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**The phytohormone mediated affair of *Triticum durum* and  
*Azospirillum brasilense*: a case study on plant-bacteria  
interaction**

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## **The phytohormone mediated affair of *Triticum durum* and *Azospirillum brasilense*: a case study on plant-bacteria interaction**

This thesis was performed at the ecology lab in FUCL and Bugworkers Laboratory | M&B – BioISI | Teclabs under the supervision of Professora Doutora Cristina Cruz and Professor Doutor Rogério Tenreiro in the scope of the Master in Applied Microbiology of the Faculty of Sciences of the University of Lisbon.

## Abstract

One of the greatest challenges that mankind faces is being able to feed a growing population while trying to minimize its environmental impact. To achieve this, we need to combine an increase in crop yield with a more eco-friendly approach in agriculture. A possible solution is through the utilization of plant growth promoting rhizobacteria (PGPR). Among those PGPR, *Azospirillum* is considered as one of the most important rhizobacterial genus. Since it was discovered, more than 40 years ago, the genus *Azospirillum* became the most studied plant growth-promoting bacteria, being used worldwide for improving plant growth and crop yield. It has been reported that inoculation with *Azospirillum* leads to changes in the plant root system, with increased root proliferation. Which in turn, leads to an increase in root density, enhancing plants capacity to access water and nutrients, as it extends the area explored by the root system, the so-called sponge effect.

This work shows that nitric oxide (NO) and indole-3-acetic acid (IAA) mediate the *A. brasilense*-induced increase in the number of second order roots (SORs) in wheat, without affecting the first order root (FORs) structure. Those effects combine, lead to an increase in root density. Application of a NO scavenger – 2-(4- carboxyphenyl)-4,4,5,5, -tetramethylimidazoline-1-oxyl- 3-oxide (cPTIO) and an auxin scavenger –  $\alpha$ -(p-Chlorophenoxy) isobutyric acid (PCIB), either together or separated, completely block the *A. brasilense* effect on the root system. While the application of a NO donor - sodium nitroprusside (SNP) and a synthetic auxin – Naphthaleneacetic acid (NAA), either together or separated, mimic the effects of inoculation.

It was also showed that NO and IAA mediate the *A. brasilense* direct increase of the wheat biomass production, through the stimulation of the plant photosynthetic activity. Application of cPTIO completely blocked the *A. brasilense* effects, while application of PCIB only partially blocked the effects of the inoculation. The application of SNP and NAA, together or separated, mimic the effects of the bacterial inoculation.

The IAA production by *A. brasilense* is very dependent on the availability of tryptophan and increases with a higher initial inoculum concentration. Whereas denitrification might be actual a positive feature for bacterial inoculants, as it produced NO which is fundamental for the *A. brasilense* plant growth promoting (PGP) effects. Although these phytohormones are not just produced as part of the symbiotic relationship between plants and the bacteria, as a novel of possible other roles for the pathways involved in the production of IAA and NO, have been discovered and proposed in the last few years.

The PGP effects of *A. brasilense* seem to be broad and not limited to some varieties, as from the 2 varieties of *Triticum durum* tested (Marialva and Preto Amarelo), inoculation resulted in positive effects on the number of SORs and in the biomass production of both varieties, despite the differences between them. With the more ancestral variety (Preto Amarelo) being more responsive to *A. brasilense* inoculation.

These effects are positive for plant growth and development as well as for the environment. Since increasing root density leads to increased nutrient use efficiency and therefore decreased agricultural environmental impacts. While the direct increase in the biomass production due to the inoculation of *A. brasilense*, leads to an increased crop yield.

**Keywords:** *Azospirillum brasilense*, indole-3-acetic acid, nitric oxide, phytohormones, wheat.

## Resumo

Um dos maiores desafios que a humanidade enfrenta é conseguir alimentar uma população crescente e simultaneamente minimizar o seu impacto no ambiente. Para atingir tais objetivos é necessário combinar o aumento da produtividade agrícola com sistemas mais “amigos do ambiente”. Uma possível solução é através da utilização de rizobactérias promotoras do crescimento vegetal. Entre essas rizobactérias, o género *Azospirillum* é considerado um dos mais importantes. Desde a sua descoberta, há mais de 40 anos no Brasil, este género tornou-se no género de bactérias promotoras de crescimento vegetal mais estudado, sendo usado para aumentar o crescimento das plantas e a sua produtividade, um pouco por todo o mundo. Apesar da sua capacidade para fixar azoto atmosférico, está descrito na bibliografia que os principais efeitos resultantes da inoculação de plantas com *Azospirillum* ocorrem ao nível do sistema radicular, com um aumento na proliferação de raízes. Que por sua vez, aumenta a densidade do sistema radicular que conseqüentemente a capacidade das plantas de absorverem água e nutrientes, visto que expande o volume de solo explorado pelo sistema radicular. Este efeito é apelidado de “efeito esponja”, sendo postulado que se deve à produção de substâncias promotoras de crescimento (fito-hormonas) por parte das bactérias.

Mas para se poder utilizar culturas de *A. brasilense* como inoculantes para a agricultura, numa escala muito superior à usada atualmente, é necessário um conhecimento mais profundo sobre os efeitos da inoculação das plantas com *A. brasilense* e sobre os mecanismos responsáveis por esses efeitos,. Será também desejável que os estudos que venham a ser realizados, utilizem plantas importantes a nível económico e não plantas modelo.

De modo a tentar responder a estas perguntas foi delineado um conjunto de experiências para descrever e quantificar o efeito da inoculação de sementes de trigo (*Triticum durum*) com *Azospirillum* na germinação. Foram testados os efeitos da inoculação em trigo de duas estirpes de *A. brasilense*, Sp245 e ARG2, e foi avaliado o papel do óxido nítrico e do ácido indol-acético, duas fitohormonas produzidas pelas estirpes em estudo, nos efeitos observados. Para bloquear os efeitos do óxido nítrico e das auxinas produzidas por *A. brasilense*, foram usados "scavengers", 2-(4-carboxifenil)-4,4,5,5-tetrametilimidazolina-1-oxi-3-óxido (cPTIO) no caso do óxido nítrico e Ácido  $\alpha$ - (p-clorofenoxil) isobutírico (PCIB), para as auxinas. Enquanto para mimetizar os efeitos do óxido nítrico e auxinas produzidas aquando da inoculação, foram usados um dador de óxido nítrico – nitroprussiato de sódio (SNP) e uma auxina sintética – Ácido 1-naftalenoacético (NAA).

Os resultados deste trabalho mostraram que o óxido nítrico e as auxinas produzidas pelas estirpes de *A. brasilense* Sp245 e ARG2, atuam como moléculas mediadoras do aumento do número de raízes de segunda ordem, resultante da inoculação com *A. brasilense*, mas sem influenciar o número ou o comprimento das raízes de primeira ordem. O que resulta num aumento da densidade radicular. A aplicação de cPTIO e PCIB, tanto conjuntamente como separadamente, levou a um bloqueio de todos os efeitos da inoculação com *A. brasilense* no sistema radicular. Enquanto que a aplicação de SNP e de NAA, tanto conjuntamente como separadamente, mimetizou os efeitos da inoculação.

Para além dos efeitos na estrutura radicular, foi também demonstrado que a inoculação com *A. brasilense* tem um efeito direto na produção de biomassa do trigo, aumentando-a. Este aumento foi provavelmente o resultado de mudanças no balanço fito-hormonal das plantas, que levaram a uma estimulação da atividade fotossintética das plantas. A aplicação de cPTIO bloqueou todos os efeitos da inoculação com *A. brasilense* no sistema radicular enquanto que a aplicação de PCIB apenas bloqueou parte dos efeitos da inoculação. Quer a aplicação de SNP quer a de NAA, tanto conjuntamente como separadamente, mimetizou os efeitos da inoculação no aumento de biomassa das plantas de trigo.

Através da aplicação da metodologia de Salkowski para a quantificação da produção de ácido indolacético, foi possível determinar que essa produção por parte de *A. brasilense* é muito dependente da presença de triptofano e do número de células bacterianas. Apesar disto, as plantas resultantes da inoculação de sementes com diferentes concentrações de inóculo (de  $10^6$  a  $10^9$  CFU.semente<sup>-1</sup>) não tiveram diferenças entre si no número de raízes de segunda ordem. Em termos da acumulação de biomassa só houve diferenças mínimas, com todas as concentrações testadas a terem efeitos positivos no trigo. Várias hipóteses foram propostas e discutidas para explicar esta ausência de influência das diferentes concentrações de inóculo, mas infelizmente não foi possível chegar a uma conclusão.

Os resultados experimentais fortalecem a teoria que a desnitrificação pode até ser uma característica positiva em inóculos bacterianos, que apesar de reduzir a quantidade de azoto disponível para a planta devido à redução de  $\text{NO}_3^-$ , vai produzir óxido nítrico como um dos produtos resultantes desse processo redutor. E como é demonstrado neste trabalho, o óxido nítrico é essencial para os efeitos promotores do crescimento vegetal resultantes da inoculação com *A. brasilense*.

Ambas as fitohormonas, óxido nítrico e ácido indolacético, são fundamentais para os efeitos de *A. brasilense* no trigo, mas é muito provável que não sejam produzidas pelas bactérias simplesmente com o objetivo de promover o crescimento da planta, mesmo que “em troca” a planta forneça à bactéria exsudados radiculares, que servem como fonte de carbono e nutrientes. Neste trabalho é discutido que as vias metabólicas que levam à produção de óxido nítrico e ácido indolacético são ativadas como respostas a diversos stresses, quer ambientais, quer nutricionais, quer energéticos, e as fito-hormonas produzidas como resultado, são exportadas das células bacterianas para a planta. Consequentemente levam à ativação de um conjunto de processos fisiológicos que resultam na estimulação do crescimento da planta e da exsudação de nutrientes. Provavelmente com o passar do tempo esta relação entre *A. brasilense* e as plantas foi evoluindo em direção a uma simbiose mutualista, que apesar de não ser essencial, ambas beneficiam, e muito, da sua ocorrência.

Um dos maiores problemas relacionados com o uso de biofertilizantes na agricultura é a especificidade da resposta de diferentes espécies ou variedades de plantas. Neste trabalho foi demonstrado que das duas variedades de *Triticum durum* testadas, Marialva e Preto Amarelo, apesar de muito diferentes, ambas reagem positivamente à inoculação com *A. brasilense*. Sendo que o aumento do número de raízes de segunda ordem é semelhante entre as duas variedades, enquanto que em termos da produção de biomassa a variedade mais ancestral (Preto Amarelo) teve um maior aumento, principalmente na biomassa radicular.

Os efeitos da inoculação com *A. brasilense* são positivos tanto para o crescimento da planta como para o ambiente. Visto que aumentar a densidade do sistema radicular é uma estratégia para aumentar a eficiência do uso de água e nutrientes, dado que existe uma maior absorção por parte da planta. Por conseguinte, uma menor quantidade de água e fertilizantes será necessária, o que leva a uma diminuição dos impactos ambientais da agricultura. Enquanto que o aumento de biomassa que advém dos efeitos estimuladores resultantes da inoculação com *A. brasilense* na fotossíntese, leva a um aumento da produtividade das culturas agrícolas.

**Palavras chave:** ácido indolacético, *Azospirillum brasilense*, fitohormonas, óxido nítrico, trigo.

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## List of abbreviations

ARFs – auxin response factors

cm – centimetres

cPTIO – 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide

FORs – first order roots

IAA – indole-3-acetic acid

IaaH – IAM hydrolase

IAAld – indole-3-acetaldehyde

IaaM – Trp-2-monooxygenase

IAD – indole-3-acetaldoxime

IAM – indole-3-acetamide

IAN – indole-3-acetonitrile

ipdC – indole-3-pyruvate decarboxylase

IPyA – indole-3-pyruvic acid

K<sup>+</sup> – potassium

KNO<sub>3</sub> – potassium nitrate

min – minutes

N – nitrogen

N<sub>2</sub> – atmospheric nitrogen

NA – nutrient agar

NAA – 1-Naphthaleneacetic acid

Nap – periplasmatic nitrate reductase

Nar – dissimilatory nitrate reductase

Nas – assimilatory nitrate reductase

NB – nutrient broth

Nir – assimilatory nitrite reductase

Nirk - dissimilative nitrite reductase

NH<sub>2</sub>OH – hydroxylamine

NH<sub>4</sub><sup>+</sup> – ammonium

NO – nitric oxide

NO<sub>2</sub><sup>-</sup> – nitrite

NO<sub>3</sub><sup>-</sup> – nitrate

N<sub>2</sub>O – nitrous oxide

NOS – NO synthase

O<sub>2</sub> – oxygen

OD – optical density

PCIB –  $\alpha$ -(p-Chlorophenoxy) isobutyric acid

PGP – plant growth promotion

PGPR – plant growth promotion rhizobacteria

QS – quorum sensing

SNP – sodium nitroprusside

SORs – second order roots

SORFC – SORs founder cells

SORP – SORs primordia

T6SS – type VI secretion system

TAM – tryptamine

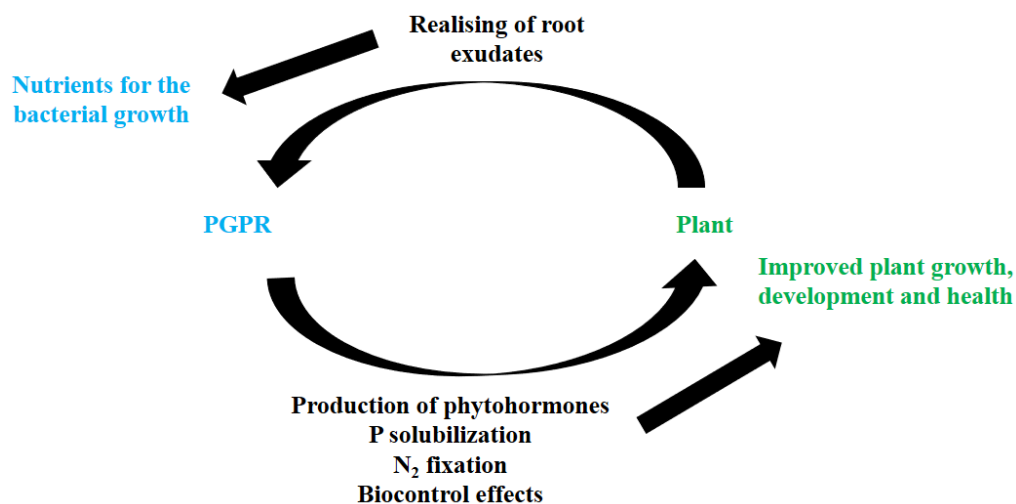
Trp – tryptophan

XPP – xylem poles pericycle

## Chapter 1 – Introduction

### 1.1 – Rhizosphere: the meeting point between PGPR and plants

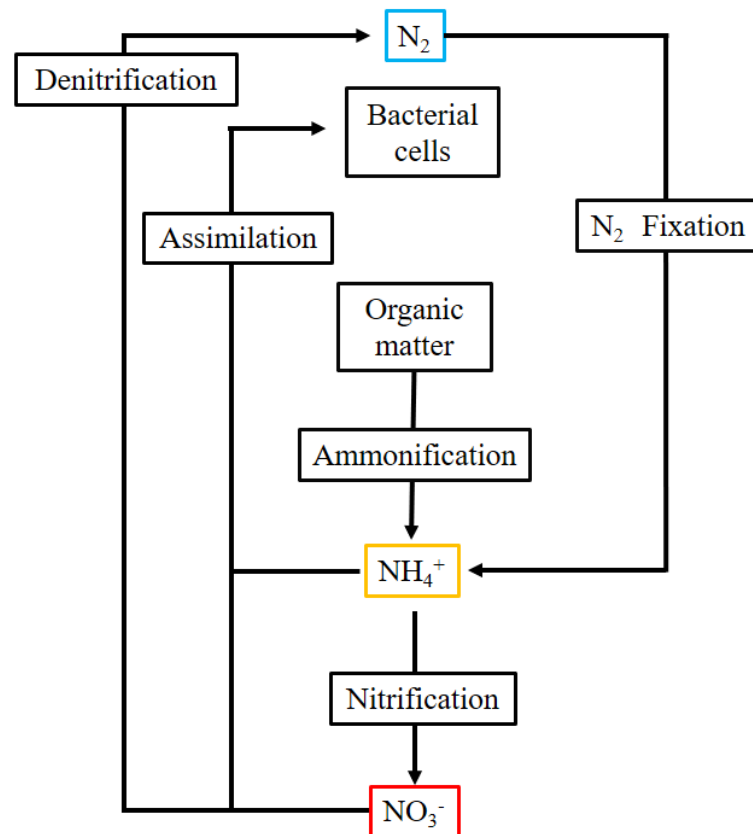
Plant development and growth are strongly influenced by biotic and abiotic factors encountered by roots within soils. Among the biotic factors, the plant-microbe interactions stand out. Some of those interactions can be symbiotic<sup>1</sup> mutualistic<sup>2</sup>, as plants secrete root exudates that will provide nutrients for the soil microbial populations and as compensation some of those populations can be beneficial for plants by improving their growth, development and health. Representative beneficial microbes include plant growth promoting rhizobacteria (PGPR), which establish associative symbiotic interactions with their host plant (Raaijmakers et al., 2009; Richardson et al., 2009; Spaepen et al., 2009). PGPR colonize the rhizosphere<sup>3</sup> of many plant species and some can, even, colonize the plant itself, enhancing plant growth through a variety of mechanisms, direct and indirect. The direct mechanisms include increases on nutrients availability/uptake by plants, such as, atmospheric nitrogen (N<sub>2</sub>) fixation and phosphorus solubilisation (biofertilizers<sup>4</sup>), and the production of phytohormones (phytostimulators<sup>5</sup>), while the indirect mechanisms consist in the production of metabolites (including antibiotics) having “biocontrol” effect on plant diseases and protecting them from pathogens (Richardson et al., 2009; Yang et al., 2009). A simplified model of the symbiotic relationship between PGPR and plants, is outlined in Fig 1.1.



**Figure 1.1** – A simplified model of the symbiotic relationship between PGPR and plants. PGPR can produce phytohormones, solubilize P, fixate N or have biocontrol effects against pathogens. These effects will improve plant growth, development and health. While the plant supplies nutrients for the bacterial growth. Abbreviations: P, Phosphate, N<sub>2</sub>, Nitrogen.

Due to the PGPR capacity to act as biofertilizers and/or phytostimulators, the potential utilization of those PGPR as bacterial inoculum to plant crops have been receiving an increased attention, as they might contribute to solve one of the mankind greatest challenges: being able to feed a growing population while minimizing farming inputs and environment impact. To achieve this, we need to combine an increase in crop yield with a more eco-friendlier approach in agriculture. PGPR have the potential to diminish the application of synthetic fertilizers in agriculture, which leads to severe environmental and economic problems, such as, water eutrophication and increasing costs of food production (Bhardwaj et al., 2014). This matter is of critical importance, because the human population is growing exponentially, and food demand is increasing faster than crop yields.

Among PGPR, *Azospirillum* is considered as one of the most important genus and it is used worldwide for improving plant growth and crop yield, at a commercial level (Bashan et al., 2004; Jacoud et al., 1998; Okon & Labandera-Gonzalez, 1994). It was rediscovered in the mid-1970s in Brazil as a plant-associated bacteria and characterized by having the ability to fixate  $N_2$  and producing phytohormones (Tien et al., 1979). Since then, more than 20 proposals have been suggested regarding the potential mechanisms responsible for the plant growth promotion (PGP) effects of *Azospirillum* (Bashan & De-Bashan, 2010). Besides the ability to fixate  $N_2$ , strains of *Azospirillum* can be involved in many other roles in the nitrogen (N) cycle, as in the denitrification, nitrification, ammonification and assimilation processes (Fig 1.2).



**Figure 1.2** – Multiple roles that *Azospirillum* can have in the N cycle. Can fixate  $N_2$  into  $NH_4^+$ , which in turn can be nitrified to  $NO_3^-$  or assimilated into amino acids of the bacterial cells. The  $NO_3^-$  which result from the nitrification process can also be assimilated into amino acids of the bacterial cells or alternatively be denitrified to  $N_2$ , although during this process many more products are formed, one of which being  $NO$ . *Azospirillum* can also take part in the ammonification process which convert organic nitrogen back into  $NH_4^+$ . Abbreviations:  $NO_3^-$ , nitrate;  $N_2$ , atmospheric nitrogen;  $NH_4^+$ , ammonium.

### 1.2 – Major plant growth promotion mechanisms of *Azospirillum*

As *Azospirillum* possess the ability to fixate  $N_2$ , the first plant growth promoting (PGP) mechanism that was proposed as responsible for improving plant growth was the supplying of N to the host-plant (Lin et al., 1983). Host-plants would benefit from enhanced  $N_2$  fixation in their roots while *Azospirillum* spp. would benefit from carbon provided by the plant host, that could be used as an energy source (Christiansen-Weniger, 1998). But various experiments (Table 1.1) show that positive responses to plant *Azospirillum* inoculation are associated with enhanced root and plant growth without increased plant N content, and may be attributed to plant growth regulating substances (phytohormones<sup>6</sup>) produced by *Azospirillum* strains (Inbal & Feldman, 1982; Tien et al., 1979). Which would increase root proliferation and elongation, leading to a "sponge" effect, that enhances plant capacity to access water and nutrients, such as, nitrate ( $NO_3^-$ ), phosphate and potassium ( $K^+$ ), essential to the plants growth and development

(Richardson et al., 2009). These effects (Fig 1.3) have been reported in several plants including maize, rice, sorghum and wheat inoculated with *Azospirillum brasilense* (Salamone et al., 2012; Kapulnik et al., 1985; Lin et al., 1983), although those effects varied with the strain (Jain & Patriquin, 1984).

**Table 1.1** – Evidences which support that the positive responses to plant *Azospirillum* inoculation are due to the production of phytohormones and not through fixation of N<sub>2</sub>.

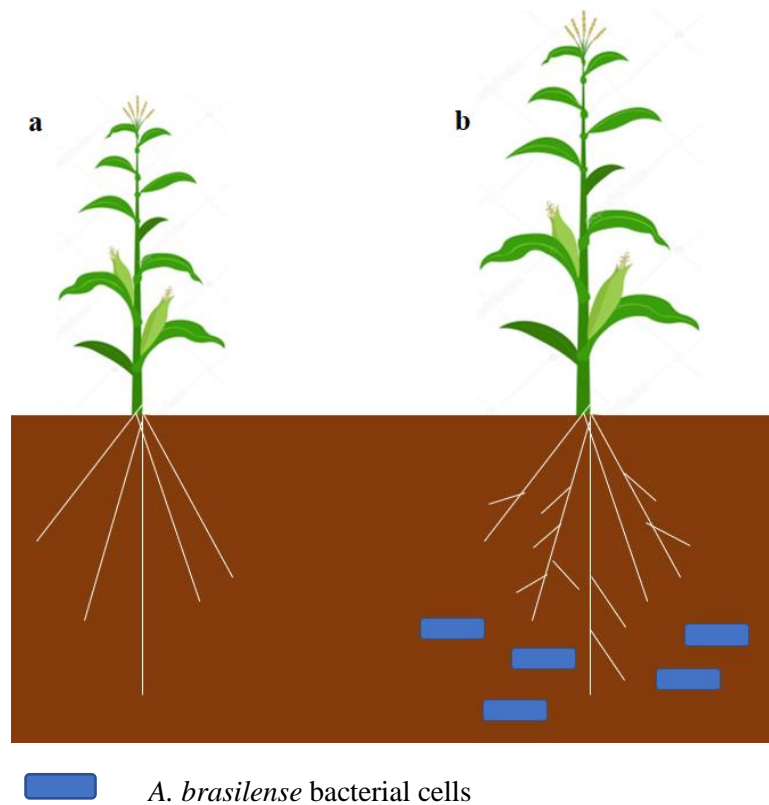
Tested plant	Evidences	Reference
Wheat	Inoculation of hormonal mutant wheat plants with 5 <i>Azospirillum</i> strains increased the proportion of plants which exhibit normal phenotype. This effect was similar to that obtained after foliar treatment with exogenous auxin; No differences in the protein content of the grain, between the inoculated and non-inoculated treatments.	(Inbal & Feldman, 1982)
Pearl Millet	Inoculated plants had more lateral roots and root hairs; In both, N-free closed systems and open systems, <i>A. brasilense</i> significantly increased the dry weight of the plants; Acetylene reduction assays (which measures N fixation) of plants grown in N-free Hoagland solution were negative for both inoculated and uninoculated treatments.	(Tien et al., 1979)
Corn Sorghum	Inoculation with <i>A. brasilense</i> Cd or Sp7 increased the uptake of several mineral ions: NO <sub>3</sub> <sup>-</sup> , K <sup>+</sup> , and H <sub>2</sub> PO <sub>4</sub> <sup>-</sup> ; Increased shoot dry matter accumulation.	(Lin et al., 1983)

### 1.3 – Production of phytohormones by *Azospirillum*

Phytohormones are organic substances that, at very low concentration (pM-nM) promote plant physiological responses (Van Overbeek, 1944). They play an important role as regulators of plant growth and development and contribute to the coordination of diverse physiological processes in plants, including the regulation of root quiescence centre, root formation, florescence, branching, fruit ripening, and seed germination. They also mediate plant response to biotic and abiotic factors through the induction or suppression of specific genes expression and the consequent synthesis of enzymes, pigments and metabolites (Tsavkelova et al., 2006).

