1 did not affect the senescence rate, when compared with the control (no bacteria added). However, the presence of strain BXF1 pRKACC delayed flower senescence to a considerable extent, when compared to results obtained with the wild-type strain of *S. quinivorans* BXF1 or the control. This difference was obtained only past 4 days after inoculation, suggesting that ethylene senescence effects only start to take effect in latter periods after the flower being cut. After 8 days of incubation, carnation flowers inoculated with BXF1 pRKACC presented less senescence symptoms when compared to flowers inoculated with the wild-type BXF1 (Figure 28). These results suggest that the expression of ACC deaminase is vital for the endophyte ability to regulate flower ethylene levels. Similar results were obtained by Ali et al., [104], showing that flowers treated with wild-type ACC deaminase-containing endophytic strains exhibited the most significant delay in flower senescence, while flowers treated with the ACC deaminase minus mutants senesced at a rate similar to the control.

![Figure 27](image.png) - Variation of the senescence symptoms presented by carnation flowers inoculated with BXF1 and BXF1 pRKACC, during 8 days. Statistical significant differences (P < 0.05) are marked with *.

![Figure 28](image.png) – Carnation flowers after 8 days of incubation. Flowers treated with A) BXF1 pRKACC and B) BXF1.
Despite the knowledge on the role of endophyte’s ACC deaminase in decreasing the senescence rate of carnation, not much is understood about its impact in the endophytic colonization abilities of these bacteria. Hence, the endophytic colonization abilities of both wild-type and BXF1 pRKACC were studied. In this sense, the PCR method was employed in order to qualitatively confirm the presence of strains BXF1 wild-type and pRKACC inside the carnation flowers, after 48 hours of incubation. Results presented in Figure 29 show that both wild-type and BXF1 pRKACC could be detected inside the shoot of carnation flowers at least 10 cm away from the inoculation point.

Figure 29 – Results obtained after gel electrophoresis of the amplified DNA products. Caption: 1 – Negative Control; 2 and 3 – BXF1 (0-1cm); 4 and 5 – BXF1 pRKACC (0-1cm); 6 and 7 – BXF1 (10-11cm); 8 and 9 – BXF1 pRKACC (10-11cm); 10 – Positive Control. The DNA ladder used was 1Kb Plus (Invitrogen).

These results suggest that, despite being a qualitatively analysis, at this time point (48 hours) the presence of ACC deaminase does not influence the colonization abilities of strain BXF1, since both strains are present. This is consistent with the results described above, showing a similar senescence rate between flowers inoculated with wild-type or BXF1 pRKACC, after 48 hours. Nevertheless, more robust studies with increased sampling points and incubation times, are necessary to confirm this hypothesis.
Conclusions and Future prospects

Overall, this work confirmed the beneficial effects of the application of an exogenous ACC deaminase in promoting general plant growth and development, in increasing plant resistance to both abiotic and biotic stresses, in enhancing the nodulatory capacities of rhizobia, and in prolonging the shelf-life of an ethylene sensitive flower. Thus, the broad spectrum of results obtained indicate that ACC deaminase is involved in many different aspects of the plant life cycle, through complex interactions with bacteria and plant, many of which still remain unknown. Furthermore, the diversity of plants used in this work demonstrates that treatment with ACC deaminase-producing bacteria is transversal to many different types of plants and legumes, which may point to an extensive range of industrial applications and solutions. Hence, the main findings presented in this work support the application of PGPB producers of ACC deaminase as a competitive and eco-friendly answer to many environmental problems raised by the application of chemical fertilizers in crops.

Further work and investigation must, therefore, be done in order to better understand how to convert these laboratorial findings to a large scale commercialization and industrial application of inoculum. Hence, on the topic of the co-inoculation of endophytic producers of ACC deaminase and rhizobia, new studies could arise with the aim of finding the best combination of both types of bacteria to better suit the development of a determined legume. Moreover, further studies regarding the behavior of acdS-transformed rhizobia in the nodulation of legumes could also be performed. A more profound investigation concerning the acdS gene could be considered as well, namely with respect to better understanding the regulatory regions of the acdS gene of β-rhizobia, or to map the existence of the acdS gene in endophytes, with the aim of understanding its function phylogeny and origin. Furthermore, a deeper investigation on the genes responsible for endophytic colonization is also relevant, since this field of work is almost unexplored.

Finally, the present work offers a new approach to the applications of bacterial ACC deaminase as a suitable commercial inoculum for many agricultural crops, thriving as a clean solution for the environmental future of our planet.
References


bacteria on crown gall formation in tomato plants infected by *Agrobacterium tumefaciens* or *A. vitis,* "*Plant Pathol.*, vol. 59, no. 6, pp. 1023–1030, 2010.


**Visited Websites**

- **Population Institute:** [www.populationinstitute.org](http://www.populationinstitute.org)
- **US Census Bureau:** [www.census.gov](http://www.census.gov)
Appendix A – Bacterial Strains used in this work

Table 3 – Bacterial strains used in this work.