As it was reported by many authors, *Azospirillum* species can enhance plant growth due to the self-production of phytohormones and/or by inducing synthesis of these compounds in the plant tissues (Chamam et al., 2013; A. C. Cohen et al., 2015; Duca et., 2014). The described effects of *Azospirillum* inoculation on plant root systems are mainly attributed to the production of phytohormones by *Azospirillum*: auxins, mostly indole-3-acetic acid (IAA), and/or nitric oxide (NO) (Molina-Favero et., 2008; Prigent-Combaret et al., 2008; Steenhoudt & Vandereyden, 2000). Besides those phytohormones, the production of gibberellins, abscisic acid, cytokinins and ethylene by *Azospirillum*, have also been reported (Cassán et al., 2009; Tien et al., 1979).

More recently, it has been proposed that phytohormones may also act as signaling molecules for the bacteria as they influence gene expression of both plants and bacteria (Koul et al., 2015a). As IAA and NO, seem to be the main phytohormones produced by *A. brasilense* that will have a PGP effect, we review the effects of those phytohormones in plants and bacterial cells, as well as the mechanisms and metabolic pathways leading to their production.



**Figure 1.3** – Simple schematic representation of the described effects of *A. brasilense* in plants. (a) non-inoculated plant, (b) inoculated plant in which the phytohormones produced by the *A. brasilense* increase root proliferation, which increases nutrient uptake and consequently plant growth.

### 1.3.1 – Nitric oxide

#### NO functions on plants and bacteria

NO is a lipophilic free radical, a small, volatile, highly diffusible gas and a ubiquitous bioactive molecule. Its chemical properties make NO a versatile signal molecule that functions through interactions with cellular targets via either redox or additive chemistry, although it is toxic above certain concentrations. In plants, NO plays a role in a broad spectrum of physiological processes, such as, regulation of plant growth and development, signalling, pathogen defence and abiotic stress responses, being involved in the promotion of seed germination, photomorphogenesis, mitochondrial activity, leaf expansion, root growth, stomatal closure, fruit maturation, senescence and iron metabolism. In most of these physiological processes this is achieved through its interaction with other phytohormones – IAA, gibberellins, abscisic acid, cytokinins and ethylene (Cohen et al., 2009; Lamattina et al., 2003; Molina-Favero et al., 2008; Sanz et al., 2015). But one of its major and more described roles, is in the IAA signalling pathways, where NO acts downstream of the signalling pathway that leads to an IAA-induced second order roots (SORs) and adventitious root development in tomato (Correa-Aragunde et al., 2006; Correa-Aragunde et al., 2003). NO was also shown to influence bacterial gene expression (Koul, Adholeya, et al., 2015a) and to promote biofilm formation by *A. brasilense* (Arruebarrena et al., 2013).

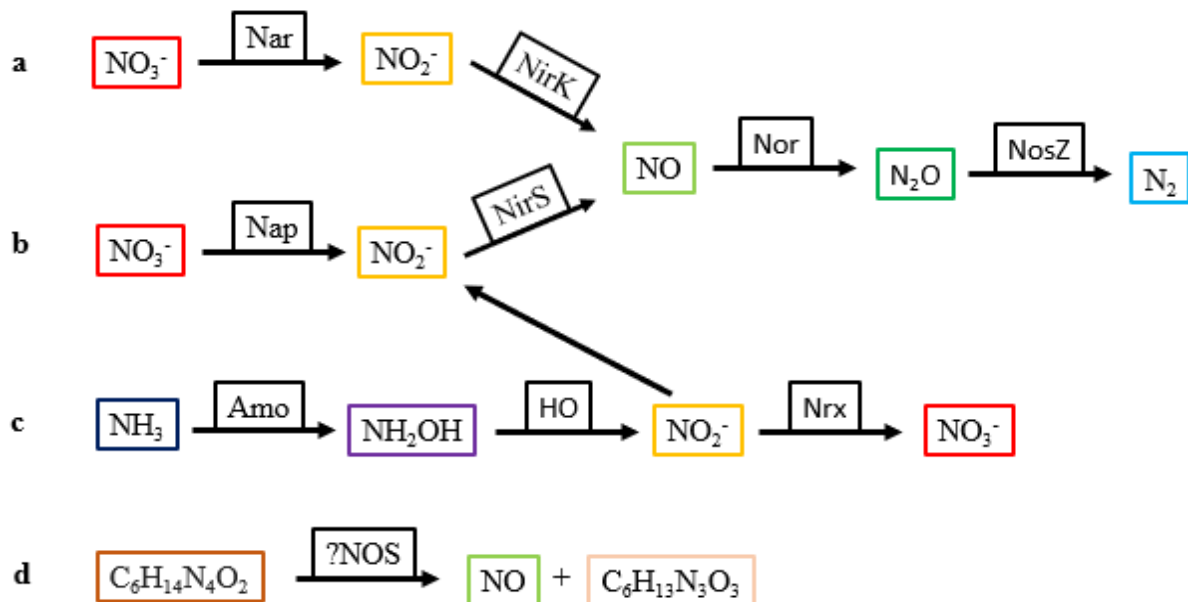
#### NO production pathways of *A. brasilense*

Many *Azospirillum* strains, including *A. brasilense* Sp245, can produce NO *in vitro*, under anaerobic and aerobic conditions (Creus et al., 2005). Although NO is produced during the middle and late

logarithmic phases of growth, is in the late logarithmic phase of growth that the amount of NO produced is higher (Molina-Favero et al., 2008; Molina-Favero et al., 2007). In anaerobic conditions, NO is produced through anaerobic denitrification – NO<sub>3</sub><sup>-</sup> respiration (Zimmer et al., 1984) but aerobic denitrification also produces NO (Steenhoudt et al., 2001a). Evidences for NO production by *A. brasilense* through other pathways (Fig 1.4) include: heterotrophic nitrification (Molina-Favero et al., 2008) and NO synthase (Molina-Favero et al., 2008).

### Anaerobic denitrification

Anaerobic denitrification (Fig 1.4a) is the dissimilative reduction of NO<sub>3</sub><sup>-</sup> to nitrite (NO<sub>2</sub><sup>-</sup>), NO, nitrous oxide (N<sub>2</sub>O), and N<sub>2</sub> by the corresponding N oxides reductases, dissimilatory nitrate reductase (Nar), which is membrane-bound, nitrite reductase, nitric oxide reductase and nitrous oxide reductase, respectively. In this process, NO<sub>3</sub><sup>-</sup> is used instead of O<sub>2</sub> as a final electron acceptor in respiration. This pathway allows denitrifiers to generate energy and to grow under low O<sub>2</sub> levels or anaerobic conditions. The most widespread pathway is the reduction of NO<sub>3</sub><sup>-</sup> to NO, although some microorganism can do a complete denitrification, from NO<sub>3</sub><sup>-</sup> to N<sub>2</sub> (Zumft, 1997) with NO being an obligatory intermediary of this process (Ye et al., 1994). Zimmer and associates, showed that *Azospirillum* has the ability to perform a complete denitrification from NO<sub>3</sub><sup>-</sup> to N<sub>2</sub> (W. Zimmer et al., 1984). In anaerobic conditions



**Figure 1.4** – Pathways involved in the production of NO by *A. brasilense*. (a) anaerobic denitrification, (b) aerobic denitrification, (c) heterotrophic nitrification, (d) putative NO synthase. Abbreviations: ?NOS, putative NO synthase; Amo, ammonium monooxygenase; ; C<sub>6</sub>H<sub>14</sub>N<sub>4</sub>O<sub>2</sub>, arginine; C<sub>6</sub>H<sub>13</sub>N<sub>3</sub>O<sub>3</sub>, L-citruline; HO, hydroxylamine oxidoreductase; N<sub>2</sub>, nitrogen; Nap, periplasmatic nitrate reductase; Nar, dissimilatory nitrate reductase; NH<sub>4</sub>, ammonium; ; NirS, nitrite reductase; nirK nitrite reductase; NO<sub>3</sub><sup>-</sup>, nitrate; NO<sub>2</sub><sup>-</sup>, nitrite; NO, nitric oxide, N<sub>2</sub>O, nitrous oxide; NH<sub>2</sub>OH, hydroxylamine; Nor, nitric oxide reductase; NosZ, nitrous oxide reductase; Nrx, nitrite oxidoreductase.

the major known source of NO in bacteria is a periplasmic-located enzyme, the dissimilative nitrite reductase (Nirk), that catalyzes the reduction of NO<sub>2</sub><sup>-</sup> mainly to NO (Cutruzzolà, 1999) and, only in minor quantities, to N<sub>2</sub>O (Ye et al., 1994). It was found that *A. brasilense* Sp245 did not contain only one copy of *nirK* but 2, in 2 different plasmids (Pothier et al., 2008). The expression of the *nirK* gene is stimulated by high levels of N oxides and low levels of O<sub>2</sub> (Baek & Shapleigh, 2005).

### Aerobic denitrification

Aerobic denitrification (Fig 1.4b) occurs when denitrification genes are activated in aerobic conditions (Zumft, 1997). A periplasmic nitrate reductase (Nap) was identified in *A. brasilense* Sp245, which is neither repressed nor inactivated by O<sub>2</sub> (Steenhoudt et al., 2001a). The NO<sub>2</sub><sup>-</sup> resulting from that step is further reduced to NO. From that step on, the dissimilatory pathway for aerobic and anaerobic denitrification is the same. It was proven by Molina-Favero and associates that aerobic denitrification can be the major source of NO in these bacteria. Under aerobic conditions, a Nap- knockout mutant of *A. brasilense* Sp245 (strain Faj164) produced only 5 % of the NO relatively to the wild-type level, due to the lack of a functional periplasmic nitrate reductase (Molina-Favero et al., 2008). Nap is thought to be involved in maintaining an optimal redox balance by dissipation of the reducing equivalent stress, during aerobic growth (Steenhoudt et al., 2001a; Steenhoudt et al., 2001b). In agreement with this, it was reported that, the more reduced the carbon source available for the bacterium is (butyrate or caproate), the higher the Nap activity (Robertson & Kuenen, 1988).

It is commonly assumed that denitrification represents a loss of N from the system, because it reduces plant N available (W. Zimmer et al., 1984). But, the observation that denitrification is one of the major sources of NO in *A. brasilense*, was a turning point. In fact NO production may target many rhizosphere processes associated with PGPR traits (Creus et al., 2005; Molina-Favero et al., 2008), meaning that PGPR ability to denitrification may be considered as a desirable characteristic.

### **Heterotrophic nitrification**

Heterotrophic nitrification (Fig 1.4c) can be a significant source of NO from bacteria living in aerobic and microaerobic soil and water (Anderson et al., 1993; Papen et al., 1989). Nitrification is the biological oxidation of ammonium (NH<sub>4</sub><sup>+</sup>) to NO<sub>3</sub><sup>-</sup>. The first step of this pathway is the oxidation of NH<sub>4</sub><sup>+</sup> to hydroxylamine (NH<sub>2</sub>OH), which is catalysed by the enzyme ammonium monooxygenase. Next, NH<sub>2</sub>OH is oxidized to NO<sub>2</sub><sup>-</sup> by a hydroxylamine oxidoreductase. This NO<sub>2</sub><sup>-</sup> can enter the denitrification pathway and be reduced to NO<sup>•</sup> by a nitrite reductase (Stein, 2011) or be oxidized to NO<sub>3</sub><sup>-</sup> by nitrite oxidoreductase (Wrage et al., 2001). Heterotrophic nitrifiers can oxidize organic forms of N such as urea (Papen et al., 1989). There are evidences for the presence of this pathway in *Azospirillum*, as it was reported that in the strain *A. brasilense* Sp245, the addition of hydroxylamine, a nitrification intermediate, increases NO production by 1.5 fold comparing with NH<sub>4</sub><sup>+</sup> as the N source (Molina-Favero et al., 2008).

### **NO synthase**

The NO synthase (NOS) (Fig 1.4d) is an enzyme that can produce NO aerobically by oxidizing arginine, in the presence of O<sub>2</sub>, to L-citruline and NO (Stuehr, 1997). There are some reports suggesting the presence of a NOS-like activity in *A. brasilense* Sp245 (Creus et al., 2005; Molina-Favero et al., 2008). They reported a higher production of NO when the bacteria were grown in NH<sub>4</sub><sup>+</sup> plus arginine relatively to when were grown in NH<sub>4</sub><sup>+</sup> alone, although the NOS gene (encoding a N synthase) is absent of the *A. brasilense* Sp245 sequenced genome (Koulet al., 2015b).

## **1.3.2 – Indole-3-acetic acid**

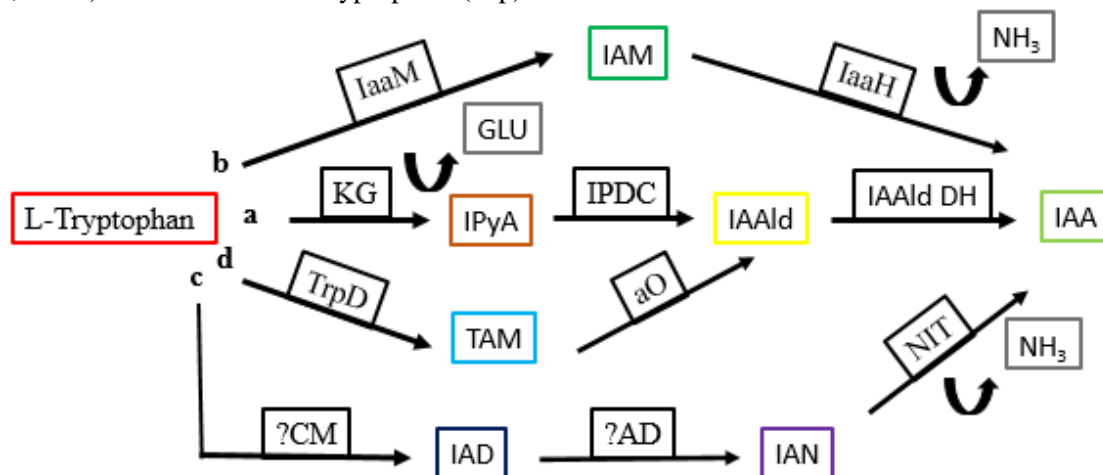
### **IAA influence on plants and bacteria**

In plants, IAA is responsible for the regulation of the cell cycle, division and extension, and differentiation of cells and tissues. Phytohormones of this group increase the rate of xylem and root formation, control processes of vegetative growth, apical dominance, tropism, florescence, fructification

of plants and their senescence. It also affects photosynthesis, pigment formation, biosynthesis of various metabolites and even other phytohormones, such as cytokinins and gibberellins. It can also increase resistance to biotic stress factors (Costacurta & Vanderleyden, 1995; Ilyas & Bano, 2010; Patten & Glick, 1996). Besides its effects on the plants, IAA was also reported to affect *A. brasilense* gene expression (van Puyvelde et al., 2011). They report that inactivation of the *ipdC* gene or the addition of IAA results in many transcriptional changes. This *ipdC* gene encodes a indole-3-pyruvate decarboxylase (*ipdC*) which is the rate limiting step of the indole-3-pyruvic acid pathway (Zakharova et al., 1999), which provides 90 % of the IAA synthesized by *Azospirillum* (Duca et al., 2014). When exposed to IAA, bacteria adapt to the plant rhizosphere, by changing its arsenal of transport proteins and cell surface proteins, for example by upregulating the expression of type VI secretion system (T6SS). The T6SS is specifically involved in bacteria–eukaryotic host interactions and although it is mostly reported in relation to virulence, its presence on symbiotic strains of *Rhizobium* has already been reported (Bladergroen et al., 2003).

### IAA production pathways of *A. brasilense*

*Azospirillum* can produce IAA during all growth stages (Malhotra & Srivastava, 2009), but the release of large amounts of IAA by *Azospirillum* spp. cultures is probably controlled by the stationary phase of the bacteria cells after depletion of the carbon source in the medium used in the batch culture (Ona et al., 2005). The absence of tryptophan (Trp) from the culture medium decreases the level of IAA



**Figure 1.5** – Known pathways of IAA biosynthesis dependent on Trp in *A. brasilense*. (a) indole-3-pyruvic acid, (b) indole-3-acetamide, (c) indole-3-acetonitrile, (d) tryptamine. Abbreviations: ?AD, putative aldoxime dehydratase; ?CM, putative cytochrome; aO, amine oxidase; GLU, glutamine; IAA, indole-3-acetic acid; IAD, to indole-3-acetaldoxime; IaaH, IAM hydrolase; IAAld, indole-3-acetaldehyde; IAAld DH, IAAld dehydrogenase; IaaM, tryptophan-2-monooxygenase; IAM, indole-3-acetamide; IAN, indole-3-acetonitrile; IPDC, indole-3-pyruvate decarboxylase; IPyA, indole-3-pyruvic acid; KG, aminotransferase; NH<sub>3</sub>, ammonia; NIT, nitrilase; TAM, Tryptamine; TrpD, tryptophan decarboxylase monooxygenase.

synthesis by *Azospirillum*, while the addition of exogenous Trp may augment IAA biosynthesis by at least an order of magnitude (Zakharova et al., 1999). There are 5 known pathways for IAA biosynthesis in *Azospirillum* (Duca et al., 2014), 4 of them (Fig 1.5) are related to Trp metabolism (an amino acid frequently found in root exudates (Patten & Glick, 1996)): (a) indole-3-pyruvic acid (IPyA), (b) indole-3-acetamide (IAM), (c) indole-3-acetonitrile (IAN) (Bashan & De-Bashan, 2010) and (d) tryptamine (TAM) (Hartmann et al., 1983). A Trp independent pathway was also reported (Prinsen et al., 1993)

#### Indole-3-pyruvic acid pathway (Fig 1.5a)

The first step of this pathway is the transamination of L-Trp to indole-3-pyruvic acid (IPyA) by an aminotransferase, with the release of glutamine for the N metabolism. Next, IPyA is decarboxylated to indole-3-acetaldehyde (IAAld) by an ipdC, with this step being the rate-limiting step. In the last step IAAld is oxidized to IAA by an IAAld dehydrogenase (Zakharova et al., 1999). This pathway is the major responsible for the IAA production in *Azospirillum*, providing 90 % of the IAA synthesized (Duca et al., 2014). The ipdC is encoded by the *ipdC* gene, which expression was showed to be Trp-dependent (Zimmer et al., 1998). The addition of IAA, 1-Naphthaleneacetic acid (NAA), 2,4-dichlorophenoxypropionic acid or p-chlorophenoxyacetic acid, also upregulated the expression of the *A. brasilense* Sp245 *ipdC* gene, *in vitro* (Broek et al., 1999).

#### **Indole-3-acetamide pathway (Fig 1.5b)**

In this 2-step pathway, Trp is first converted to IAM by the enzyme Trp-2-monooxygenase (IaaM). In the second step IAM is converted to IAA by an IAM hydrolase (IaaH), with the release of NH<sub>3</sub> for the N metabolism (Zakharova et al., 1999).

#### **Indole-3-acetonitrile pathway (Fig 1.5c)**

The first step in this pathway is the conversion of Trp to indole-3-acetaldoxime (IAD). In bacteria the enzyme that catalyses this process as not been characterized, although in *Arabidopsis*, a cytochrome P450 monooxygenase catalyses this reaction (Mikkelsen et al., 2000). In the next step the aldoxime group (-CH=NOH) of the IAD is dehydrated to a nitrile (-C≡N) by an aldoxime dehydratase resulting indole-3-acetonitrile (IAN). In *Bacillus* sp. OxB-1, this reaction is catalysed by a aldoxime dehydratase (Kato et al., 2000). The last step is the conversion of IAN to IAA trough the activity of nitrilases, with the release of NH<sub>3</sub> for the N metabolism (Patten et al., 2013).

#### **Tryptamine pathway (Fig 1.5d)**

The first step of this pathway is the decarboxylation of Trp to TAM by a Trp decarboxylase. Next TAM is directly converted to IAAld by an amine oxidase. The last step is common to the IPyA pathway, in which the IAAld is oxidized to IAA by an IAAld dehydrogenase (Spaepen et al., 2007).

#### **Trp-independent pathway**

There was a report that a bacterial Trp-independent pathway was demonstrated in *A. brasilense* by feeding experiments with labelled precursors (Prinsen et al., 1993). When no Trp was supplied to the medium, this pathway provided 90 % of the IAA synthesized by the bacteria. However, the mechanisms of this pathway are largely unknown (Spaepen et al., 2007).

Other factors also affect IAA production by *Azospirillum*: low O<sub>2</sub> levels and low pH stimulate IAA production (Ona et al., 2005). Very low levels of B vitamins, especially pyridoxine and nicotinic acid, also increased IAA production by *A. brasilense* (Zakharova et al., 2000). It was also proposed (Patten et al., 2013) that genes involved in IAA synthesis are downregulated when preferred N sources are available, for example, NH<sub>4</sub><sup>+</sup>. When in the stationary phase of growth, after preferred N sources have been exhausted, deamination of Trp can provide N, through the activation of the IAA biosynthetic pathways. In agreement with this it was reported a higher IAA accumulation in cultures of *A. brasilense* SM, when grown in 50 % less ammonium sulphate (Malhotra & Srivastava, 2009).

## 1.4 – Importance of auxins and NO on SORs development and plant growth

### 1.4.1 – SORs development

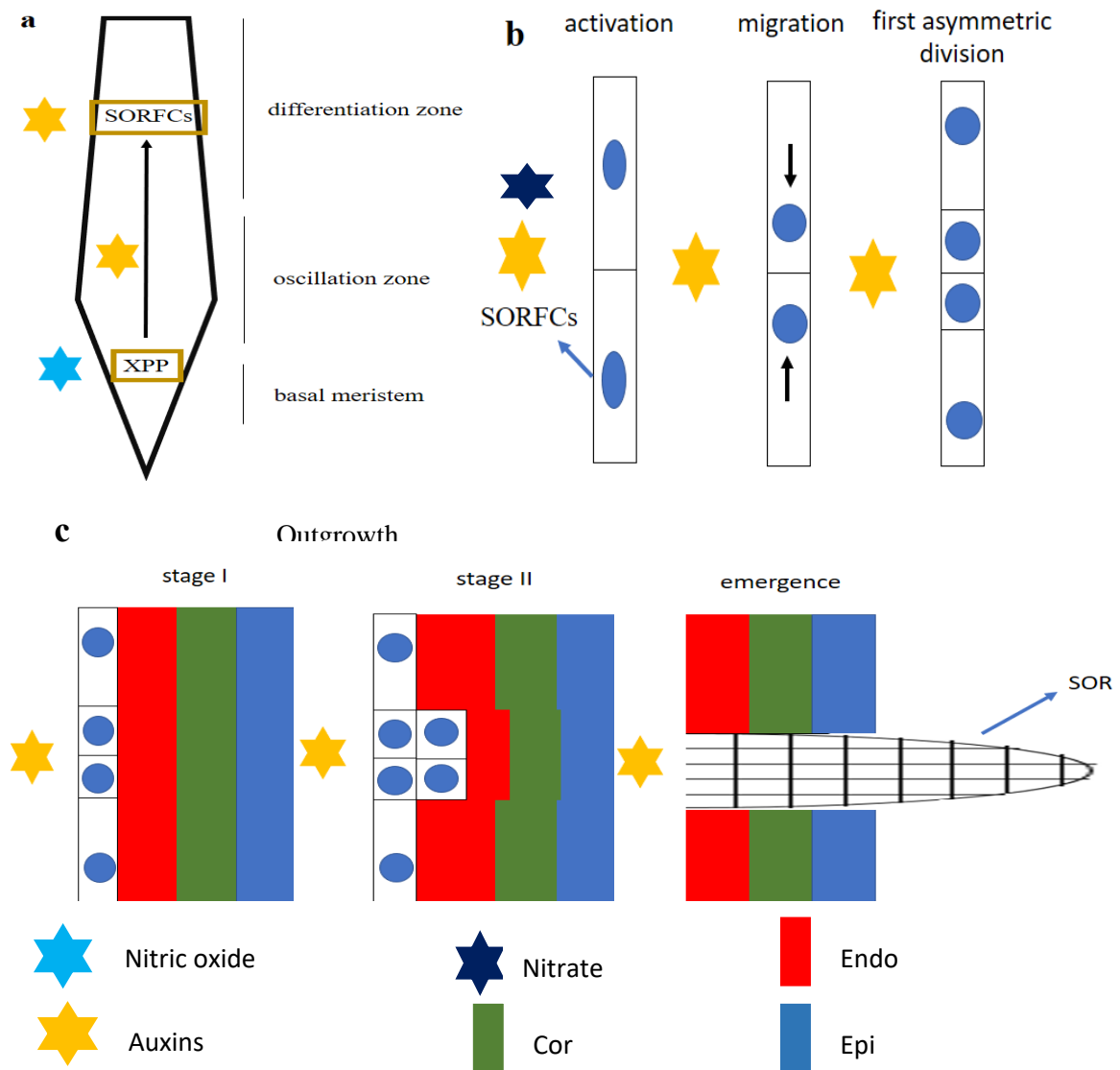
The formation of SORs, comprises 4 stages (Du & Scheres, 2018; Fig 1.6): (1) positioning, (2) initiation, (3) outgrowth and (4) emergence.

The positioning stage (Fig 1.6a) determines the spatial distribution of SORs primordia (SORP) and SORs along FORs. This stage includes the oscillation and specification of the SORs founder cells (SORFCs). Auxins modulate the oscillation of the SORs initiation sites and auxin signaling participate in regulating SORFCs specification (Dubrovsky et al., 2008). SORs positioning initiates in the basal meristem and progresses until the xylem poles pericycle (XPP) cells reach the differentiation zone, where they are specified to SORFCs. Besides auxins, it was demonstrated that NO acts during the earlier stages of SORs development through the activation of cell division in the pericycle, where NO induces the expression of the cell cycle regulatory genes *CDKA1*, *CYCD3* and *CYCA*, while repressing the expression of the gene encoding the cyclin-dependent kinase inhibitor *KRP2* (Correa-Aragunde et al., 2006).

The initiation stage (Fig 1.6b) encompasses the activation of nuclear migration in specified SORFCs until the first asymmetric cell division. Specified SORFCs are assumed to subsequently receive activating signals to start nuclear migration toward the central common cell wall. During this migration SORFCs start to swell and are transformed to nearly round shape, which is observed before the first division (Smet et al., 2006). With IAA being involved in all the steps of this stage, activation, migration and first asymmetric cell division (Du & Scheres, 2018).  $\text{NO}_3^-$  also seem to be involved in this stage, with low concentrations promoting initiation, while high inhibiting it, although the transduction mechanisms involved auxins (Sun et al, 2017).

The outgrowth stage (Fig 1.6c), involves the “primordium-intrinsic” patterning of *de novo* organ tissues and a meristem. Cell growth and subsequent rounds of anticlinal, periclinal, and tangential cell divisions are launched to establish a dome-shaped primordium that emerges as a SOR at the final stage of the outgrowth (Wangenheim et al., 2016). With each layers of cells in the primordia, being considered a different stage, but all included in the outgrowth (Malamy & Benfey, 1997). Auxin is also required in this stage, with, auxin-responses genes being expressed (Marin et al., 2010).

In the emergence stage (Fig 1.6c), there is an interaction between SORP and the overlaying tissues (endodermis, cortex, and epidermis) to allow passage through the cell layers (Lee & Kim, 2013). Auxins are also involved in this stage, with auxin transport and signalling control the emergence from the cortex and epidermis (Porco et al., 2016).

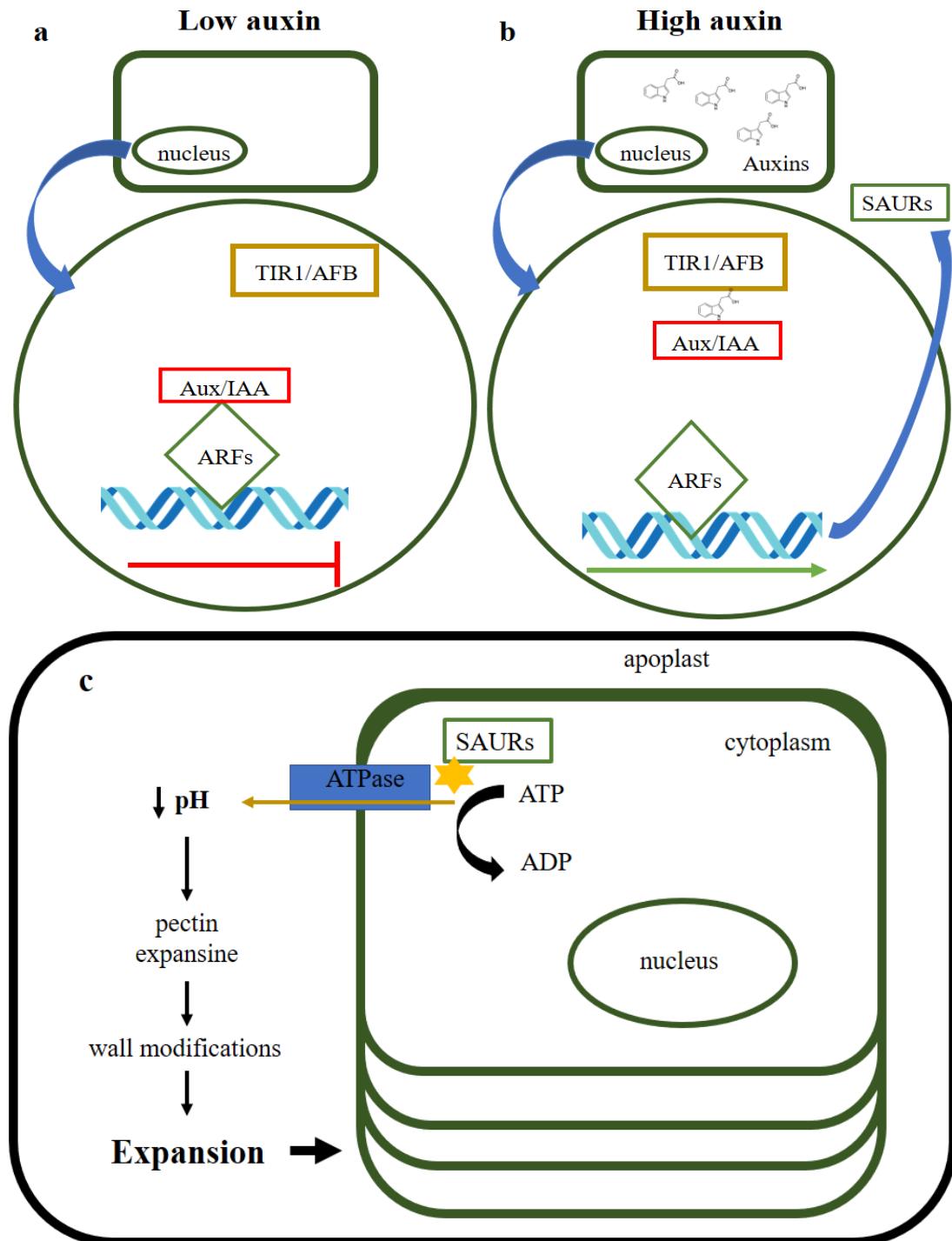


**Figure 1.6** – Simplified model of the auxin and NO-induced formation of SORs. (a) positioning stage – NO leads to the activation of cell division in pericycle – XPP, which oscillate until the differentiation zone where they specified to SORFC, through the action of auxins, (b) initiation stage – through the action of auxins the SORFCs start to swell and migrate, after which they divided asymmetrically, (c) outgrowth and emergence stages – through the action of auxins, rounds of anticlinal, periclinal, and tangential cell divisions are launched to establish a dome-shaped primordium that emerges as a SOR at the final stage of the outgrowth and emerges from the FORs after passing the endo, cor and epi cellular layers. Abbreviations: Cor, cortex; Endo, endodermis; Epi, epidermis; SOR, second order roots; SORFCs, second order roots founder cells; XPP, xylem poles pericycle.

### 1.4.2 – Plant growth

Auxins contribute to plant growth, through acidification of cells that leads to an altered growth, with an increased in cell division and proliferation of the plants tissues (Roosjen et al., 2018). As outlined in Fig 1.7, auxins act on gene expression through a short nuclear pathway that converges upon the activation of a family of DNA-binding transcription factors – auxin response factors (ARFs). Under low auxin levels, the Aux/IAA transcriptional co-repressors prevent ARFs from controlling auxin-regulated genes. But, when auxin levels increase, auxin serves as “molecular glue” between the TIR1/AFB receptor and the Aux/IAA protein. This leads to subsequent ubiquitination and degradation of the Aux/IAA protein, releasing ARFs from inhibition (Roosjen et al., 2018). Subsequently this ARFs promote the expression of a family of short-lived proteins, the small auxin up-rna, which in turn cause the activation of the

plasma membrane H<sup>+</sup>-ATPase through phosphorylation, leading to a decrease in apoplastic pH and

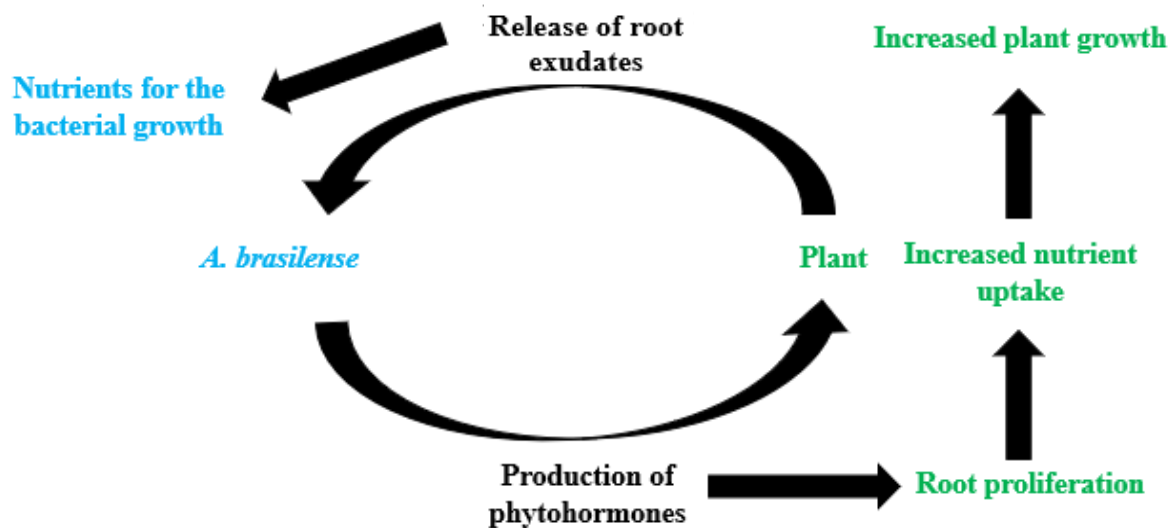


**Figure 1.7** – Simplified model of the auxin- driven cell growth. (a) Under low auxin levels, the Aux/IAA transcriptional co-repressors prevent ARFs from controlling auxin-regulated genes, (b) when auxin levels increase, auxin serves as “molecular glue” between the TIR1/AFB receptor and the Aux/IAA protein, releasing ARFs from inhibition, which in turn promote the expression of SAURs, (c) This SAURs, cause activation of the plasma membrane H<sup>+</sup>-ATPase through phosphorylation, leading to a decrease in apoplastic pH and consequent acidification, which will activate pectins and expansines, promoting a reduction in wall rigidity, leading to cell expansion. Abbreviations: ARFs, auxin response factors; Aux/IAA, Auxin/Indole-3-acetic acid; SAUR, small auxin up-rna; TIR1/AFB, Transport Inhibitor Resistant 1/Auxin Signalling F-Box.

consequent acidification (Spartz et al., 2014). This acidification, leads to a reduction in wall rigidity, due to the action of modifying agents (pectin, expansine) found in the apoplast, resulting in cell elongation, expansion and consequently, division (Arsuffi & Braybrook, 2018).

### 1.5 – Where are we and where do we want to be

As summarized above, there are multiple pathways leading to IAA and NO production in *A. brasilense*. In a simplified model of this symbiotic relationship (Fig 1.8) these phytohormones may work as a bacterial reward for plants root exudates (Raaijmakers et al., 2009).



**Figure 1.8** – A simplified model of the symbiotic relationship between *A. brasilense* and plants. The phytohormones produced by the *A. brasilense* increase root proliferation, which increases nutrient uptake and consequently plant growth. While the plant supplies nutrients for the bacterial growth.

So far, the main reports on the *A. brasilense* PGP effects and the involved pathways, are mainly on model plants, like *Arabidopsis*. Although they supply a good base of study for those mechanisms, if we want to use bacterial inoculants in large scale agriculture we need, not only a more profound knowledge about which phytohormones contribute the most for the *A. brasilense* PGP effects and which pathways are involved, but also studies using economically more important crops.

In this work the tested plant was wheat (*Triticum durum*), which is the most important food grain source for humans, providing 19 % of our available calories and being grown in more land area than any other commercial crop (source: <http://www.fao.org/resources/infographics/infographics-details/en/c/240943/>, accessed on 21 of June of 2018 at 15:32, UTC + 1 h or WEST).

### 1.6 – Objectives

In this project we propose to investigate the *A. brasilense* PGP effects on wheat (*Triticum durum*) and specially the role of IAA and NO produced by *A. brasilense* as potential mediators of those PGP effects. Also, understand if the pathways which produce IAA and NO might have another role in *A. brasilense* and try to clarify whether denitrification might be a desirable feature for PGP or not.

### 1.7 – Hypotheses

Through the course of this work the following experimental hypotheses were tested: (1) if inoculation with *A. brasilense* alters the root structure of wheat, (2) if inoculation with *A. brasilense* increases the biomass production of wheat, (3) if the NO and auxins produced by *A. brasilense* are involved as mediators in the potential PGP effects on wheat, (4) if the inoculum concentration influences the potential PGP effects, (5) if the IAA production of *A. brasilense* is influenced by the presence of Trp,

the plant exudates and the inoculum concentration, (6) if denitrification is involved in the NO production of *A. brasilense* and (7) if the potential effects of *A. brasilense* vary with the wheat variety.

### **1.8 – Experimental strategy**

In this work to try to answer to the experimental hypotheses, an experimental approach based on the utilization of Petri dishes where seeds were grown in sterile and control conditions, was used.

Seeds have a large amount of reserves, which are stored in the endosperm (Lopes & Larkins, 1993), that can be used for plants to start growing just after germination. Seedling development is characterized by an initial intensive root growth followed by shoot development. Root development is very dependent on the plant hormonal balance, namely on NO and IAA concentrations, and therefore may be an important indicator of plant microbial interaction. In this work we will use the SORs to report the interaction between plant and bacteria during early stages of plant development.

The plant (seedling) dry weight will be assessed as the biomass production and interpreted as a consequence of changed plant hormonal balance. Since plant development (in the experimental setup) was completely dependent on the reserves present in the endosperm (no nutrients were added to the media) differences in plant biomass among treatments will be interpreted as changes in the plant hormonal balance able to stimulate photosynthesis.

The hypothesis related with the IAA production by *A. brasilense* will be assessed with a colorimetric method based on the utilization of the Salkowski reagent.

## Chapter 2 – Materials and Methods

### 2.1 – Bacterial strains and growth conditions

Two *Azospirillum* strains were used in this work, *A. brasilense* Sp245 and *A. brasilense* ARG2, which ability to produce auxins (IAA) and presence of the denitrifying pathways were already experimentally confirmed. Relatively to the strain SP245, the key genes for the NO production, *Nap* and *nirk* are localized on plasmids and the *ipdC* gene, essential to the IAA production, is localized on the bacterial chromosome ([https://www.genome.jp/dbget-bin/www\\_bget?gn:T02152](https://www.genome.jp/dbget-bin/www_bget?gn:T02152), accessed on 11 of August of 2018 at 23:47, UTC + 1 h or WEST).

They were recovered from - 80 °C by inoculation in Nutrient Agar (NA) medium, followed by incubation at 28 °C. Liquid cultures were initiated by inoculating 10 mL of Nutrient Broth (NB) medium, in 50 mL Erlenmeyer flasks, with an isolate bacterial colony from the growth on the solid NA medium, which were incubated overnight at 28 °C with constant agitation (100 rpm) in an orbital shaker (Thermo scientific Max Q 400, Model No SHKE4000 – 8CE). These cultures were used as pre-inocula.

From the pre-inocula, the working inocula were grown in 100 mL Erlenmeyer flasks containing 20 mL of NB medium with constant agitation (100 rpm) at 28 °C (initial optical density (OD) = 0.1 at 600 nm). After 6 h, the bacterial cells were harvested while at the logarithm phase of the growth curve (Molina-Favero et al., 2008) by centrifugation at 4000 rpm for 10 minutes (min) with a centrifuge (Eppendorf 5810 R). Harvested cells were washed 3 times with equal volumes of saline solution (0.85 % NaCl), by centrifugation at 4000 rpm for 10 min and densities were adjusted to the required final concentrations.

### 2.2 – Growth curves determination

For each strain 3 replicates were prepared from the pre-inocula, a cell suspension was transferred to 100 mL Erlenmeyer flasks containing 20 mL of NB medium and growth for 24 h with constant agitation (100 rpm) at 28 °C. The growth of the bacterial cultures was followed by measuring the OD at 600 nm using a spectrophotometer (Unicam UV/Vis Spectrometer). An aliquot of 1 mL was aseptically collected, 2 h after the beginning of the experiment, and every h, until cultures reached the stationary phase. One last aliquot was taken 24 h after the beginning of the experiment to assure that the OD had not increase (and that the stationary phase was reached).

### 2.3 – Bacterial cells viability

To test if the NO and/or auxin scavengers, 2-(4- carboxyphenyl)-4,4,5,5,-tetramethylimidazoline-1-oxyl- 3-oxide (cPTIO) and scavenger –  $\alpha$ -(p-Chlorophenoxy) isobutyric acid (PCIB), respectively – affected bacterial growth (when the bacterial strains were in contact with the seed and with the scavengers), the bacterial strains were grown in liquid NB medium supplemented with the scavengers and, the growth curves compared with the control ones (no scavengers addition). This experiment consisted of 8 treatments, 4 for each strain grown in NB medium: 1 (control) – no scavenger supplementation; 2 – supplemented with 10  $\mu$ M of cPTIO; 3 – supplemented with 10  $\mu$ M of PCIB; 4 – supplemented with 10  $\mu$ M of cPTIO plus 10  $\mu$ M of PCIB. Each treatment was performed in triplicates and started with the inoculation of 9 mL of NB medium (using Erlenmeyer flasks of 50 mL) with 1 mL of pre-inocula. Cultures were grown for 24 h with constant agitation (100 rpm) at 28 °C. Aliquots of 1 mL were taken from each culture at 5 and 24 h after the beginning of the experiment for OD determination.

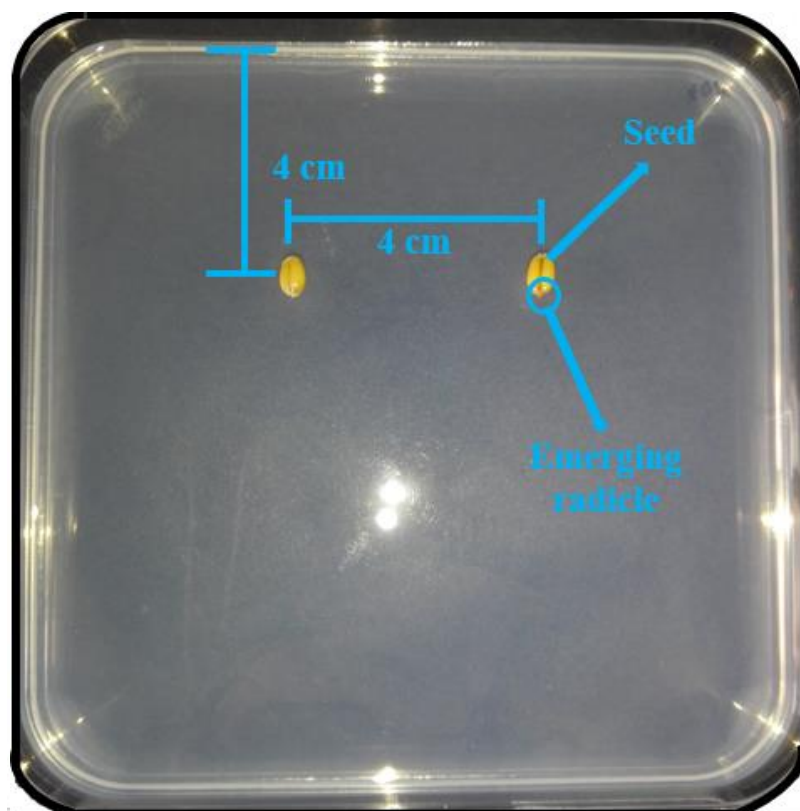
## 2.4 – Petri dish experiments

### 2.4.1 – Chosen plant

Two varieties of *Triticum durum* were used: Preto Amarelo, a conservation variety from Alentejo and Ribatejo, this variety was used in one experiment; and Marialva, a good quality semi-precocious wheat (eligible for high quality product compensation measures), used in all experiments. Only seeds with no deformities and a weight ranging between 40 and 47 mg, were selected. They were submerged for 12 h in distilled water and then surface sterilized with 70 % ethanol for 1 min and 2.5 % (v/v) sodium hypochlorite for 3 min, followed by 3 washes of 3 min in sterile distilled water.

### 2.4.2 – Plant growth conditions

Square Petri dishes (12×12 centimetres (cm)) were filled with 50 mL of medium containing 1.5 % (w/v) agar and a pH of 7.0. Depending on the treatments, they were supplemented or not with a NO donor (sodium nitroprusside – SNP) with a final concentration of 1  $\mu\text{M}$ , 10  $\mu\text{M}$  or 100  $\mu\text{M}$  (Correa-Aragunde et al., 2004), a synthetic auxin (NAA) with a final concentrations of 10 nM, 50 nM or 100 nM (Correa-Aragunde et al., 2004), a NO scavenger (cPTIO) with a final concentration of 10  $\mu\text{M}$ , a auxin scavenger (PCIB) with a final concentration of 10  $\mu\text{M}$ , or potassium nitrate ( $\text{KNO}_3$ ) with a final concentration of 1 mM. Two wheat seeds were placed 4 cm apart from each other and from the top of the Petri dish, with the emerging radicle facing down (Fig. 2.1).



**Figure 2.1** – Schematic representation of the experimental design of the petri dishes experiment. In each petri dish, 2 seeds were placed with 4 cm apart from each other and another's 4 cm from the top, with the emerging radicle facing down. Abbreviators: cm, centimetres.

Inoculated seeds received 0.1 mL of a bacterial suspension with a concentration of  $10^7 \text{ CFU.mL}^{-1}$ ,  $10^8 \text{ CFU.mL}^{-1}$ ,  $10^9 \text{ CFU.mL}^{-1}$  or  $10^{10} \text{ CFU.mL}^{-1}$ , so each seed received a total of  $10^6 \text{ CFU}$ ,  $10^7 \text{ CFU}$ ,  $10^8 \text{ CFU}$  or  $10^9 \text{ CFU}$ , according to the treatment. In the non-inoculated treatments 0.1 mL of saline solution (NaCl 0.85 %) was added to each seed.

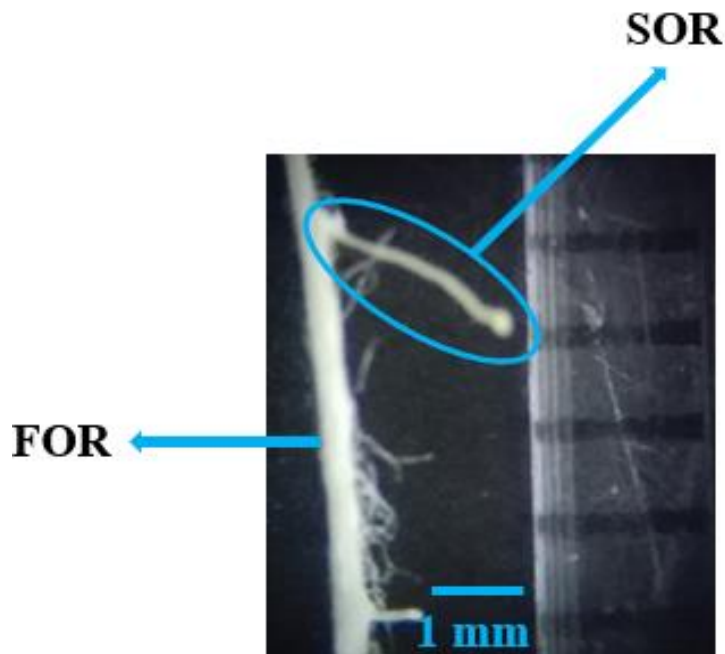
Petri dishes were kept under controlled and sterilized conditions. During the first 24 h of the experiment, the Petri dishes were left in the dark at 28 °C in a horizontal position. After those first 24 h they were placed in a growth chamber (Table 2.1), with an inclination of 45 ° and the part of the Petri dish where were the roots was covered with an aluminum foil, to protect the roots from the light. Each treatment was replicated 15 times and the experiment was terminated 8 days after inoculation.

**Table 2.1** – Conditions of the growth chamber (aralab, model: 5000EH) where the experiments took place.

Parameters	Conditions
Temperature (°C)	day: 28/night: 20
Light intensity ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	350 at plant level
Photoperiod (h)	16/8
Humidity (%)	60

### 2.4.3 – Data collection

Every 24 h photographs were taken from each Petri dish and the photographs were analyzed using ImageJ software (version 1.8.0\_122, developed by National Institutes of Health). For every plant of every image, the number of the first order roots (FORs<sup>7</sup>) and SORs<sup>8</sup> was counted (Fig 2.2), and the length of the FORs was measured, through the analysis of the number of pixels of each root and conversion to cm. The number of SORs only included those that were at least 1 mm long (Correa-Aragunde et al., 2004).



**Figure 2.2** – Part of the wheat root structure, amplified 10 times with a magnifying glass. It is possible to visualize the outgrowth of the SORs from the FORs. Abbreviations: FOR, First order root; mm, millimetre; SOR, Second order root.

At the end of each experiment, plants were removed from the Petri dishes and the root systems were gently rinsed. The plants were then cut above the base root emergence, to separate shoots and roots. Excess water was removed by blotting with tissue paper, before weighing shoots and roots separately. Shoots and roots were kept in envelopes at 60 °C until constant weight (Siddiqui, 2004; Tian et al.,

2015). Plant biomass was assessed based on total plant dry weight and the root to shoot ratio based on the respective dry weights.

#### **2.4.4 – Bacteria effects**

This experiment consisted of 3 treatments to test the potential PGP effects of *A. brasilense* on wheat based on Agar medium without any supplement: 1 – non-inoculated seeds (control); 2 – inoculated seeds with *A. brasilense* Sp245; 3 – inoculated seeds with *A. brasilense* ARG2. Each seed was inoculated with  $10^7$  CFU of the respective strain.