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Serratia quinivorans</em> BXF1</td>
<td>Wild type, Endophytic strain from the <em>Enterobacteriaceae</em> family</td>
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<td>pRKACC</td>
<td>BXF1 strain overexpressing ACC deaminase</td>
</tr>
<tr>
<td>p519ngfp</td>
<td>BXF1 strain expressing Green Fluorescence Protein</td>
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<tr>
<td><em>Pseudomonas fluorescens</em> YsS6</td>
<td>Wild type, Endophytic strain from the <em>Pseudomonaceae</em> family</td>
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<tr>
<td>acdS-</td>
<td>YsS6 with a knock-out of the <em>acdS</em> gene</td>
</tr>
<tr>
<td><em>Cupriavidus taiwanensis</em> STM894</td>
<td>Wild type, Rhizospheric bacteria from the ( \beta )-Proteobacteria family</td>
</tr>
<tr>
<td>pRKACC</td>
<td>STM894 overexpressing ACC deaminase</td>
</tr>
<tr>
<td><em>Rhizobium tropici</em> CIAT 899</td>
<td>Wild type, Rhizospheric bacteria from the ( \alpha )-Proteobacteria family</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>pRKACC, Strain used as donor (carries the plasmid of interest) in the triparental conjugation method</td>
</tr>
<tr>
<td><em>E. coli</em> MT616</td>
<td>pRK600, Strain used as helper (carries a conjugative plasmid) in the triparental conjugation method</td>
</tr>
</tbody>
</table>
Appendix B – Scale of disease symptoms in common bean

Figure 30 – Scale of disease symptoms on common bean, caused by the pathogen Curtobacterium flaccumfaciens.
Appendix C – Bacterial Characterization

1. Methods of bacterial characterization

Table 4 – Methods of bacterial characterization

<table>
<thead>
<tr>
<th>Property</th>
<th>Method</th>
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<tr>
<td><strong>Cellulase activity</strong></td>
<td>To determine cellulase production, 5 µL of bacterial isolates grown overnight in TSB (Tryptic Soy Broth) medium were spot-inoculated in CMC (carboxymethylcellulose) plates, and incubated overnight at 28ºC. After this period, plates were flooded with Gram’s iodine solution (2.0 g KI and 1.0 g iodine in 300 mL distilled water). The presence of zones of clearance around the bacterial colony indicated cellulase activity. Comparison between isolates assessed the production of cellulase. Testing was repeated three times [105].</td>
</tr>
<tr>
<td><strong>Protease activity</strong></td>
<td>Aiming to estimate bacterial protease production, 20 µL of bacterial isolates grown overnight in TSB (Tryptic Soy Broth) medium were spot-inoculated in NA (Nutrient Agar) plates supplemented with 5% lactose-free milk, and incubated for 2 days at 28ºC. Protease activity was assessed based on a clearance area formed around the bacterial colony. Comparison between isolates assessed the production of cellulase and testing was repeated three times.</td>
</tr>
<tr>
<td><strong>Phosphate solubilization activity</strong></td>
<td>To evaluate bacterial phosphate solubilization, it was used PDYA-CaP medium (potato-dextrose agar supplemented per liter with 5 g yeast extract, 10% K₂HPO₄ and 10% CaCl₂), in which 5 µL of each bacterial isolate grown overnight in TSB medium was spot-inoculated, and incubated for 48 hours, at 28ºC. Phosphate solubilization was determined based on the comparison between the clearance zone developed around the colony and colony diameter. Testing was repeated three times for each isolate [106].</td>
</tr>
<tr>
<td>Siderophore production</td>
<td>Qualitative measuring of siderophore production was assessed based on a change of the medium color, according to the method of Schwyn and Neilands [107]. Bacteria-produced siderophores remove iron from a complex formed with the blue dye chrome azurol S (CAS). A change in CAS medium from blue to orange indicates a positive reaction. To qualitatively measure the production of siderophores, 5 mL of an overnight bacterial culture grown in King's B medium was spot-inoculated onto a CAS agar plate, and incubated at 28°C for 48 h. The procedure was repeated three times for each bacterial strain.</td>
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<tr>
<td>Indole-3-acetic acid production</td>
<td>Bacterial ability to produce IAA was measured by a minor variant of the method described by Glickmann and Dessaux [108]. A 5 mL aliquot of TSB medium supplemented with tryptophan (500 µg/mL) was inoculated with 20 µL of an overnight grown bacterial culture, and incubated at 28°C for 24h. The same procedure was applied to the control, which consisted of inoculated TSB medium without addition of tryptophan. Cultures were then centrifuged, and 1 mL of the supernatant was added to 4 mL of Salkowski's reagent (2 mL 0.5M FeCl₃ and 49 mL perchloric acid in 49 mL distilled water). Following 20 minutes of incubation at room temperature, absorbance was measured at OD₅₃₅. The concentration of IAA was quantified based on a standard curve, with a range from 1 to 25 µg/mL. This experimental procedure was repeated three times for each isolate.</td>
</tr>
<tr>
<td>Antibiotic resistance</td>
<td>Bacterial isolates were tested for resistance to four different antibiotics, them being, ampicillin (Amp) (50 µg/mL), kanamycin (Kan) (50 µg/mL), Streptomycin (Strp) (50 µg/mL), and tetracyclin (Tet) (15 µg/mL). Each antibiotic was added separately to 5 mL TSB to which 20 mL of an overnight culture test culture was added. Bacterial cultures were then incubated for 2 days at 28°C. After this period, bacterial resistance to the different antibiotics was evaluated based on their growth in each medium.</td>
</tr>
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</table>
## 2. Results

Table 5 – Results of bacterial characterization.

Caption: n.q – not quantified; R - resistant; N- not resistant; Tet – Tetracycline; Amp – Ampiciline; Kan – Kanamycin; Strep – Streptomycin

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