#### **2.4.5 – NO effects**

This experiment consisted of 8 treatments to test the role of NO in the PGP effects of *A. brasilense* on wheat: 1 – non-inoculated seeds and with no medium supplementation (control); 2 – non-inoculated seeds and the medium supplemented with 1  $\mu$ M of SNP; 3 – non-inoculated seeds and the medium supplemented with 10  $\mu$ M of SNP; 4 – non-inoculated seeds and the medium supplemented with 100  $\mu$ M of SNP; 5 – non-inoculated seeds and the medium supplemented with 10  $\mu$ M of cPTIO; 6 – non-inoculated seeds and the medium supplemented with 10  $\mu$ M of SNP plus 10  $\mu$ M of cPTIO; 7 – inoculated seeds with *A. brasilense* Sp245 and the medium supplemented with 10  $\mu$ M of cPTIO; 8 – inoculated seeds with *A. brasilense* ARG2 and the medium supplemented with 10  $\mu$ M of cPTIO. Each seed was inoculated with  $10^7$  CFU of the respective strain.

#### **2.4.6 – Auxin effects**

This experiment consisted of 8 treatments to test the role of auxins in the PGP effects of *A. brasilense* on wheat: 1 – non-inoculated seeds and with no medium supplementation (control); 2 – non-inoculated seeds and the medium supplemented with 10 nM of NAA; 3 – non-inoculated seeds and the medium supplemented with 50 nM of NAA; 4 – non-inoculated seeds and the medium supplemented with 100 nM of NAA; 5 – non-inoculated seeds and the medium supplemented with 10  $\mu$ M of PCIB; 6 – non-inoculated seeds and the medium supplemented with 100 nM of NAA plus 10  $\mu$ M of PCIB; 7 – inoculated seeds with *A. brasilense* Sp245 and the medium supplemented with 10  $\mu$ M of PCIB; 8 – inoculated seeds with *A. brasilense* ARG2 and the medium supplemented with 10  $\mu$ M of PCIB. Each seed was inoculated with  $10^7$  CFU of the respective strain.

#### **2.4.7 – Different inoculum concentrations experiment**

This experiment consisted of 9 treatments to test if different concentrations of inoculum lead to different PGP effects of *A. brasilense* on wheat: 1 – non-inoculated seeds and with no medium supplementation (control); 2 – inoculated seeds with  $10^6$  CFU.seed<sup>-1</sup> of *A. brasilense* Sp245; 3 – inoculated seeds with  $10^7$  CFU.seed<sup>-1</sup> of *A. brasilense* Sp245; 4 – inoculated seeds with  $10^8$  CFU.seed<sup>-1</sup> of *A. brasilense* Sp245; 5 – inoculated seeds with  $10^9$  CFU.seed<sup>-1</sup> of *A. brasilense* Sp245; 6 – inoculated seeds with  $10^6$  CFU.seed<sup>-1</sup> of *A. brasilense* ARG2; 7 – inoculated seeds with  $10^7$  CFU.seed<sup>-1</sup> of *A. brasilense* ARG2; 8 – inoculated seeds with  $10^8$  CFU.seed<sup>-1</sup> of *A. brasilense* ARG2; 9 – inoculated seeds with  $10^9$  CFU.seed<sup>-1</sup> of *A. brasilense* ARG2.

#### **2.4.8 – Additive effect experiment**

This experiment consisted of 8 treatments to test if the application of exogenous auxins and/or NO alters the PGP effects of *A. brasilense* on wheat: 1 – non-inoculated seeds and with no medium supplementation (control); 2 – non-inoculated seeds and the medium supplemented with 100 nM of NAA plus 10  $\mu$ M of SNP; 3 – inoculated seeds with *A. brasilense* Sp245 and the medium supplemented with 10  $\mu$ M of SNP; 4 – inoculated seeds with *A. brasilense* ARG2 and the medium supplemented with 10  $\mu$ M of SNP; 5 – inoculated seeds with *A. brasilense* Sp245 and the medium supplemented with 100

nM of NAA; 6 – inoculated seeds with *A. brasilense* ARG2 and the medium supplemented with 100 nM of NAA; 7 – inoculated seeds with *A. brasilense* Sp245 and the medium supplemented with 10 µM of SNP plus 100 nM of NAA; 8 – inoculated seeds with *A. brasilense* ARG2 and the medium supplemented with 10 µM of SNP plus 100 nM of NAA. Each seed was inoculated with 10<sup>7</sup> CFU of the respective strain.

#### **2.4.9 – Denitrification experiment**

This experiment consisted of 4 treatments to test if the presence of NO<sub>3</sub> increases the *A. brasilense* PGP effects on wheat: 1 – non-inoculated seeds and with no medium supplementation (control); 2 – non-inoculated seeds and the medium supplemented with 1 mM of KNO<sub>3</sub>; 3 – inoculated seeds with *A. brasilense* Sp245 and the medium supplemented with 1 mM of KNO<sub>3</sub>; 4 – inoculated seeds with *A. brasilense* ARG2 and the medium supplemented with 1 mM of KNO<sub>3</sub>. Each seed was inoculated with 10<sup>7</sup> CFU of the respective strain.

#### **2.4.10 – Preto Amarelo wheat experiment**

This experiment consisted of 6 treatments to test if the test the PGP effects of *A. brasilense* on the Marialva variety and the role of NO on those effects was also verified for the Preto Amarelo variety: 1 – non-inoculated seeds and with no medium supplementation (control); 2 – inoculated seeds with *A. brasilense* Sp245; 3 – inoculated seeds with *A. brasilense* ARG2; 4 – non-inoculated seeds and the medium supplemented with 10 µM of SNP; 5 – inoculated seeds with *A. brasilense* Sp245 and the medium supplemented with 10 µM of cPTIO; 6 – inoculated seeds with *A. brasilense* ARG2 and the medium supplemented with 10 µM of cPTIO. Each seed was inoculated with 10<sup>7</sup> CFU of the respective strain.

### **2.5 – Determination of the IAA production**

The quantification of the IAA production was assessed using the colorimetric method described by several authors, (Gordon & Weber, 1951; Gutierrez et al., 2009; Kielak et al., 2016; Shrestha et al., 2014; Singh et al., 2013), with some adaptations.

This experiment consisted of 32 different treatments: the 2 *A. brasilense* strains, Sp245 and ARG2 x 4 different concentrations of bacterial inoculum x addition or no addition of Trp x addition or no addition of naringenin. The 4 different concentrations of bacterial inoculum (10<sup>3</sup> CFU.mL<sup>-1</sup>, 10<sup>5</sup> CFU.mL<sup>-1</sup>, 10<sup>7</sup> CFU.mL<sup>-1</sup> and 10<sup>9</sup> CFU.mL<sup>-1</sup>), were to evaluate how the production of IAA varies with the initial bacterial concentration. The presence or absence of Trp from the medium was to evaluate the stimulation of the IAA production by Trp. It was reported that the absence of Trp from the culture medium decreases the level of IAA synthesis by *Azospirillum*, while the addition of exogenous Trp may augment IAA biosynthesis by at least an order of magnitude (Zakharova et al., 1999). The presence or absence naringenin from the medium, which mimics plant presence, was to evaluate how would the presence of the plant influence IAA production. To highlight, that Trp and naringenin are both present in the wheat exudates of the tested wheat varieties (data not shown).

There were 4 different medium composition: T1) control – only NB medium with no supplementation; T2) Trp – NB medium supplemented with 2 mg mL<sup>-1</sup> of L-Trp; T3) naringenin – NB medium supplemented with 1 µg mL<sup>-1</sup> of naringenin and T4) Trp + naringenin – NB medium supplemented with 2 mg.mL<sup>-1</sup> of L-Trp plus 1 µg.mL<sup>-1</sup> of naringenin. Liquid cultures were initiated by inoculating 9.7 mL of the respective medium, in 50 mL Erlenmeyer flasks, with an 0.3 mL aliquot from the bacterial suspensions previously prepared with the concentrations of 3.3 x 10<sup>4</sup> CFU.mL<sup>-1</sup>, 3.3 x 10<sup>6</sup> CFU.mL<sup>-1</sup>, 3.3 x 10<sup>8</sup> CFU.mL<sup>-1</sup> and 3.3 x 10<sup>10</sup> CFU.mL<sup>-1</sup>, so the initial concentrations of the liquid cultures were 10<sup>3</sup> CFU.mL<sup>-1</sup>, 10<sup>5</sup> CFU.mL<sup>-1</sup>, 10<sup>7</sup> CFU.mL<sup>-1</sup> and 10<sup>9</sup> CFU.mL<sup>-1</sup>, respectively. The cultures were

incubated during 48 h at 28 °C with constant agitation (100 rpm) in an orbital shaker (Thermo scientific Max Q 400, Model No SHKE4000 – 8CE).

After 6 h, 24 h and 48 h of incubation, a 2 mL aliquot (1 mL for the determination of the number of CFU.mL<sup>-1</sup> and 1 mL for the determination of the IAA production) was taken aseptically from the broth. For the determination of the IAA production, samples were centrifuged at 14000 rpm for 10 min with a centrifuge (Microfuge18, Beckman Coulter™). The supernatant was collected and transferred to a test tube where 2 mL of Salkowski reagent was added. After room temperature incubation in the dark (tubes were covered with an aluminum foil) for 30 min, the presence of IAA was established by the pink coloration and quantified by measuring absorbance at 530 nm using a spectrophotometer (Unicam UV/Vis Spectrometer). Each treatment was assessed in triplicate, with the negative control being constituted by the respective medium alone. IAA concentrations were determined by comparison with a triplicate standard calibration curve spiked with IAA, with the concentrations of 0, 5, 10, 20, 50 and 100 µg.mL<sup>-1</sup> of IAA (Sigma®), diluted in basal medium, from a stock solution of 1000 µg.mL<sup>-1</sup> of IAA (dissolved in acetone).

## 2.6 – Statistical analysis

To try to reduce the variability associated with the seeds biological variability, the control treatment used in the statistical analysis, and display in the graphics, was the combination from all the controls treatments from all the different experiments. All the control treatments were validated by convenient residual analyses that did not show departure from the normal distribution according to the Shapiro-Wilks normality test ( $P>0.05$ ), neither significant differences between them (ANOVA,  $P>0.05$ ).

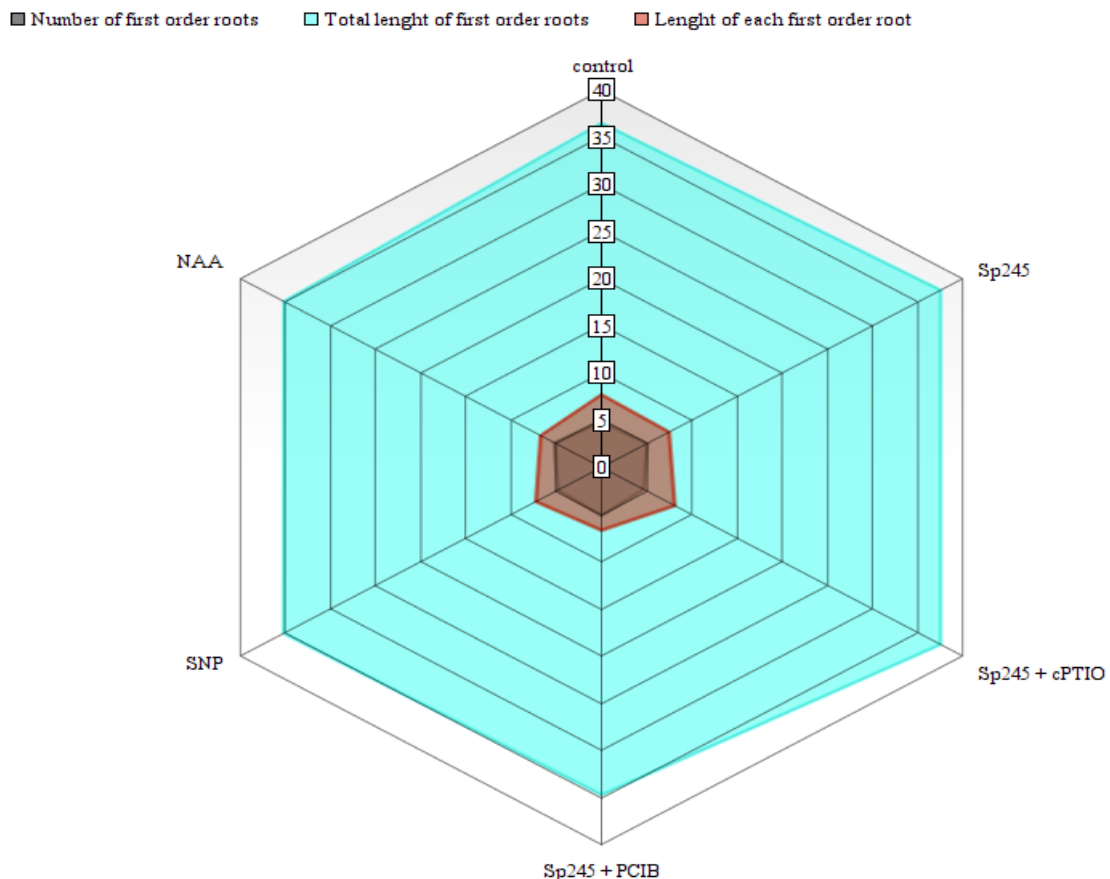
All analyses were validated by convenient residual analyses that did not show departure from the normal distribution according to the Shapiro-Wilks normality test ( $P>0.05$ ). The data was analysed using ANOVA and means comparison were estimated values of Tukey's test, when factors and interactions were significant ( $P\leq 0.05$ ). To compare the effects of the different treatments in the 2 wheat varieties, Marialva and Preto Amarelo; the effects of the 2 strains of *A. brasilense*, Sp245 and ARG2, and the IAA production of the 2 strains, a 2-way ANOVA were performed. When factors and interactions were significantly different ( $P\leq 0.05$ ), a Student's t-test was applied to determine which treatments were indeed significantly different ( $P\leq 0.05$ ). All statistical tests were performed using the statistical program PAST3 software (version 3.20; <https://folk.uio.no/ohammer/past/>, accessed on 17 of April of 2018 at 17:09, UTC + 1 h or WEST).

## Chapter 3 – Results and discussion

### 3.1 – NO and auxin production are required for the *A. brasilense* PGP effects on wheat

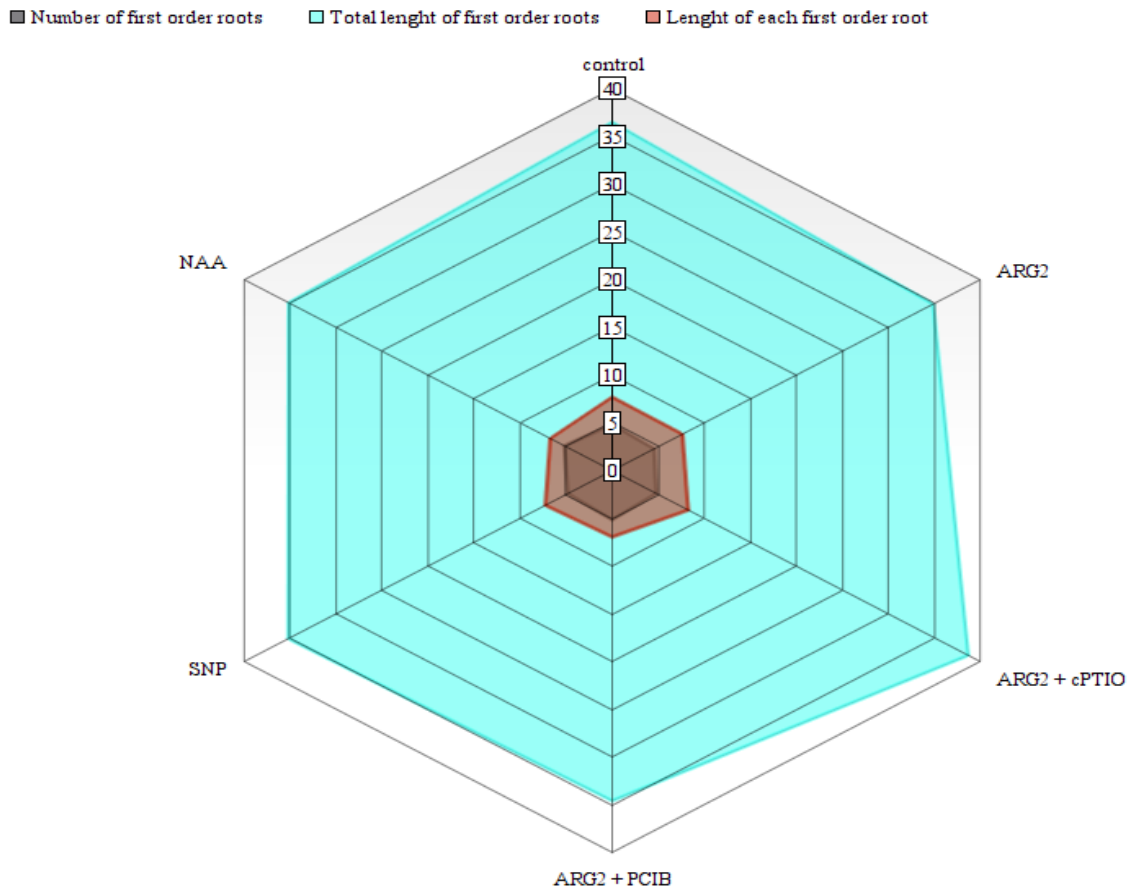
#### 3.1.1 – Effects on the root structure

The number of FORs or their length (Figs 3.1 and 3.2) did not change in response to seed inoculation with *A. brasilense* Sp245 or ARG2 (ANOVA,  $P>0.05$ ). But the number of SORs per plant increased relatively to those of the control (Fig 3.3; Tukey's test,  $P\leq 0.05$ ) in the plants developed from inoculated seeds. Showing that seed inoculation with *A. brasilense* increased the number of SORs, without affecting the structure of FORs.

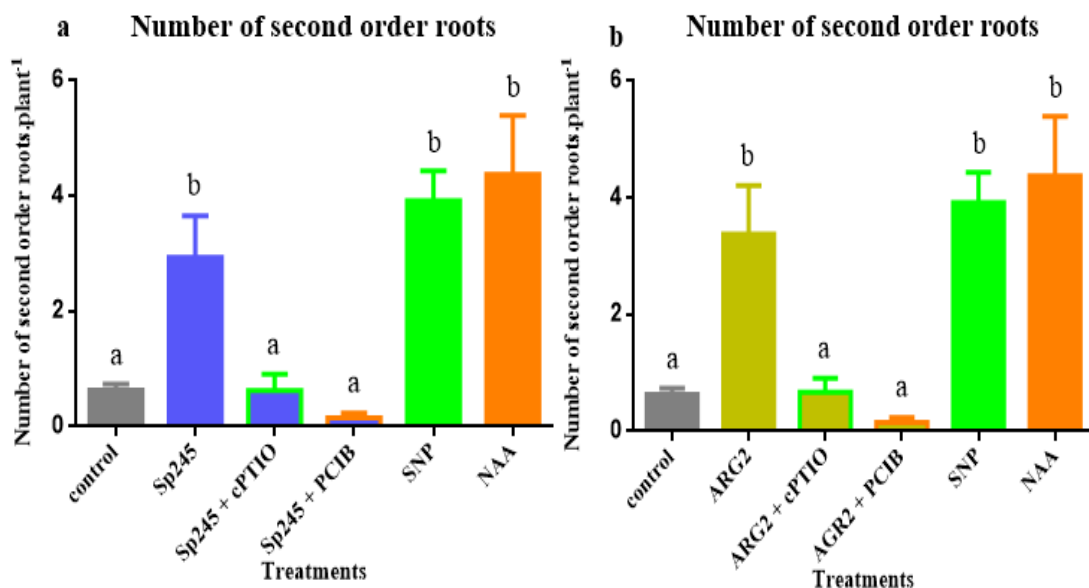


**Figure 3.1** – Inoculation with *A. brasilense* Sp245 did not influence the FORs of wheat. 6 different treatments: control, inoculated with *A. brasilense* Sp245, inoculated with *A. brasilense* Sp245 plus 10  $\mu\text{M}$  of cPTIO, inoculated with *A. brasilense* Sp245 plus 10  $\mu\text{M}$  of PCIB, 10  $\mu\text{M}$  of SNP, 100 nM of NAA. Number of FORs (ANOVA,  $F=1.37$ ,  $P>0.05$ ), total length of FORs (cm. plant<sup>-1</sup>; ANOVA,  $F=0.81$ ,  $P>0.05$ ), length of each FOR ((cm.(root.plant)<sup>-1</sup>); ANOVA,  $F=2.04$ ,  $P>0.05$ ).  $n=15$

When seeds were simultaneously inoculated with bacteria and NO scavenger (cPTIO) or auxin scavenger (PCIB), the number of SORs per plant was similar to that of the control (Tukey's test,  $P>0.05$ ). While the addition of external sources of NO (SNP) or auxins (NAA), increased the number of SORs to the levels observed in plants inoculated with bacteria only (Fig 3.3).

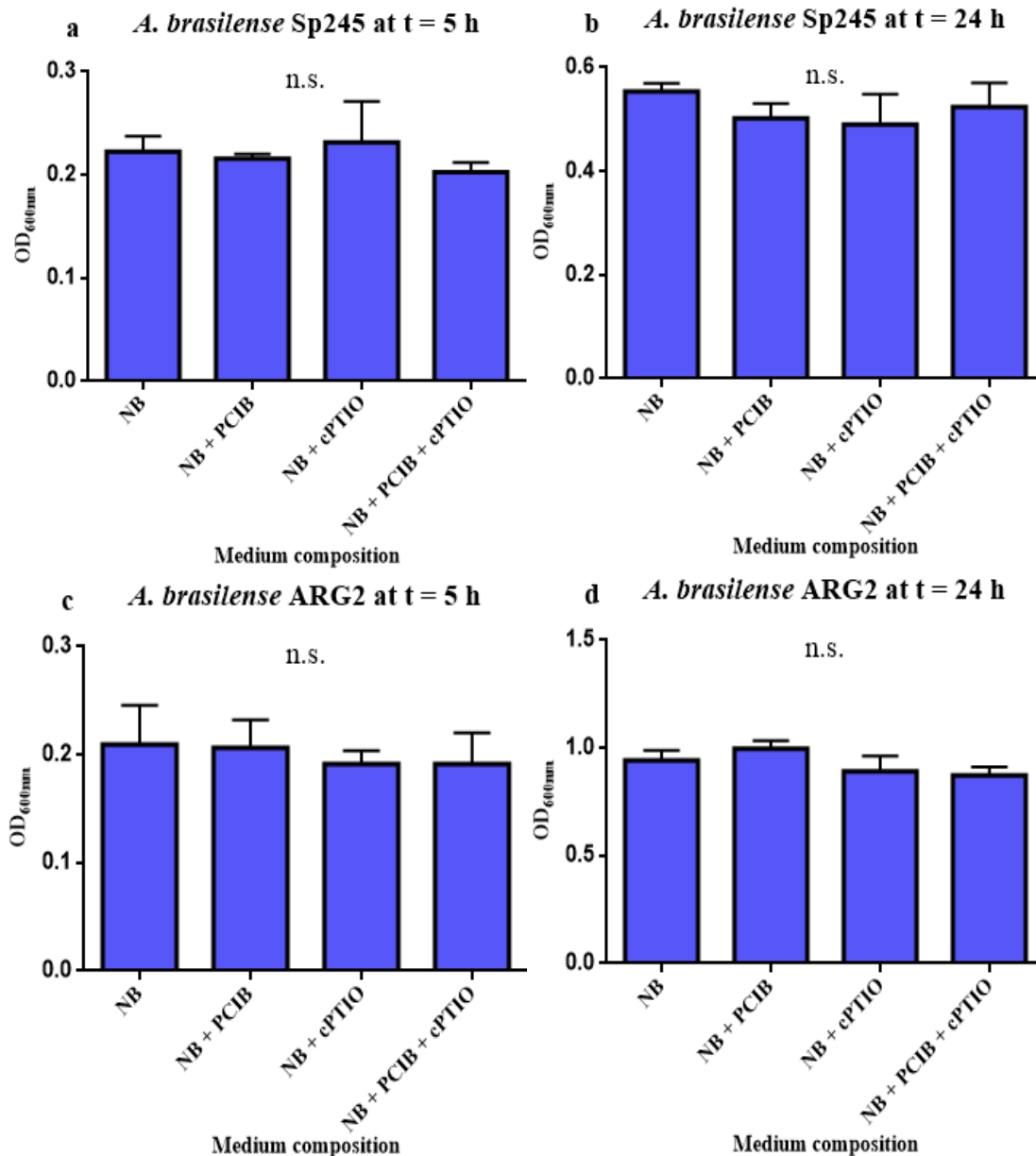


**Figure 3.2** – Inoculation with *A. brasilense* ARG2 did not influence the FORs of wheat. 6 different treatments: control, inoculated with *A. brasilense* ARG2, inoculated with *A. brasilense* ARG2 plus 10  $\mu\text{M}$  of cPTIO, inoculated with *A. brasilense* ARG2 plus 10  $\mu\text{M}$  of PCIB, 10  $\mu\text{M}$  of SNP, 100 nM of NAA. number of FORs (ANOVA,  $F=1.37$ ,  $P>0.05$ ), total length of FORs (cm. plant<sup>-1</sup>; ANOVA,  $F=0.81$ ,  $P>0.05$ ), length of each FOR (cm.(root. plant)<sup>-1</sup>; ANOVA,  $F=2.04$ ,  $P>0.05$ ).  $n=15$



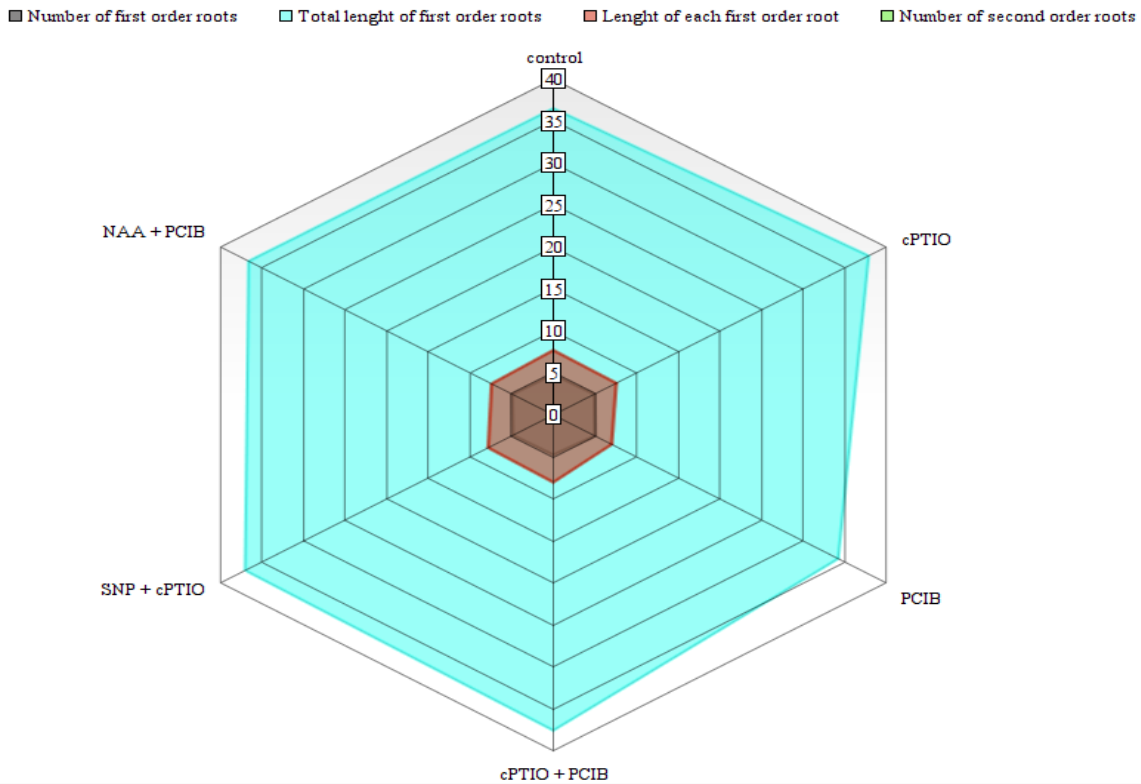
**Figure 3.3** – Effects of the inoculation with *A. brasilense* Sp245 or ARG2 on number of SORs of wheat are due to the production of NO and IAA by the bacterium. (a) 6 different treatments: control, inoculated with *A. brasilense* Sp245, inoculated with *A. brasilense* Sp245 plus 10  $\mu\text{M}$  of cPTIO, inoculated with *A. brasilense* Sp245 plus 10  $\mu\text{M}$  of PCIB, 10  $\mu\text{M}$  of SNP, 100 nM of NAA (ANOVA,  $F=24.45$ ,  $P\leq 0.001$ ). (b) 6 different treatments: control, inoculated with *A. brasilense* ARG2, inoculated with *A. brasilense* ARG2 plus 10  $\mu\text{M}$  of cPTIO, inoculated with *A. brasilense* ARG2 plus 10  $\mu\text{M}$  of PCIB, 10  $\mu\text{M}$  of SNP, 100 nM of NAA. (ANOVA,  $F=24.96$ ,  $P\leq 0.001$ ). Different small letters at the top of each bar indicated significant differences (Tukey's test,  $P\leq 0.05$ ). Each bar represents mean of  $n=15 \pm \text{SE}$ .

Controls were performed to assess possible toxic effects of the tested products on bacteria development (Fig 3.4). But the OD of the bacterial cultures with or without the scavengers, when grown in liquid NB medium was identical (ANOVA,  $P>0.05$ ).



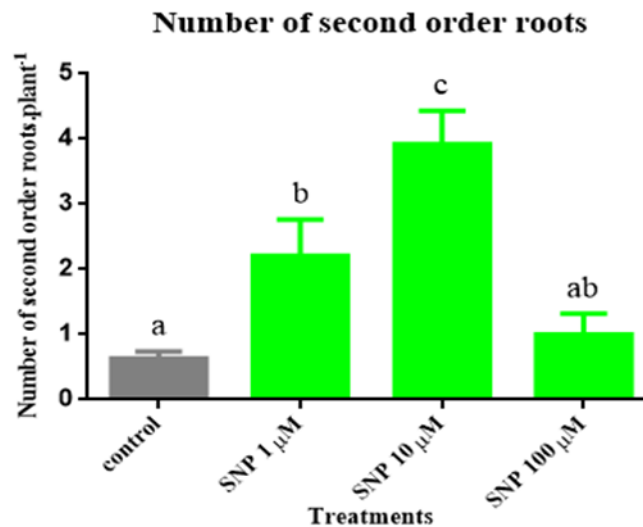
**Figure 3.4** – The NO scavenger (cPTIO) and the auxin scavenger (PCIB) do not affect the growth of the *A. brasilense* strains Sp245 and ARG2 in liquid NB medium. 4 different medium compositions were tested: only NB (control), NB + 10 μM of cPTIO, NB + 10 μM of PCIB and NB + 10 μM of cPTIO + 10 μM of PCIB. (a) OD of the cultures of *A. brasilense* Sp245 at t=5 h (ANOVA,  $F = 0.59$ ), (b) OD of the cultures of *A. brasilense* Sp245 at t = 24 h (ANOVA,  $F=0.93$ ), (c) OD of the cultures of *A. brasilense* ARG2 at t=5 h (ANOVA,  $F=0.24$ ), (d) OD of the cultures of *A. brasilense* ARG2 at t=24 h (ANOVA,  $F = 2.45$ ). n.s. treatments show no significant differences by ANOVA ( $P>0.05$ ). Each bar represents mean of  $n=3 \pm SE$

When scavengers were applied to the seed alone or in combination with the respective donor, the number of SORs per plant was similar to the control (ANOVA,  $P>0.05$ ; Fig 3.5), showing that scavengers blocked the effects of the NO donor (SNP) and synthetic auxin (NAA) on the number of SORs, but did not affect the root structure only *per se*.

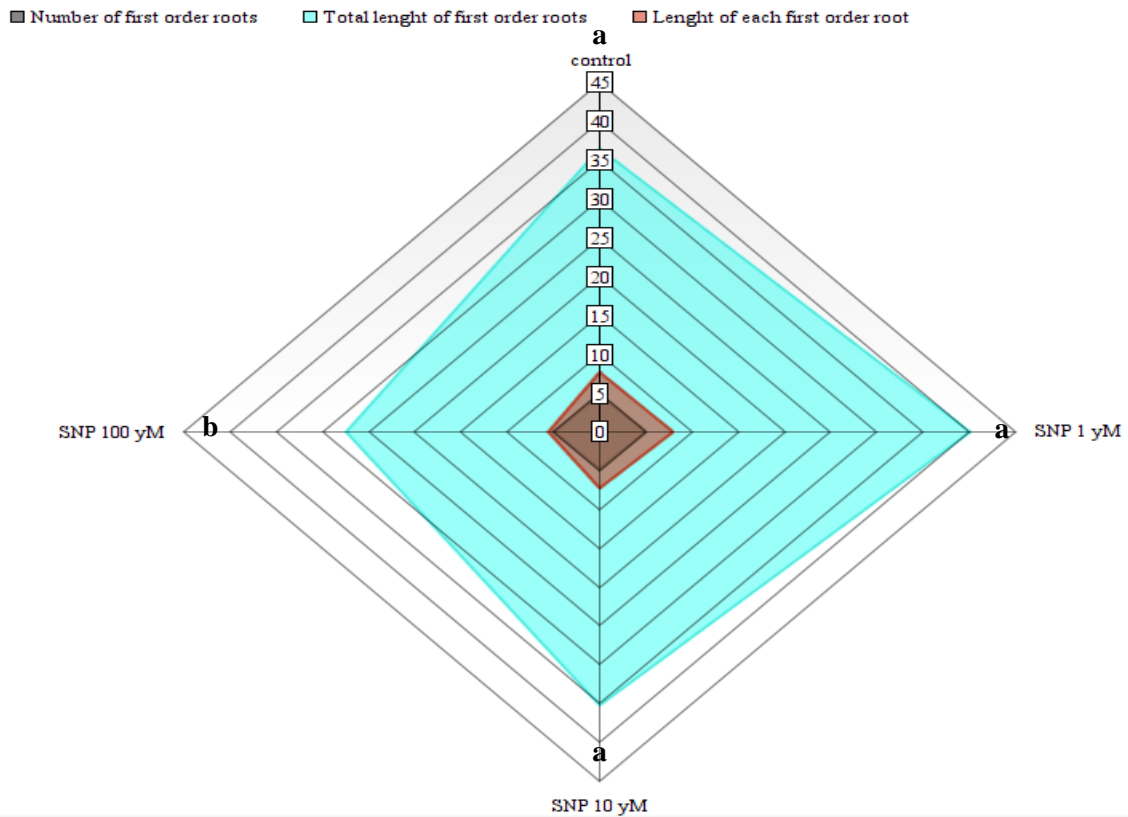


**Figure 3.5** – The NO scavenger (cPTIO) and the auxin scavenger (PCIB) do not affect the roots structure of wheat, also the addition of cPTIO and PCIB, blocks the effects of the NO donor (SNP) and the synthetic auxin (NAA), respectively, on the roots structure of wheat. 6 different treatments: nothing added to the medium (control), 10  $\mu\text{M}$  of cPTIO, 10  $\mu\text{M}$  of PCIB, 10  $\mu\text{M}$  of cPTIO plus 10  $\mu\text{M}$  of PCIB, 10  $\mu\text{M}$  of SNP plus 10  $\mu\text{M}$  of cPTIO and 100 nM NAA plus 10  $\mu\text{M}$  of PCIB. Number of FORs (ANOVA,  $F=1.06$ ,  $P>0.05$ ), total length of FORs (cm. plant<sup>-1</sup>; ANOVA,  $F=0.33$ ,  $P>0.05$ ), length of each FORs (cm.root.plant<sup>-1</sup>; ANOVA,  $F=0.41$ ,  $P>0.05$ ), number of SORs (ANOVA,  $F=0.95$ ,  $P>0.05$ ).  $n=15$

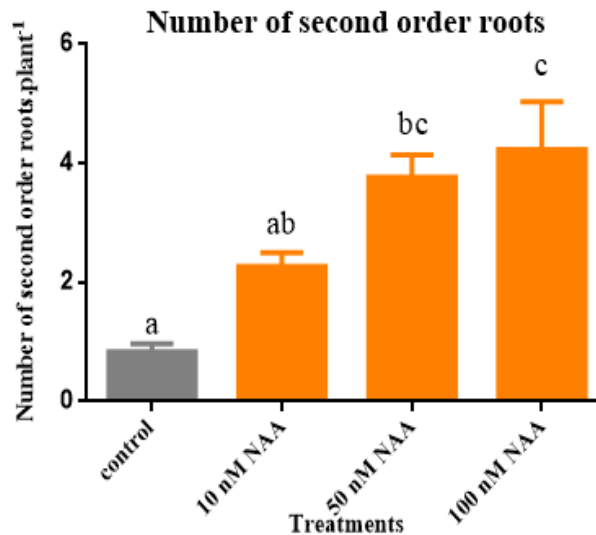
Application of SNP in the range of 1-10  $\mu\text{M}$  increased the number of SORs (Fig. 3.6; Tukey's test,  $P\leq 0.05$ ). At 100  $\mu\text{M}$ , the number of SORs was similar to that of the control (Tukey's test,  $P>0.05$ ) and the length of FORs inferior (Fig 3.7; Tukey's test,  $P>0.05$ ). These results highlight the inhibitory effects of high NO concentrations on SORS and FORs formation.



**Figure 3.6** – Different concentrations of the NO donor (SNP) have different effects on the number of SORs of wheat. 4 different treatments: control, 1  $\mu\text{M}$  of SNP, 10  $\mu\text{M}$  of SNP, 100  $\mu\text{M}$  of SNP (ANOVA,  $F=6.73$ ,  $P\leq 0.001$ ), Different small letters at the top of each bar indicated significant differences (Tukey's test,  $P\leq 0.05$ ). Each bar represents mean of  $n=15 \pm \text{SE}$ .



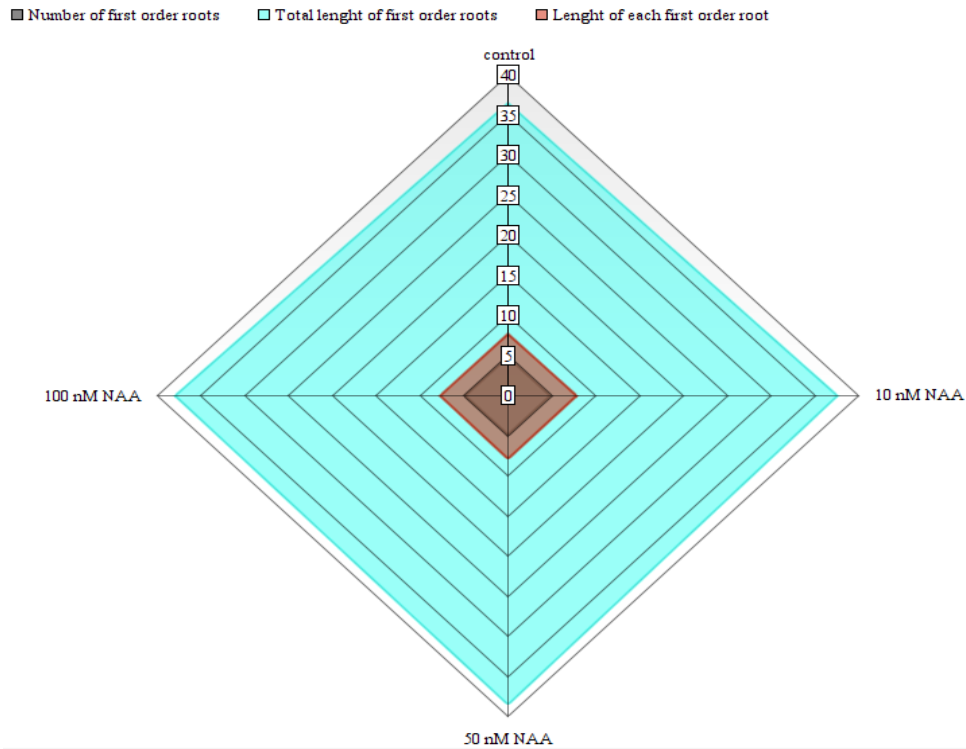
**Figure 3.7** – Different concentrations of the NO donor (SNP) have different effects on the FORs of wheat. 4 different treatments: control, 1  $\mu\text{M}$  of SNP, 10  $\mu\text{M}$  of SNP, 100  $\mu\text{M}$  of SNP. Number of FORs (ANOVA,  $F=0.89$ ,  $P>0.05$ ), total length of FORs ( $\text{cm. plant}^{-1}$ ; ANOVA,  $F=8.67$ ,  $P\leq 0.001$ ), (d) length of each FORs ( $\text{cm. (root.plant)}^{-1}$ ; ANOVA,  $F=11.23$ ,  $P\leq 0.001$ ). Different small letters at each edge indicated significant differences (Tukey's test,  $P\leq 0.05$ ).  $n=15$



**Figure 3.8** – Different concentrations of the synthetic auxin (NAA) have different effects on the number of SORs of wheat. 4 different treatments: control, 10 nM of NAA, 50 nM of NAA, 100 nM of NAA (ANOVA,  $F=42.95$ ,  $P\leq 0.001$ ) Different small letters at the top of each bar indicated significant differences (Tukey's test,  $P\leq 0.05$ ). Each bar represents mean of  $n=15 \pm \text{SE}$ .

In the range between 50-100 nM, NAA tended to stimulate the number of SORs according to the concentration (Fig. 3.8, Tukey's test,  $P\leq 0.05$ ), without influencing the FORs structure (Fig 3.9; ANOVA,  $P>0.05$ ).

These results are in line with previous works reporting that wheat inoculation with *A. brasilense* resulted in an increased number of SORs, without affecting the structure of the FORs (Malhotra & Srivastava, 2009) and that the application of an NO donor (SNP) or synthetic auxin (NAA) increased the number of SORs in a dose dependent manner in tomato seedlings (Correa-Aragunde et al., 2004).



**Figure 3.9** – Different concentrations the synthetic auxin (NAA) have different effects on the FORs of wheat. 4 different treatments: control, 10 nM of NAA, 50 nM of NAA, 100 nM of NAA. Number of FORs (ANOVA,  $F=0.11$ ,  $P>0.05$ ), total length of FORs ( $\text{cm. plant}^{-1}$ ; ANOVA,  $F=0.62$ ,  $P>0.05$ ), length of each FORs ( $\text{cm. (root.plant)}^{-1}$ ; ANOVA,  $F=0.1$ ,  $P>0.05$ ).  $n=15$

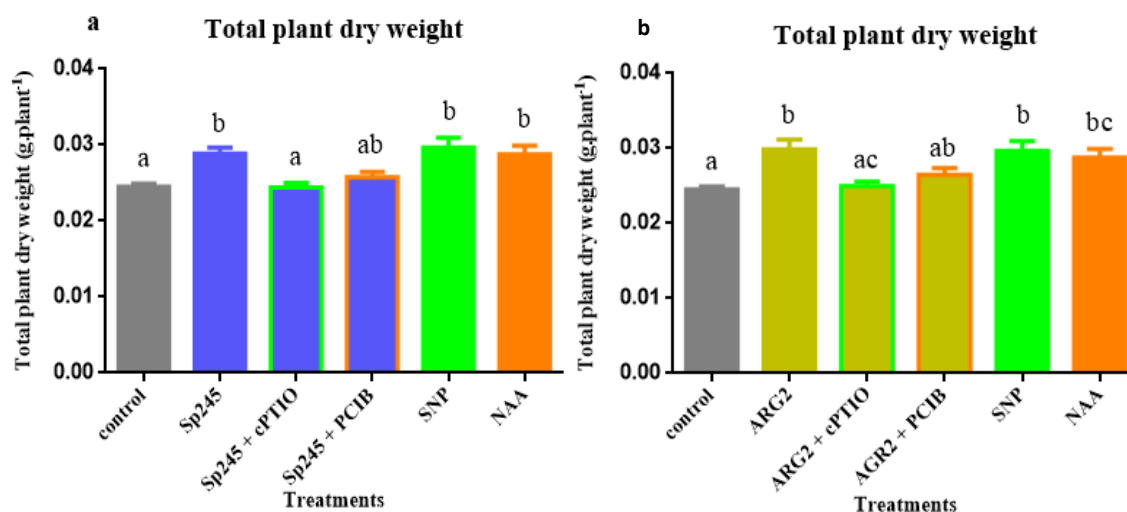
All together the results suggest that NO and IAA, are critical signalling and effector molecules mediating the stimulation of SORs after seed inoculation with *A. brasilense*. Both *A. brasilense* strains seemed to influence the wheat root structure in a similar manner (2-way ANOVA,  $P>0.05$ ). However the effect of NO and IAA was not additive and the application of SNP and NAA, separately, increased the number of SORs to values similar to those of the inoculated seeds (Fig 3.3).

Based on the mechanism involved in the formation of the SORs (Du & Scheres, 2018), both phytohormones are essential for formation of SORs. But phytohormones work in very complex networks and changes in the concentration of one, leads to changes in the relative concentration of many, which may influence many cellular processes (Sanz et al., 2015). On the other hand, many reports have highlighted many processes in plants in which NO and other phytohormones act separately to give the same response (Lamattina et al., 2003; Sanz et al., 2015). For instance auxins (application of IAA) can increase the plant own levels of NO (Chen et al., 2010), despite that no NO synthases has been identified in higher plants (Astier et al., 2017). On the other hand, it was also reported that NO can indirectly increase the plants IAA levels, by reducing IAA oxidase-driven IAA degradation (Chen et al., 2010).

In *A. brasilense* SM strain, NO and IAA biosynthesis share an extensive cross-talk (Koul et al., 2015b), where the presence of cPTIO may decrease the production of IAA by the bacteria, but that of PCIB may increase bacterial production of NO. So the internal bacterial coregulation of NO and IAA levels cannot explain why there was no additive effect of NO and IAA on SORs formation.

### 3.1.2 – Effects on the biomass production

Plant biomass production increased in response to inoculation with *A. brasilense* Sp245 or ARG2 (Fig 3.10; Tables 3.1 and 3.2; Tukey's test,  $P \leq 0.05$ ). No significant differences were observed in the root to shoot ratio between the treatments (ANOVA,  $P > 0.05$ ), showing that seed inoculation with *A. brasilense* increased plant biomass without affecting biomass distribution within the plant.



**Figure 3.10** – Effects of the inoculation with *A. brasilense* Sp245 and ARG2 on the wheat biomass are due to the production of NO and IAA by the bacterium. (a) 6 different treatments: control, inoculated with *A. brasilense* Sp245, inoculated with *A. brasilense* Sp245 plus 10  $\mu\text{M}$  of cPTIO, inoculated with *A. brasilense* Sp245 plus 10  $\mu\text{M}$  of PCIB, 10  $\mu\text{M}$  of SNP, 100 nM of NAA (ANOVA,  $F=8.92$ ,  $P \leq 0.001$ ). (b) 6 different treatments: control, inoculated with *A. brasilense* ARG2, inoculated with *A. brasilense* ARG2 plus 10  $\mu\text{M}$  of cPTIO, inoculated with *A. brasilense* ARG2 plus 10  $\mu\text{M}$  of PCIB, 10  $\mu\text{M}$  of SNP, 100 nM of NAA (ANOVA,  $F=9.48$ ,  $P \leq 0.001$ ). Different small letters at the top of each bar indicated significant differences (Tukey's test,  $P \leq 0.05$  Each bar represents mean of  $n=15 \pm \text{SE}$ ).

When seeds were simultaneously inoculated with bacteria and the medium supplemented with a NO scavenger (cPTIO), plants did not produce more biomass than control plants (Tukey's test,  $P > 0.05$ ). But seeds inoculated and supplemented with PCIB produced plants, which biomass was not different from the control plants and neither from the inoculated ones (Tukey's test,  $P > 0.05$ ). The addition of external sources of NO (SNP) or auxins (NAA), increased wheat plants biomass to levels similar to those observed in plants inoculated with bacteria only (Fig 3.10; Tukey's test,  $P > 0.05$ ).

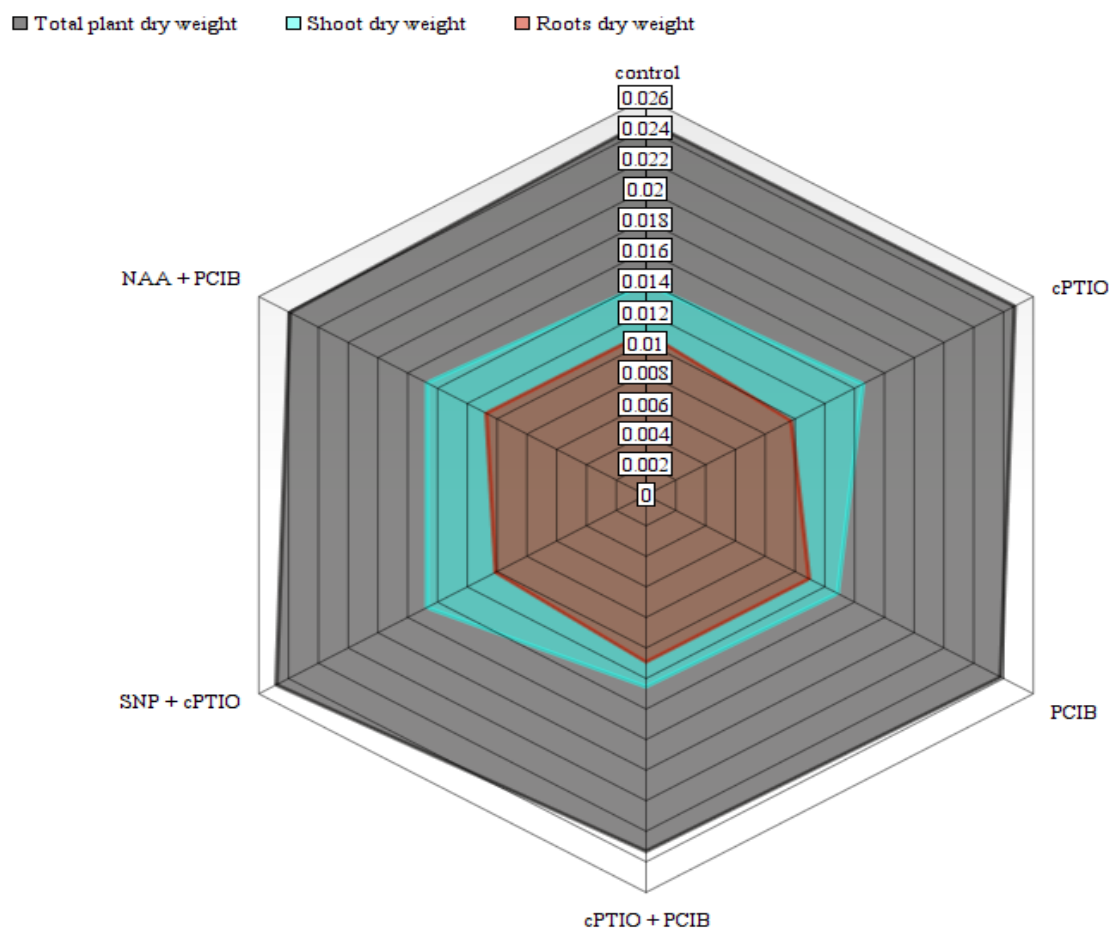
**Table 3.1** – Effects of the inoculation with *A. brasilense* Sp245 on the wheat biomass are due to the production of NO and IAA by the bacterium. 6 different treatments: control, inoculated with *A. brasilense* Sp245, inoculated with *A. brasilense* Sp245 plus 10  $\mu\text{M}$  of cPTIO, inoculated with *A. brasilense* Sp245 plus 10  $\mu\text{M}$  of PCIB, 10  $\mu\text{M}$  of SNP, 100 nM of NAA. Plant shoot dry weight in g per plant, (ANOVA,  $F=5.49$ ,  $P \leq 0.001$ ), plant roots dry weight in g per plant (ANOVA,  $F=4.92$ ,  $P \leq 0.001$ ), dry plant root to shoot weight ratio (ANOVA,  $F=0.87$ ,  $P > 0.05$ ). Different small letters in the front of each value indicated significant differences (Tukey's test,  $P \leq 0.05$ ). Each value represents mean of  $n=15 \pm \text{SE}$ .

Treatment	Shoot dry weight (g.plant <sup>-1</sup> )	Roots dry weight (g.plant <sup>-1</sup> )	Root to shoot ratio
control	0.014 $\pm$ 0.0003 <sup>a</sup>	0.011 $\pm$ 0.0002 <sup>a</sup>	0.80 $\pm$ 0.018
Sp245	0.016 $\pm$ 0.0006 <sup>b</sup>	0.012 $\pm$ 0.0003 <sup>b</sup>	0.77 $\pm$ 0.02
Sp245 + cPTIO	0.015 $\pm$ 0.0006 <sup>ab</sup>	0.011 $\pm$ 0.0006 <sup>ab</sup>	0.73 $\pm$ 0.013
Sp245 + PCIB	0.014 $\pm$ 0.0008 <sup>ab</sup>	0.011 $\pm$ 0.0005 <sup>ab</sup>	0.78 $\pm$ 0.057
SNP	0.017 $\pm$ 0.0009 <sup>b</sup>	0.013 $\pm$ 0.0004 <sup>b</sup>	0.73 $\pm$ 0.024
NAA	0.016 $\pm$ 0.0005 <sup>b</sup>	0.013 $\pm$ 0.0005 <sup>b</sup>	0.80 $\pm$ 0.024

**Table 3.2** – Effects of the inoculation with *A. brasilense* ARG2 on the wheat biomass are due to the production of NO and IAA by the bacterium. 6 different treatments: control, inoculated with *A. brasilense* ARG2, inoculated with *A. brasilense* ARG2 plus 10  $\mu$ M of cPTIO, inoculated with *A. brasilense* ARG2 plus 10  $\mu$ M of PCIB, 10  $\mu$ M of SNP, 100 nM of NAA. Plant shoot dry weight in g per plant, (ANOVA, F=6.1, P $\leq$ 0.001), plant roots dry weight in g per plant (ANOVA, F=8.45, P $\leq$ 0.001), dry plant root to shoot weight ratio (ANOVA, F=2.15, P>0.05). Different small letters in the front of each value indicated significant differences (Tukey’s test, P $\leq$ 0.05). Each value represents mean of n=15  $\pm$ SE.

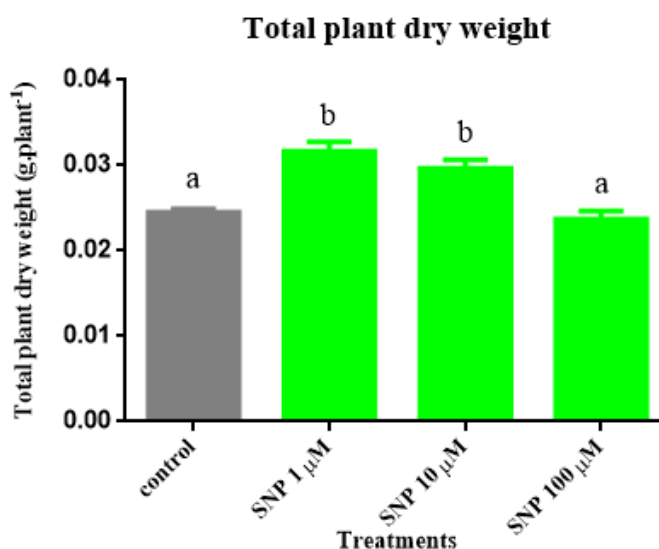
Treatment	Shoot dry weight (g.plant <sup>-1</sup> )	Roots dry weight (g.plant <sup>-1</sup> )	Root to shoot ratio
control	0.014 $\pm$ 0.0003 <sup>a</sup>	0.011 $\pm$ 0.0002 <sup>a</sup>	0.80 $\pm$ 0.018
ARG2	0.017 $\pm$ 0.0008 <sup>b</sup>	0.013 $\pm$ 0.0006 <sup>b</sup>	0.77 $\pm$ 0.031
ARG2 + cPTIO	0.015 $\pm$ 0.0007 <sup>ab</sup>	0.010 $\pm$ 0.0003 <sup>a</sup>	0.82 $\pm$ 0.045
AGR2 + PCIB	0.015 $\pm$ 0.0006 <sup>ab</sup>	0.012 $\pm$ 0.0005 <sup>ab</sup>	0.78 $\pm$ 0.044
SNP	0.017 $\pm$ 0.0009 <sup>b</sup>	0.013 $\pm$ 0.0004 <sup>b</sup>	0.73 $\pm$ 0.024
NAA	0.016 $\pm$ 0.0005 <sup>b</sup>	0.013 $\pm$ 0.0005 <sup>b</sup>	0.80 $\pm$ 0.024

The effects of the NO scavenger (cPTIO) and auxin scavenger (PCIB) were due to the prevention of the NO and IAA mediated effects. When scavengers were applied to the seed alone or in combination with the respective donor, resulted in a biomass production similar to the plants of the control (ANOVA, P>0.05; Fig 3.11), showing that scavengers blocked the effects of the NO donor (SNP) and synthetic auxin (NAA) on the biomass production, which were reduced to values similar to the control ones (t-test, P>0.05).



**Figure 3.11** – The NO scavenger (cPTIO) and the auxin scavenger (PCIB) do not affect the wheat biomass production, also the addition of cPTIO and PCIB, blocks the effects of the NO donor (SNP) and the synthetic auxin (NAA), respectively, on the wheat biomass. 6 different treatments: control, 10  $\mu$ M of cPTIO, 10  $\mu$ M of PCIB, 10  $\mu$ M of cPTIO + 10  $\mu$ M of PCIB, 10  $\mu$ M of SNP + 10  $\mu$ M of cPTIO and 100 nM NAA + 10  $\mu$ M of PCIB. total plant dry weight in g per plant (ANOVA, F=0.39, P>0.05), plant shoot dry weight in g per plant (ANOVA, F=1.6, P>0.05), plant roots dry weight in g per plant (ANOVA, F=0.69, P>0.05), dry plant roots/shoot weight ratio (ANOVA, F=2.19, P>0.05). n=15

The application of SNP in the range of 1-10  $\mu\text{M}$  increased biomass production (Fig 3.12 and Table 3.3; Tukey's test,  $P \leq 0.05$ ). But at 100  $\mu\text{M}$  of SNP biomass production was similar to that of the control plants (Tukey's test,  $P > 0.05$ ), which highlights inhibitory effects of high NO concentrations (Koul et al., 2015b). No significant differences were observed in the root to shoot ratio between treatments (ANOVA,  $P > 0.05$ ), showing that the distinct NO concentrations did not affect the biomass distribution within the plant.

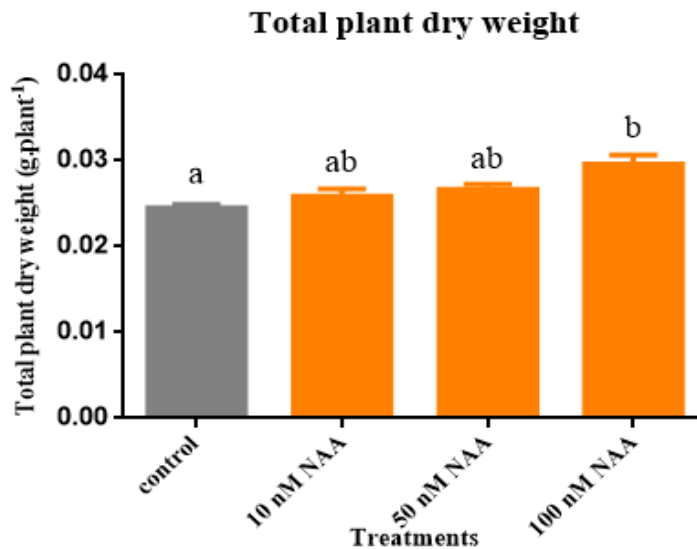


**Figure 3.12** – Different concentrations of the NO donor (SNP) have different effects on wheat biomass. 4 different treatments: control, 1  $\mu\text{M}$  of SNP, 10  $\mu\text{M}$  of SNP, 100  $\mu\text{M}$  of SNP (ANOVA,  $F = 10.21$ ,  $P \leq 0.001$ ). Different small letters at the top of each bar indicated significant differences (Tukey's test,  $P \leq 0.05$ ). Each bar represents mean of  $n = 15 \pm \text{SE}$ .

**Table 3.3** – Different concentrations of the NO donor (SNP) have different effects on the wheat biomass. 4 different treatments: control, 1  $\mu\text{M}$  of SNP, 10  $\mu\text{M}$  of SNP, 100  $\mu\text{M}$  of SNP. Plant shoot dry weight in g per plant (ANOVA,  $F = 15.25$ ,  $P \leq 0.001$ ), plant roots dry weight in g per plant (ANOVA,  $F = 3.62$ ,  $P \leq 0.05$ ), dry plant root to shoot weight ratio (ANOVA,  $F = 2.32$ ,  $P > 0.05$ ). Different small letters in the front of each value indicated significant differences (Tukey's test,  $P \leq 0.05$ ). Each value represents mean of  $n = 15 \pm \text{SE}$ .

Treatment	Shoot dry weight (g.plant <sup>-1</sup> )	Roots dry weight (g.plant <sup>-1</sup> )	Root to shoot ratio
Control	$0.014 \pm 0.0003$ <sup>a</sup>	$0.011 \pm 0.0002$ <sup>a</sup>	$0.80 \pm 0.018$
SNP 1 $\mu\text{M}$	$0.019 \pm 0.0008$ <sup>b</sup>	$0.013 \pm 0.0004$ <sup>b</sup>	$0.68 \pm 0.02$
SNP 10 $\mu\text{M}$	$0.017 \pm 0.0009$ <sup>b</sup>	$0.013 \pm 0.0004$ <sup>b</sup>	$0.73 \pm 0.074$
SNP 100 $\mu\text{M}$	$0.013 \pm 0.0005$ <sup>a</sup>	$0.011 \pm 0.0005$ <sup>a</sup>	$0.82 \pm 0.024$

By testing the effects of distinct external auxin concentrations in the biomass production (Fig 3.13 and Table 3.4) the application of 100 nM of NAA increased biomass production (Tukey's test,  $P \leq 0.05$ ). While in the concentration range of 10 – 50 nM of NAA the biomass production was similar to that of the control plants (Tukey's test,  $P > 0.05$ ). No significant differences were observed in the root to shoot ratio between treatments (ANOVA,  $P > 0.05$ ).



**Figure 3.13** – Different concentrations of the synthetic auxin (NAA) have different effects on the wheat biomass. 4 different treatments: control, 10 nM of NAA, 50 nM of NAA, 100 nM of NAA (ANOVA,  $F=8.23$ ,  $P\leq 0.001$ ). Different small letters at the top of each bar indicated significant differences (Tukey’s test,  $P\leq 0.05$ ). Each bar represents mean of  $n=15 \pm SE$ .

**Table 3.4** – Different concentrations of the synthetic auxin (NAA) have different effects on the wheat biomass. 4 different treatments: control, 10 nM of NAA, 50 nM of NAA, 100 nM of NAA. Plant shoot dry weight in g per plant (ANOVA,  $F=3$ ,  $P\leq 0.001$ ), plant roots dry weight in g per plant (ANOVA,  $F=14.1$ ,  $P\leq 0.05$ ), dry plant root to shoot weight ratio (ANOVA,  $F=2.51$ ,  $P>0.05$ ) Different small letters in the front of each value indicated significant differences (Tukey’s test,  $P\leq 0.05$ ). Each value represents mean of  $n=15 \pm SE$ .

Treatment	Shoot dry weight (g.plant <sup>-1</sup> )	Roots dry weight (g.plant <sup>-1</sup> )	Root to shoot ratio
Control	$0.014 \pm 0.0003$ <sup>a</sup>	$0.011 \pm 0.0002$ <sup>a</sup>	$0.80 \pm 0.018$
10 nM NAA	$0.014 \pm 0.0005$ <sup>a</sup>	$0.012 \pm 0.0005$ <sup>ab</sup>	$0.85 \pm 0.046$
50 nM NAA	$0.014 \pm 0.0004$ <sup>a</sup>	$0.013 \pm 0.0004$ <sup>ab</sup>	$0.90 \pm 0.020$
100 nM NAA	$0.016 \pm 0.0005$ <sup>b</sup>	$0.013 \pm 0.0005$ <sup>b</sup>	$0.80 \pm 0.024$

All these evidences suggest that NO and IAA, are involved in the stimulatory effects leading to increased plant biomass production. Both *A. brasilense* strains seemed to influence the wheat biomass accumulation in a similar manner (2-way ANOVA,  $P>0.05$ ).

It was observed that inoculation with any of the 2 strains or medium supplementation with SNP or NAA increased the roots dry weight relatively to the control (Tables 3.1, 3.2, 3.3 and 3.4; Tukey’s test,  $P\leq 0.05$ ). However no significant differences were observed among treatments relatively to the root length (Figs 3.1 and 3.2) and the weight of the SORs in the inoculated or SNP and NAA supplemented mediums were too small to account for the differences. It is possible that this increase in the roots dry weight is related with increases in the total volume of the roots and/or its density, as result from the action of phytohormones, either the ones produced by the bacterial strains, either the ones supplied exogenously, through supplementation with SNP or NAA.

At first sight, these increases in the biomass production of the inoculated treatments and the ones supplemented with SNP or NAA, could be due to an increased uptake of water and nutrients from the growth medium, resulting from the increased number of SORs, the so-called sponge effect. But the nutritional effect cannot be the reason behind this increase in the biomass production, as the plant growth medium did not contain any carbon source or any other nutrients. All the nutrients available were then

provided by the seed endosperm (Lopes & Larkins, 1993). The most probable effect is through phytohormonal stimulation of the photosynthesis.

NO seems to be essential for the bacterial induced increase in plant biomass accumulation as the application of its scavenger (cPTIO), blocked the bacterial effects. IAA also play a role in the bacterial induced increase in the wheat biomass production, as the application of its scavenger (PCIB) block part of the bacterial effects. This can mean that NO besides having a role in promoting plant biomass accumulation is also required for the IAA produced by *A. brasilense* to have a PGP effect on wheat. Alternately, as cPTIO can decrease the IAA production of *A. brasilense* (Koul et al., 2015b), it is possible that the supplementation with cPTIO in the inoculated treatments had 2 effects, blocking the NO-mediated effects while decreasing the IAA production.

Auxins (IAA) contribute to plant growth, through acidification of cells that leads to an altered growth, with an increased in cell division and proliferation of the plants tissues (Roosjen et al., 2018).

It was reported that NO is involved in water stress mitigation, through induced stomatal closure (García-Mata & Lamattina, 2001). But, neither one of the treatments showed signs of hydric stress, so water stress mitigation mediated by NO was not the responsible for the increased biomass accumulation (Fig 3.10, Tables 3.1 and 3.2). But then again NO can indirectly increase the plants IAA levels (Chen et al., 2010) and thus contributing to higher IAA levels, which in turn will lead to an increase in cellular division and consequently plant growth.

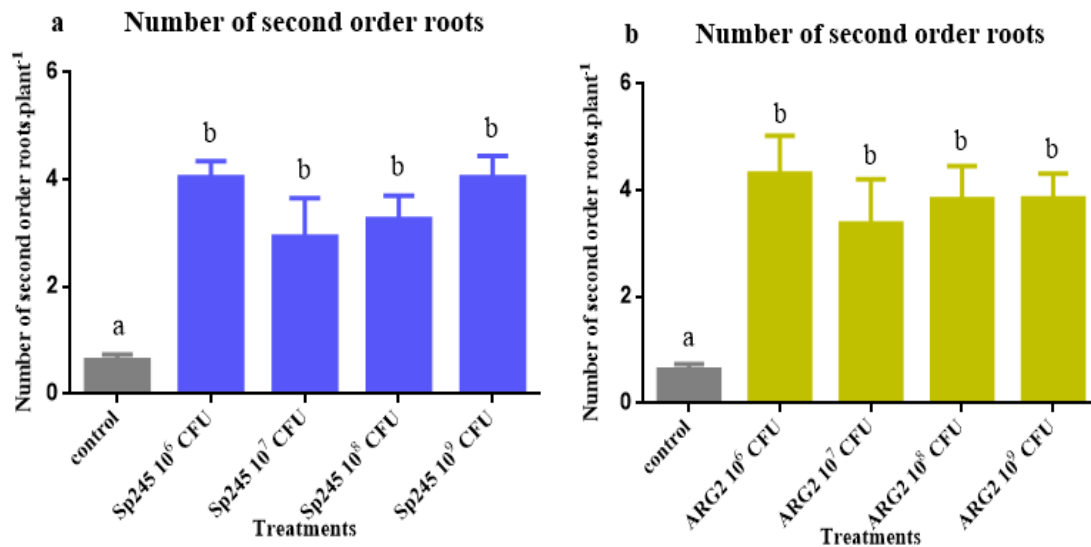
### **3.2 – Does the inoculum concentration influence the *A. brasilense* PGP effects?**

#### **3.2.1 – Effects on the root structure**

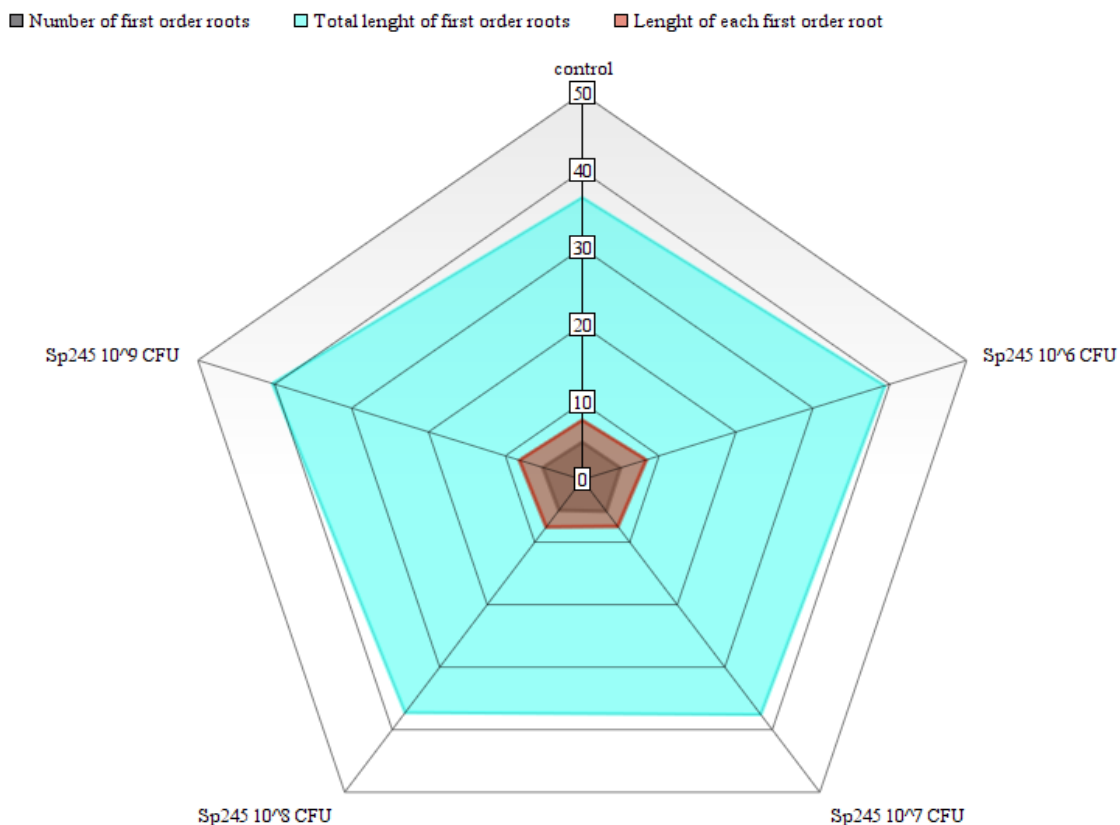
Seed inoculation with *A. brasilense* increased the number of SORs per plant relative to those of the non-inoculated seeds (Tukey's test,  $P \leq 0.05$ ), but the number of SORs was not different between the distinct inoculum concentrations ( $10^6$  -  $10^9$  CFU seed<sup>-1</sup>; Fig 3.14; ANOVA,  $P > 0.05$ ). While the number of FORs or their length (Figs 3.15 and 3.16) did not change in response to seed inoculation (ANOVA,  $P > 0.05$ ).

Since from all the compounds produced by the *A. brasilense* strains, NO and IAA are the main inducers of SOR, it is possible that: 1 - due to nutrient limitations the quantities of NO and IAA produced by the bacterial cells is the same independently of the cell concentration; 2 - only the cells in contact with the roots will produce NO and IAA, and this number is not dependent on the inoculum concentration; 3 - the distinct inoculum concentrations produce distinct amounts of NO and IAA, but the concentrations produced by the lower inoculum concentrations are above the root response threshold, and therefore no dose response relation was observed; and /or 4 - SNP and/or NAA may decrease/repress the bacterial synthesis of NO and/or IAA through a negative feedback.

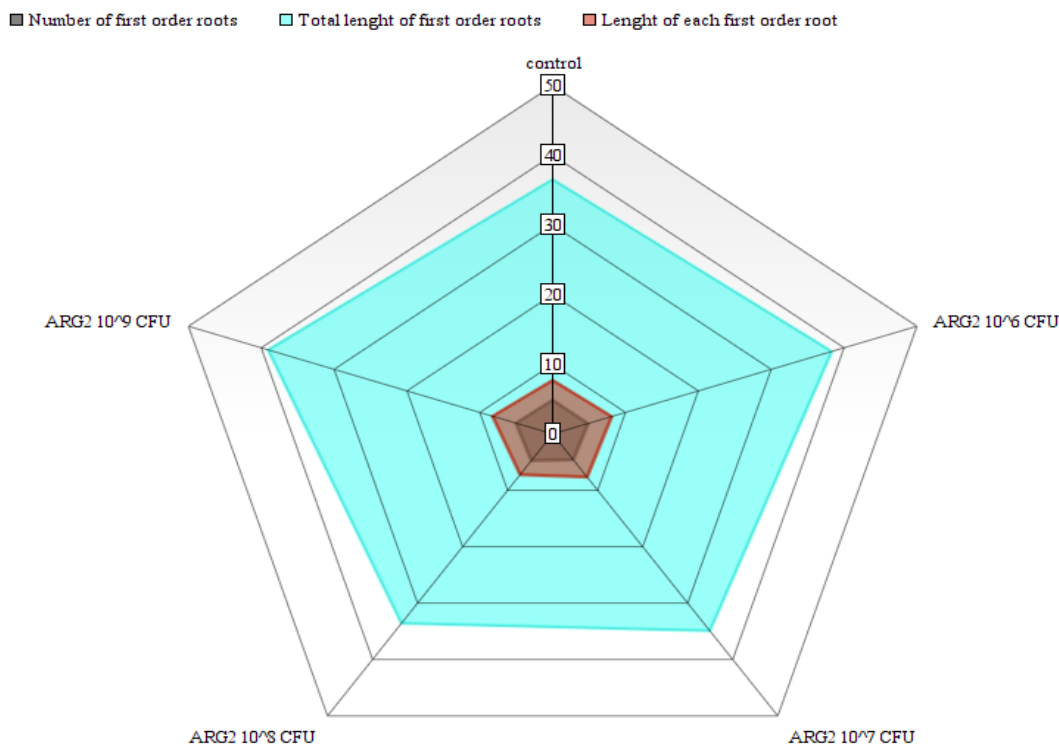
In favor of the first hypothesis it can be argued that under the experimental conditions the inoculant growth and metabolism were completely dependent on the root exudates, which may be a limiting factor for bacterial production of NO and IAA. In fact the production of high amounts of NO and IAA in *A. brasilense*, is dependent on the presence of nitrate (Molina-Favero et al., 2008) and Trp (Duca et al., 2014), respectively.



**Figure 3.14** – Effects of the inoculation with different concentrations of inoculum of *A. brasilense* Sp245 or ARG2 on the number SORs of wheat. (a) 5 different treatments: control, inoculated with *A. brasilense* Sp245 with 10<sup>6</sup> CFU seed<sup>-1</sup>, inoculated with *A. brasilense* Sp245 with 10<sup>7</sup> CFU seed<sup>-1</sup>, inoculated with *A. brasilense* Sp245 with 10<sup>8</sup> CFU seed<sup>-1</sup>, inoculated with *A. brasilense* Sp245 with 10<sup>9</sup> CFU seed<sup>-1</sup> (ANOVA, F=46.54, P≤0.001). (b) 5 different treatments: control, inoculated with *A. brasilense* ARG2 with 10<sup>6</sup> CFU seed<sup>-1</sup>, inoculated with *A. brasilense* ARG2 with 10<sup>7</sup> CFU seed<sup>-1</sup>, inoculated with *A. brasilense* ARG2 with 10<sup>8</sup> CFU seed<sup>-1</sup>, inoculated with *A. brasilense* ARG2 with 10<sup>9</sup> CFU seed<sup>-1</sup> (ANOVA, F=35.94, P≤0.001). Different small letters at the top of each bar indicated significant differences (Tukey's test, P≤0.05). Each bar represents mean of n=15 ± SE.



**Figure 3.15** – Inoculation with different concentrations of inoculum of *A. brasilense* Sp245 did not influence the FORs of wheat. 5 different treatments: control, inoculated with *A. brasilense* Sp245 with 10<sup>6</sup> CFU seed<sup>-1</sup>, inoculated with *A. brasilense* Sp245 with 10<sup>7</sup> CFU seed<sup>-1</sup>, inoculated with *A. brasilense* Sp245 with 10<sup>8</sup> CFU seed<sup>-1</sup>, inoculated with *A. brasilense* Sp245 with 10<sup>9</sup> CFU seed<sup>-1</sup>. Number of FORs (ANOVA, F=1.17, P>0.05), total length of FORs (cm. plant<sup>-1</sup>; ANOVA, F=1.19, P>0.05), length of each FOR ((cm.(root.plant)<sup>-1</sup>); ANOVA, F=0.91, P>0.05). n=15



**Figure 3.16** – Inoculation with different concentrations of inoculum of *A. brasilense* ARG2 did not influence the FORs of wheat. 5 different treatments: control, inoculated with *A. brasilense* ARG2 with  $10^6$  CFUseed<sup>-1</sup>, inoculated with *A. brasilense* ARG2 with  $10^7$  CFUseed<sup>-1</sup>, inoculated with *A. brasilense* ARG2 with  $10^8$  CFUseed<sup>-1</sup>, inoculated with *A. brasilense* ARG2 with  $10^9$  CFUseed<sup>-1</sup>. Number of FORs (ANOVA,  $F=1.72$ ,  $P>0.05$ ), total length of FORs (cm. plant<sup>-1</sup>; ANOVA,  $F=1.37$ ,  $P>0.05$ ), length of each FOR ((cm.(root.plant)<sup>-1</sup>); ANOVA,  $F=0.93$ ,  $P>0.05$ ). n=15

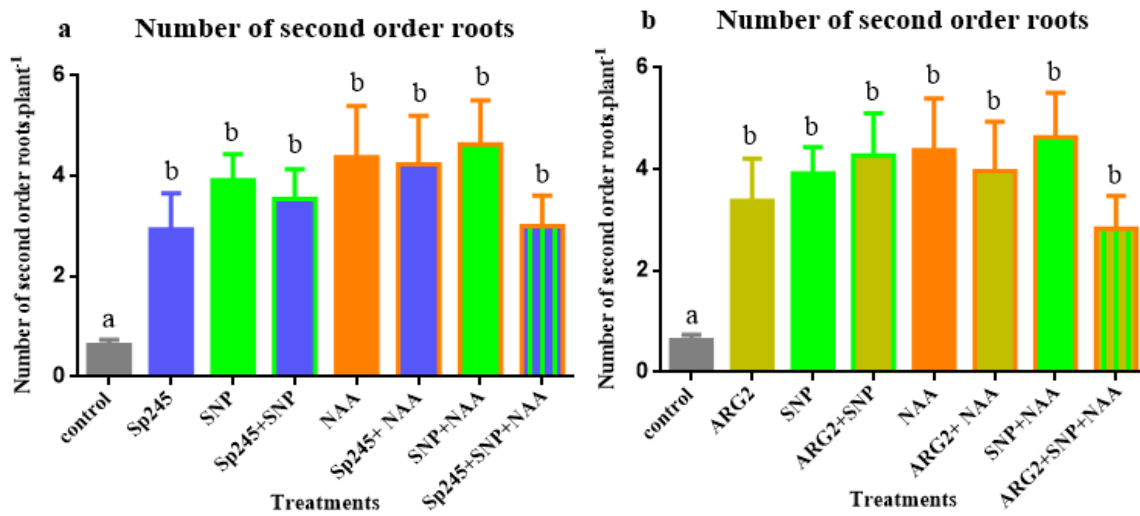
The second hypothesis, which states that only cells in contact with the root produce NO and IAA, may be supported by the need for a cross talk between bacteria and root previously to NO and IAA production and also by the chemical nature of the compounds. For instance NO has a half-life of just a few seconds (Wink & Mitchell, 1998).

In agreement with the third hypothesis, according to which there is a concentration threshold above which higher NO and IAA concentrations do not induce more SORs, are the results showing that increased concentrations of NO and IAA, obtained through the application of the NO donor (SNP, 10  $\mu$ M) and the synthetic auxin (NAA, 100 nM), either combined or apart, in both inoculated and non-inoculated seeds, did not increase the number of SORs relatively to the solo application of each compound or inoculation without supplementation (Fig 3.17; Tuckey's test,  $P>0.05$ ). With these results being corroborated by Creus et al., (2005).

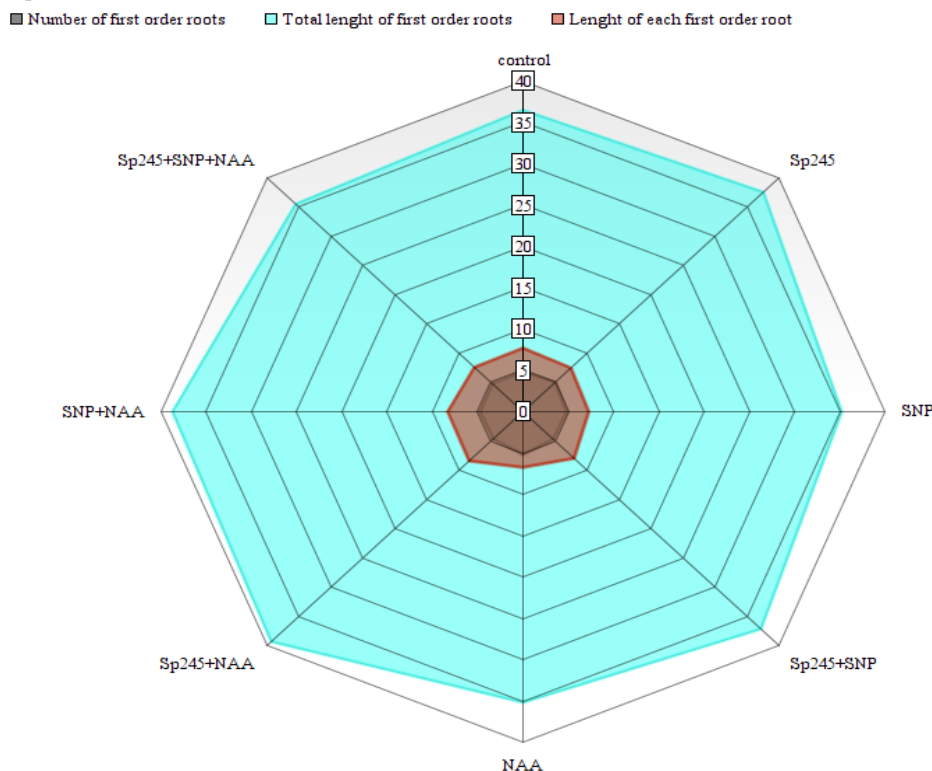
The fourth hypothesis, NO and IAA may decrease/repress the synthesis of NO and/or IAA by the bacterial strains through a negative feedback, is difficult to assess. However, this negative feedback was detected only in one case, where the addition of SNP to the culture of *A. brasilense* lead to a decrease in IAA production (Koul et al., 2015b).

On the other hand, external sources of IAA, NAA, 2,4-dichlorophenoxypropionic acid or p-chlorophenoxyacetic acid have been reported to upregulate the expression of the *A. brasilense* Sp245 *ipdC* gene (Broek et al., 1999), which encodes an indole-3-pyruvate decarboxylase (Zimmer et al., 1998) that converts IPyA to IAAld, the rate-limiting step in the IPA pathway, responsible for 90 % of the IAA synthesized in *Azospirillum* (Duca et al., 2014). The actual increase in NO production by *A. brasilense*

after addition of IAA or SNP (Koul et al., 2015b) may also be interpreted as evidence of a positive feedback.



**Figure 3.17** Application of the NO donor (SNP) and/or synthetic auxin (NAA), simultaneously with inoculation with *A. brasilense* Sp245 or ARG2 do not lead to higher effects on the number SORs of wheat relatively to those treatments applied separately. **(a)** 8 different treatments: control, inoculated with *A. brasilense* Sp245, 10  $\mu$ M of SNP, inoculated with *A. brasilense* Sp245 plus 10  $\mu$ M of SNP, 100 nM of NAA, inoculated with *A. brasilense* Sp245 plus 100 nM of NAA, 10  $\mu$ M of SNP plus 100 nM of NAA, inoculated with *A. brasilense* Sp245 plus 10  $\mu$ M of SNP and 100 nM of NAA. (ANOVA,  $F=16.7$ ,  $P\leq 0.001$ ). **(b)** 8 different treatments: control, inoculated with *A. brasilense* ARG2, 10  $\mu$ M of SNP, inoculated with *A. brasilense* ARG2 plus 10  $\mu$ M of SNP, 100 nM of NAA, inoculated with *A. brasilense* ARG2 plus 100 nM of NAA, 10  $\mu$ M of SNP plus 100 nM of NAA, inoculated with *A. brasilense* ARG2 plus 10  $\mu$ M of SNP and 100 nM of NAA. (ANOVA,  $F=15.82$ ,  $P\leq 0.001$ ). Different small letters at the top of each bar indicated significant differences (Tukey's test,  $P\leq 0.05$ ). Each bar represents mean of  $n=15 \pm SE$ .



**Figure 3.18** Application of the NO donor (SNP) and/or synthetic auxin (NAA), simultaneously with inoculation with *A. brasilense* Sp245 did not influence the FORs of wheat. 8 different treatments: control, inoculated with *A. brasilense* Sp245, 10  $\mu$ M of SNP, inoculated with *A. brasilense* Sp245 plus 10  $\mu$ M of SNP, 100 nM of NAA, inoculated with *A. brasilense* Sp245 plus 100 nM of NAA, 10  $\mu$ M of SNP plus 100 nM of NAA, inoculated with *A. brasilense* Sp245 plus 10  $\mu$ M of SNP and 100 nM of NAA. Number of FORs (ANOVA,  $F=1.4$ ,  $P>0.05$ ), total length of FORs (cm. plant<sup>-1</sup>; ANOVA,  $F=0.89$ ,  $P>0.05$ ), length of each FOR ((cm.(root.plant)<sup>-1</sup>); ANOVA,  $F=2.05$ ,  $P>0.05$ ).  $n=15$

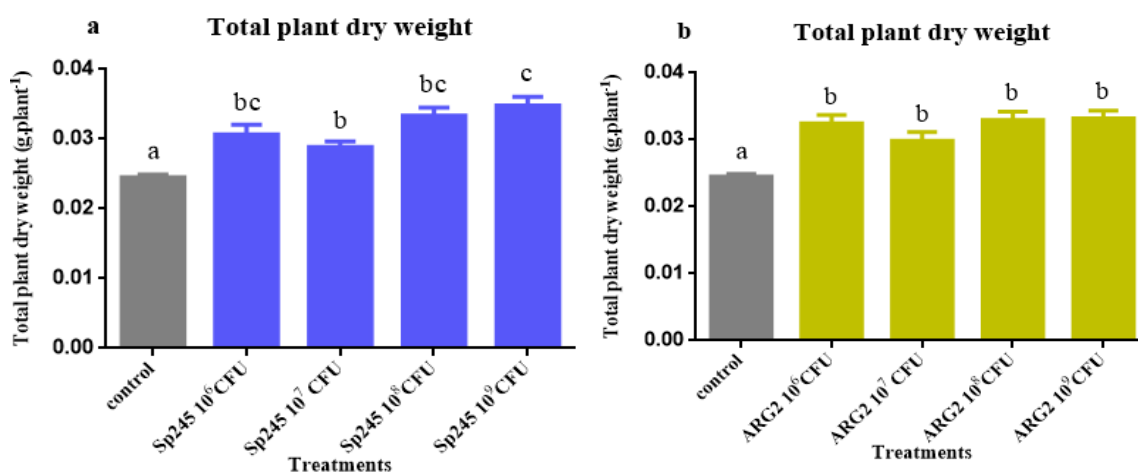


**Figure 3.19** Application of the NO donor (SNP) and/or synthetic auxin (NAA), simultaneously with inoculation with *A. brasilense* AGR2 did not influence the FORs of wheat. 8 different treatments: control, inoculated with *A. brasilense* ARG2, 10  $\mu$ M of SNP, inoculated with *A. brasilense* ARG2 plus 10  $\mu$ M of SNP, 100 nM of NAA, inoculated with *A. brasilense* ARG2 plus 100 nM of NAA, 10  $\mu$ M of SNP plus 100 nM of NAA, inoculated with *A. brasilense* ARG2 plus 10  $\mu$ M of SNP and 100 nM of NAA. Number of FORs (ANOVA,  $F=1.58$ ,  $P>0.05$ ), total length of FORs (cm. plant<sup>-1</sup>; ANOVA,  $F=0.79$ ,  $P>0.05$ ), length of each FOR ((cm.root.plant<sup>-1</sup>); ANOVA,  $F=2.01$ ,  $P>0.05$ ).  $n=15$

At this point it is not possible to disentangle the relative contribution of each of the hypotheses.

### 3.2.2 – Effects on the biomass production

Plant biomass production increased in response to inoculation (Figs 3.20; Tables 3.5 and 3.6; Tukey's test,  $P\leq 0.05$ ) without significant differences in the root to shoot ratio among treatments (ANOVA,  $P>0.05$ ).



**Figure 3.20** – Effects of the inoculation with different concentrations of inoculum of *A. brasilense* Sp245 or ARG2 on the wheat biomass. (a) 5 different treatments: control, inoculated with *A. brasilense* Sp245 with 10<sup>6</sup> CFU seed<sup>-1</sup>, inoculated with *A. brasilense* Sp245 with 10<sup>7</sup> CFU seed<sup>-1</sup>, inoculated with *A. brasilense* Sp245 with 10<sup>8</sup> CFU seed<sup>-1</sup>, inoculated with *A. brasilense* Sp245 with 10<sup>9</sup> CFU seed<sup>-1</sup>, (ANOVA,  $F=29.17$ ,  $P\leq 0.001$ ). (b) 5 different treatments: control, inoculated with *A. brasilense* ARG2 with 10<sup>6</sup> CFU seed<sup>-1</sup>, inoculated with *A. brasilense* ARG2 with 10<sup>7</sup> CFU seed<sup>-1</sup>, inoculated with *A. brasilense* ARG2 with 10<sup>8</sup> CFU seed<sup>-1</sup>, inoculated with *A. brasilense* ARG2 with 10<sup>9</sup> CFU seed<sup>-1</sup> (ANOVA,  $F=27.31$ ,  $P\leq 0.001$ ). Different small letters at the top of each bar indicated significant differences (Tukey's test,  $P\leq 0.05$ ). Each bar represents mean of  $n=15 \pm SE$ .

**Table 3.5** – Effects of the inoculation with different concentrations of inoculum of *A. brasilense* Sp245 on the wheat biomass. 5 different treatments: control, inoculated with *A. brasilense* Sp245 with  $10^6$  CFU seed<sup>-1</sup>, inoculated with *A. brasilense* Sp245 with  $10^7$  CFU seed<sup>-1</sup>, inoculated with *A. brasilense* Sp245 with  $10^8$  CFU seed<sup>-1</sup>, inoculated with *A. brasilense* Sp245 with  $10^9$  CFU seed<sup>-1</sup>. Plant shoot dry weight in g per plant (ANOVA, F=16.62, P≤0.001), plant roots dry weight in g per plant (ANOVA, F=32.32, P≤0.05), dry plant root to shoot weight ratio (ANOVA, F=1.27, P>0.05) Different small letters in the front of each value indicated significant differences (Tukey's test, P≤0.05). Each value represents mean of n=15 ±SE.

Treatment	Shoot dry weight (g.plant <sup>-1</sup> )	Roots dry weight (g.plant <sup>-1</sup> )	Root to shoot ratio
Control	0.014 ± 0.0003 <sup>a</sup>	0.011 ± 0.0002 <sup>a</sup>	0.80 ± 0.018
Sp245 10 <sup>6</sup> CFU	0.017 ± 0.0010 <sup>b</sup>	0.014 ± 0.0006 <sup>bc</sup>	0.85 ± 0.039
Sp245 10 <sup>7</sup> CFU	0.016 ± 0.0006 <sup>b</sup>	0.012 ± 0.0003 <sup>b</sup>	0.77 ± 0.020
Sp245 10 <sup>8</sup> CFU	0.018 ± 0.0005 <sup>b</sup>	0.016 ± 0.0006 <sup>c</sup>	0.88 ± 0.028
Sp245 10 <sup>9</sup> CFU	0.020 ± 0.0010 <sup>c</sup>	0.015 ± 0.0005 <sup>c</sup>	0.80 ± 0.032

**Table 3.6** – Effects of the inoculation with different concentrations of inoculum of *A. brasilense* ARG2 on the wheat biomass. 5 different treatments: control, inoculated with *A. brasilense* ARG2 with  $10^6$  CFU seed<sup>-1</sup>, inoculated with *A. brasilense* ARG2 with  $10^7$  CFU seed<sup>-1</sup>, inoculated with *A. brasilense* ARG2 with  $10^8$  CFU seed<sup>-1</sup>, inoculated with *A. brasilense* ARG2 with  $10^9$  CFU seed<sup>-1</sup>. Plant shoot dry weight in g per plant (ANOVA, F=13.69, P≤0.001), plant roots dry weight in g per plant (ANOVA, F=33.54, P≤0.05), dry plant root to shoot weight ratio (ANOVA, F=1.47, P>0.05) Different small letters in the front of each value indicated significant differences (Tukey's test, P≤0.05). Each value represents mean of n=15 ±SE.

Treatment	Shoot dry weight (g.plant <sup>-1</sup> )	Roots dry weight (g.plant <sup>-1</sup> )	Root to shoot ratio
Control	0.014 ± 0.0003 <sup>a</sup>	0.011 ± 0.0002 <sup>a</sup>	0.80 ± 0.018
ARG2 10 <sup>6</sup> CFU	0.018 ± 0.0010 <sup>b</sup>	0.015 ± 0.0005 <sup>b</sup>	0.87 ± 0.045
ARG2 10 <sup>7</sup> CFU	0.017 ± 0.0008 <sup>b</sup>	0.013 ± 0.0006 <sup>b</sup>	0.77 ± 0.031
ARG2 10 <sup>8</sup> CFU	0.018 ± 0.0008 <sup>b</sup>	0.015 ± 0.0006 <sup>b</sup>	0.85 ± 0.036
ARG2 10 <sup>9</sup> CFU	0.018 ± 0.0006 <sup>b</sup>	0.015 ± 0.0007 <sup>b</sup>	0.88 ± 0.026

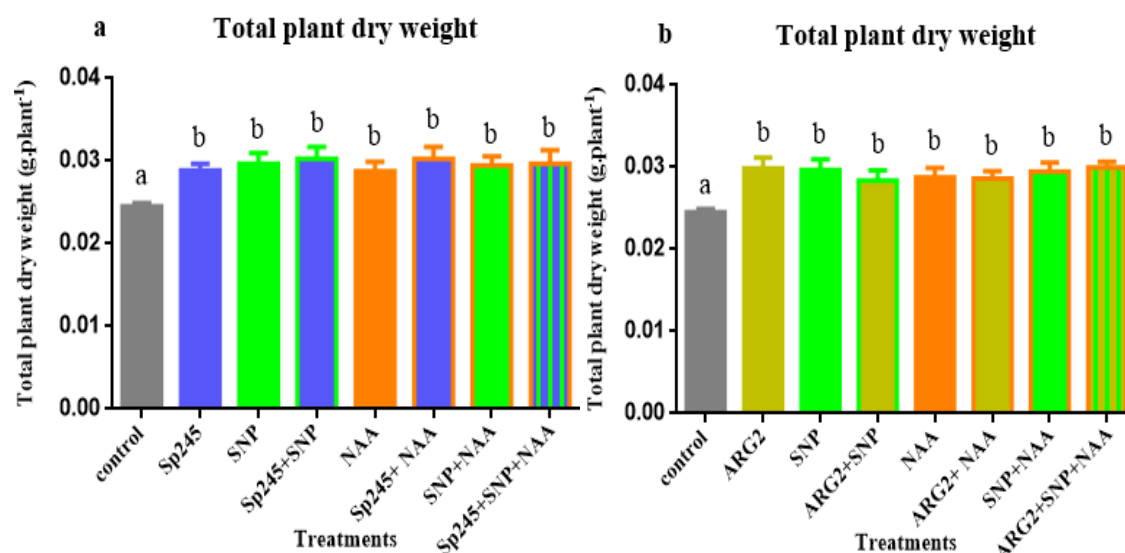
Seed inoculation with distinct concentrations of the strain Sp245 (Fig 3.20) resulted in some differences in plant biomass accumulation (Tukey's test, P≤0.05). While no differences in plant biomass accumulation were observed when seeds were inoculated with different concentrations of *A. brasilense* ARG2 (Fig 3.20, Tukey's test, P>0.05). Despite this difference, there were no significant differences in the wheat biomass production when comparing each initial inoculum concentration, between the 2 strains (2-way ANOVA, P>0.05).

In the case of the strain Sp245, seed inoculation with  $10^9$  resulted in a significantly higher plant biomass in comparison with seed inoculation with  $10^7$  CFU.seed<sup>-1</sup> (Fig 3.20; Tukey's test, P≤0.05).

Which may be due to more production of IAA and NO in the most concentrated inoculum. However, no differences were detected in plant biomass accumulation when NO and IAA levels were manipulated by addition of SNP and/or NAA to seeds inoculated with the strain Sp245 or left non-inoculated (Fig 3.21; Table 3.7; Tuckey's test, P>0.05), suggesting that increased wheat biomass accumulation was not due to higher levels of NO and IAA.

Possible explanations could be related with: 1 – a metabolite that was accumulated in the bacterial cells during its growth in liquid medium, so the higher the inoculum concentration, the higher the

concentration of that metabolite; and/or 2 – a metabolite that is only produced when the bacterial cells reach a high density. The production of that hypothetical metabolite would be regulated through a mechanism of quorum sensing (QS). This phenomenon consists in the regulation of gene expression in response to fluctuations in cell-population density (Miller & Bassler, 2001). There are evidences that QS mechanisms are involved in the PGP effects of *A. brasilense*, as it was reported that the QS mechanism of *A. brasilense* Ab-V5 are essential for the bacteria PGP effects on maize. (Fukami et al., 2018). Although, for both hypotheses, that hypothetical metabolic would not be NO or IAA.



**Figure 3.21** – Application of the NO donor (SNP) and/or synthetic auxin (NAA), simultaneously with inoculation with *A. brasilense* Sp245 or AGR2 do not lead to higher effects on the wheat biomass relatively to those treatments applied separately. **(a)** 8 different treatments: control, inoculated with *A. brasilense* Sp245, 10  $\mu$ M of SNP, inoculated with *A. brasilense* Sp245 plus 10  $\mu$ M of SNP, 100 nM of NAA, inoculated with *A. brasilense* Sp245 plus 100 nM of NAA, 10  $\mu$ M of SNP plus 100 nM of NAA, inoculated with *A. brasilense* Sp245 plus 10  $\mu$ M of SNP and 100 nM of NAA. (ANOVA,  $F=9.17$ ,  $P\leq 0.001$ ). **(b)** 8 different treatments: control, inoculated with *A. brasilense* ARG2, 10  $\mu$ M of SNP, inoculated with *A. brasilense* ARG2 plus 10  $\mu$ M of SNP, 100 nM of NAA, inoculated with *A. brasilense* ARG2 plus 100 nM of NAA, 10  $\mu$ M of SNP plus 100 nM of NAA, inoculated with *A. brasilense* ARG2 plus 10  $\mu$ M of SNP and 100 nM of NAA. (ANOVA,  $F=9.91$ ,  $P\leq 0.001$ ). Different small letters at the top of each bar indicated significant differences (Tukey’s test,  $P\leq 0.05$ ). Each bar represents mean of  $n=15 \pm SE$ .

**Table 3.7** Application of the NO donor (SNP) and/or synthetic auxin (NAA), simultaneously with inoculation with *A. brasilense* Sp245 do not lead to higher effects on the wheat biomass relatively to those treatments applied separately. 8 different treatments: control, inoculated with *A. brasilense* Sp245, 10  $\mu$ M of SNP, inoculated with *A. brasilense* Sp245 plus 10  $\mu$ M of SNP, 100 nM of NAA, inoculated with *A. brasilense* Sp245 plus 100 nM of NAA, 10  $\mu$ M of SNP plus 100 nM of NAA, inoculated with *A. brasilense* Sp245 plus 10  $\mu$ M of SNP and 100 nM of NAA. Plant shoot dry weight in g per plant (ANOVA,  $F=6.38$ ,  $P\leq 0.001$ ), plant roots dry weight in g per plant (ANOVA,  $F=11.58$ ,  $P\leq 0.05$ ), dry plant root to shoot weight ratio (ANOVA,  $F=0.5$ ,  $P>0.05$ ) Different small letters in the front of each value indicated significant differences (Tukey’s test,  $P\leq 0.05$  Each value represents mean of  $n=15 \pm SE$ ).

Treatment	Shoot dry weight (g.plant <sup>-1</sup> )	Roots dry weight (g.plant <sup>-1</sup> )	Root to shoot ratio
control	0.014 $\pm$ 0.0003 <sup>a</sup>	0.011 $\pm$ 0.0002 <sup>a</sup>	0.80 $\pm$ 0.018
Sp245	0.016 $\pm$ 0.0006 <sup>b</sup>	0.012 $\pm$ 0.0003 <sup>b</sup>	0.77 $\pm$ 0.020
SNP	0.017 $\pm$ 0.0009 <sup>b</sup>	0.013 $\pm$ 0.0004 <sup>b</sup>	0.73 $\pm$ 0.024
Sp245+SNP	0.017 $\pm$ 0.0010 <sup>ab</sup>	0.014 $\pm$ 0.0006 <sup>ab</sup>	0.82 $\pm$ 0.042
NAA	0.016 $\pm$ 0.0005 <sup>b</sup>	0.013 $\pm$ 0.0005 <sup>b</sup>	0.80 $\pm$ 0.024
Sp245+ NAA	0.017 $\pm$ 0.0007 <sup>b</sup>	0.014 $\pm$ 0.0006 <sup>b</sup>	0.77 $\pm$ 0.036
SNP+NAA	0.017 $\pm$ 0.0009 <sup>b</sup>	0.013 $\pm$ 0.0004 <sup>b</sup>	0.80 $\pm$ 0.024
Sp245+SNP+NAA	0.017 $\pm$ 0.0011 <sup>b</sup>	0.014 $\pm$ 0.0006 <sup>b</sup>	0.87 $\pm$ 0.036

For the strain *A. brasilense* ARG2, there were no differences in the wheat biomass production between the treatments with the different inoculum concentrations (Fig 3.20). A possible explanation relates with a maximum phytohormonal stimulation able to increase photosynthetic activity. By manipulating the levels of IAA and NO, through the addition of SNP and/or NAA to seeds inoculated with *A. brasilense* ARG2 or non-inoculated seeds (Fig 3.21; Table 3.8), no differences in the biomass accumulation of the plants which developed from those seeds were found (Tuckey's test,  $P>0.05$ ). Demonstrating that higher levels of NO and/or auxins did not lead to higher increases in the biomass accumulation.

**Table 3.8** Application of the NO donor (SNP) and/or synthetic auxin (NAA), simultaneously with inoculation with *A. brasilense* ARG2 do not lead to higher effects on the wheat biomass relatively to those treatments applied separately. 8 different treatments: control, inoculated with *A. brasilense* ARG2, 10  $\mu$ M of SNP, inoculated with *A. brasilense* ARG2 plus 10  $\mu$ M of SNP, 100 nM of NAA, inoculated with *A. brasilense* ARG2 plus 100 nM of NAA, 10  $\mu$ M of SNP plus 100 nM of NAA, inoculated with *A. brasilense* ARG2 plus 10  $\mu$ M of SNP and 100 nM of NAA. Plant shoot dry weight in g per plant (ANOVA,  $F=7.76$ ,  $P\leq 0.001$ ), plant roots dry weight in g per plant (ANOVA,  $F=6.63$ ,  $P\leq 0.05$ ), dry plant root to shoot weight ratio (ANOVA,  $F=0.71$ ,  $P>0.05$ ) Different small letters in the front of each value indicated significant differences (Tukey's test,  $P\leq 0.05$  Each value represents mean of  $n=15 \pm SE$ ).

Treatment	Shoot dry weight (g.plant <sup>-1</sup> )	Roots dry weight (g.plant <sup>-1</sup> )	Root to shoot ratio
control	0.014 $\pm$ 0.0003 <sup>a</sup>	0.011 $\pm$ 0.0002 <sup>a</sup>	0.80 $\pm$ 0.018
ARG2	0.017 $\pm$ 0.0008 <sup>b</sup>	0.013 $\pm$ 0.0006 <sup>b</sup>	0.77 $\pm$ 0.031
SNP	0.017 $\pm$ 0.0009 <sup>b</sup>	0.013 $\pm$ 0.0004 <sup>b</sup>	0.73 $\pm$ 0.024
ARG2+SNP	0.016 $\pm$ 0.0008 <sup>b</sup>	0.012 $\pm$ 0.0006 <sup>ab</sup>	0.78 $\pm$ 0.046
NAA	0.016 $\pm$ 0.0005 <sup>b</sup>	0.013 $\pm$ 0.0005 <sup>b</sup>	0.80 $\pm$ 0.024
ARG2+ NAA	0.017 $\pm$ 0.0005 <sup>b</sup>	0.012 $\pm$ 0.0005 <sup>ab</sup>	0.82 $\pm$ 0.058
SNP+NAA	0.017 $\pm$ 0.0009 <sup>b</sup>	0.013 $\pm$ 0.0004 <sup>b</sup>	0.80 $\pm$ 0.024
ARG2+SNP+NAA	0.018 $\pm$ 0.0007 <sup>b</sup>	0.012 $\pm$ 0.0003 <sup>b</sup>	0.72 $\pm$ 0.036

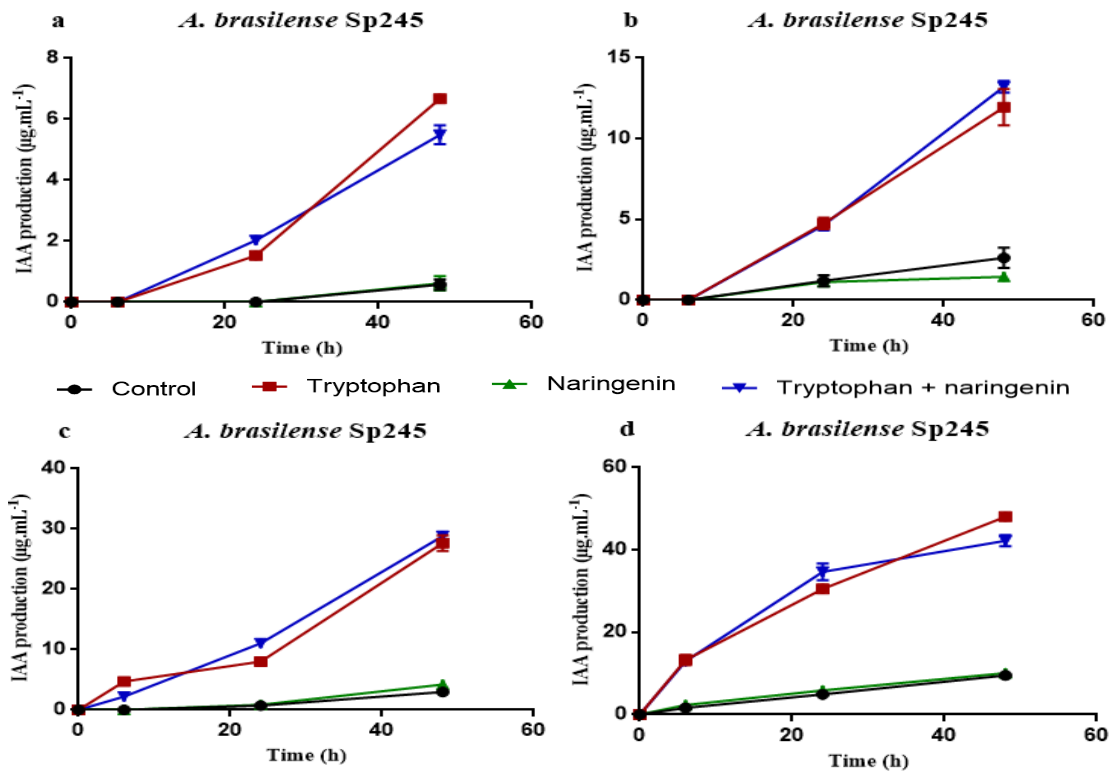
### 3.3 – Is the IAA production of *A. brasilense* influenced by the presence of Trp, the plant exudates and the inoculum concentration?

PGP effects of *A. brasilense* on wheat plants did not seem to be dependent on inoculum concentrations (Figs 3.14 and 3.20). Although it is known that IAA production is dependent on the number of bacterial cells (Zakharova et al., 1999), it may be that root exudates inhibit IAA production by bacteria. In order to analyse the effect of root exudates on IAA production by the 2 bacterial strains, naringenin was used to mimic the effects of root exudates on bacterial growth and IAA production.

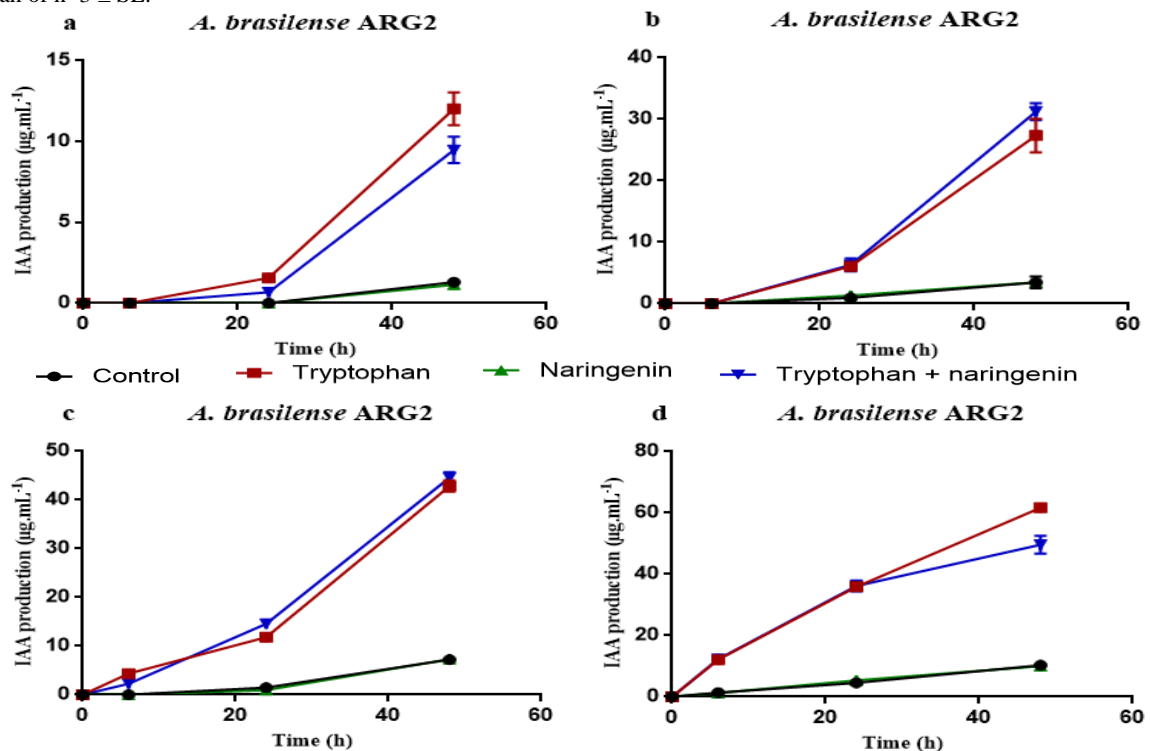
As expected, higher initial bacterial concentrations and the presence of Trp in the growth media promoted IAA production by both *A. brasilense* strains (Figs 3.22 and 3.23; Tuckey's Test,  $P\leq 0.05$ ).

A similar pattern was observed when the IAA production was expressed per bacterial cell (Figs 3.24 and 3.25), with higher initial bacterial concentrations and the presence of Trp in the growth media promoting the IAA production per cell of both *A. brasilense* strains (Tuckey's Test,  $P\leq 0.05$ ). The inoculum concentration of  $10^9$  CFU.mL<sup>-1</sup> was the exception with the IAA production per cell being the lowest.

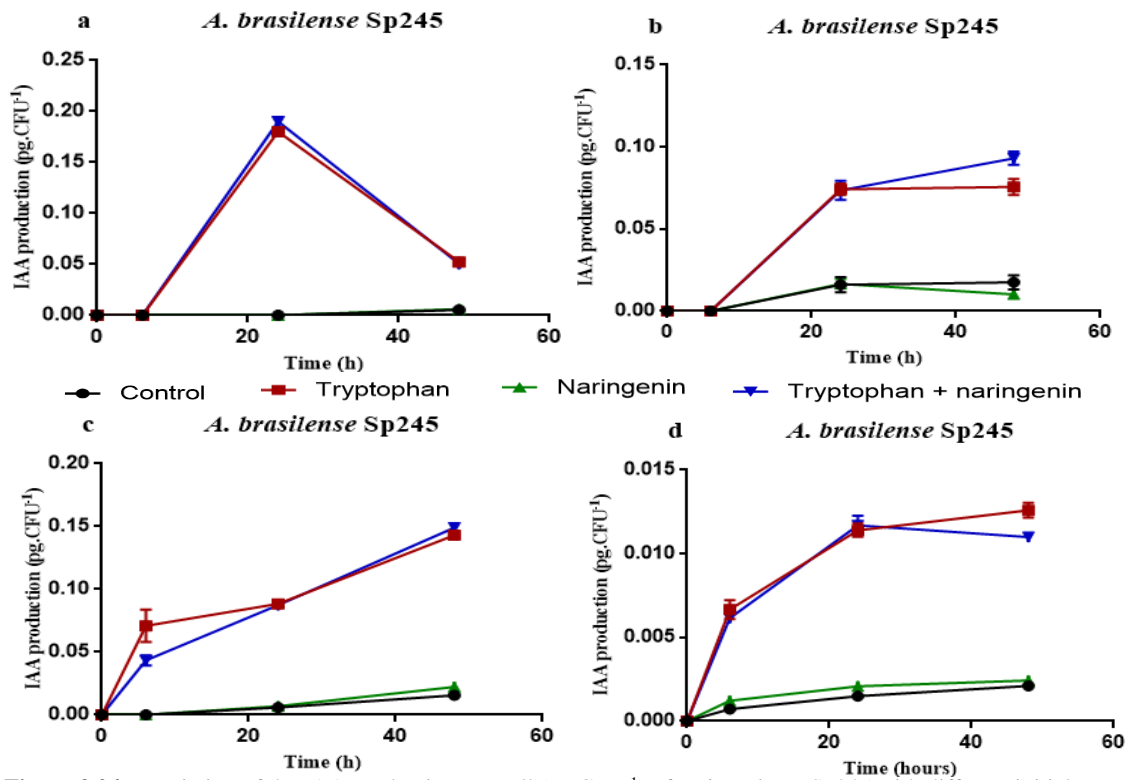
Trp may increase IAA production through 2 ways: 1 – stimulation of the expression of the *ipdC* gene, which will lead to a higher decarboxylation rate of IPyA to IAAl, which is responsible for the production of 90% of IAA in *Azospirillum* (Duca et al., 2014); and 2 – increased substrate (Trp) availability.



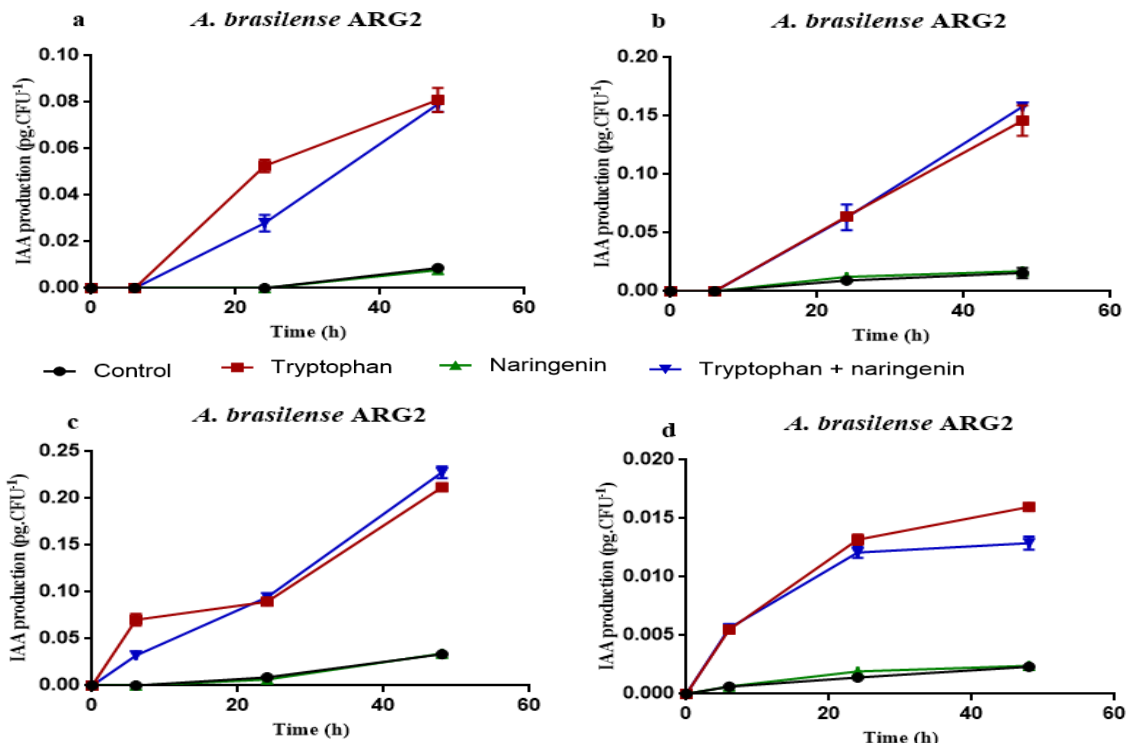
**Figure 3.22** – Variation of the IAA production kinetics of *A. brasilense* Sp245 with different initial concentrations of inoculum and the presence or absence of Trp and/or naringenin. (a)  $10^3$  CFU.mL<sup>-1</sup> (ANOVA,  $F=110.07$ ,  $P\leq 0.001$ ), (b)  $10^5$  CFU.mL<sup>-1</sup> (ANOVA,  $F=55.94$ ,  $P\leq 0.001$ ), (c)  $10^7$  CFU.mL<sup>-1</sup> (ANOVA,  $F=225.01$ ,  $P\leq 0.001$ ), (d)  $10^9$  CFU.mL<sup>-1</sup> (ANOVA,  $F=330.07$ ,  $P\leq 0.001$ ). 4 different treatments: control (black line), Trp (red line), naringenin (green line), Trp + naringenin (blue line). Different medium compositions show significant differences by ANOVA ( $P\leq 0.05$ ) Each point represents mean of  $n=3 \pm SE$ .



**Figure 3.23** – Variation of the IAA production kinetics of *A. brasilense* ARG2 with different initial concentrations of inoculum and the presence or absence of Trp and/or naringenin. (a)  $10^3$  CFU.mL<sup>-1</sup> (ANOVA,  $F=39.01$ ,  $P\leq 0.001$ ), (b)  $10^5$  CFU.mL<sup>-1</sup> (ANOVA,  $F=57.23$ ,  $P\leq 0.001$ ), (c)  $10^7$  CFU.mL<sup>-1</sup> (ANOVA,  $F=359.06$ ,  $P\leq 0.001$ ), (d)  $10^9$  CFU.mL<sup>-1</sup> (ANOVA,  $F=196.18$ ,  $P\leq 0.001$ ). 4 different treatments: control (black line), Trp (red line), naringenin (green line), Trp + naringenin (blue line). Different medium compositions show significant differences by ANOVA ( $P\leq 0.05$ ) Each point represents mean of  $n=3 \pm SE$ .



**Figure 3.24** – Variation of the IAA production per cell (pg.CFU<sup>-1</sup>) of *A. brasilense* Sp245 with different initial concentrations of inoculum and the presence or absence of Trp and/or naringenin. (a)10<sup>3</sup> CFU.mL<sup>-1</sup> (ANOVA, F=65.49, P<0.001), (b)10<sup>5</sup> CFU.mL<sup>-1</sup> (ANOVA, F=93.7, P<0.001), (c)10<sup>7</sup> CFU.mL<sup>-1</sup> (ANOVA, F=177.91, P<0.001), (d)10<sup>9</sup> CFU.mL<sup>-1</sup> (ANOVA, F=312.17, P<0.001). 4 different treatments: control (black line), Trp (red line), naringenin (green line), Trp + naringenin (blue line). Different medium compositions show significant differences by ANOVA (P<0.05) Each point represents mean of n=3 ± SE.



**Figure 3.25** – Variation of the IAA production per cell (pg.CFU<sup>-1</sup>) of *A. brasilense* ARG2 with different initial concentrations of inoculum and the presence or absence of Trp and/or naringenin. (a)10<sup>3</sup> CFU.mL<sup>-1</sup> (ANOVA, F=95.61, P<0.001), (b)10<sup>5</sup> CFU.mL<sup>-1</sup> (ANOVA, F=80.82, P<0.001), (c)10<sup>7</sup> CFU.mL<sup>-1</sup> (ANOVA, F=150.37, P<0.001), (d)10<sup>9</sup> CFU.mL<sup>-1</sup> (ANOVA, F=109.42, P<0.001). 4 different treatments: control (black line), Trp (red line), naringenin (green line), Trp + naringenin (blue line). Different medium compositions show significant differences by ANOVA (P<0.05) Each point represents mean of n=3 ± SE.

The presence of naringenin had no effect on IAA production by the bacteria in presence or absence of Trp (Tukey's Test,  $P > 0.05$ ). Which may indicate that root exudates do not influence the IAA production by the bacterial cells. However, it is also possible that the concentration of naringenin used ( $1 \mu\text{g mL}^{-1}$ ) was not adequate and therefore more experiments had to be performed.

When there were differences in the IAA production between the 2 strains (2-way ANOVA,  $P \leq 0.05$ ), it was the strain ARG2 that produce more IAA relatively to the strain Sp245 (t-test,  $P \leq 0.05$ ).

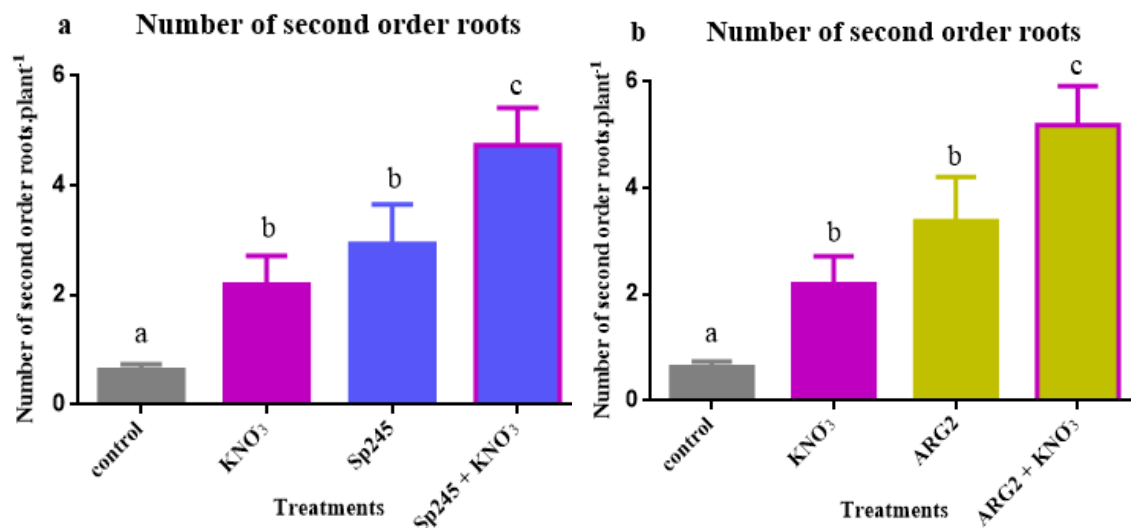
### 3.4 – Denitrification in the perspective of the NO production

Denitrification can be the major pathway for NO production by *A. brasilense* (Molina-Favero et al., 2008) and NO production is essential for the *A. brasilense*-induced PGP (Figs 3.3 and 3.10). But denitrification, from an agricultural standpoint is perceived as a negative characteristic as it reduces the amount of N available for the plants (Zimmer et al., 1984).

To try to shed some more light in this problematic, an experiment was delineated. Seeds, either non-inoculated, either inoculated, were grown in a medium supplemented with 1 mM of  $\text{KNO}_3$ . If denitrification is indeed a positive characteristic for PGPR it is expected that the PGP effects of the bacterial strains in the medium supplemented with  $\text{KNO}_3$  were higher relatively to the medium without supplementation, especially in the number of SORs. It is known that the production of considerable amounts of NO in *A. brasilense* is dependent on the presence of  $\text{NO}_3^-$  (Molina-Favero et al., 2008).

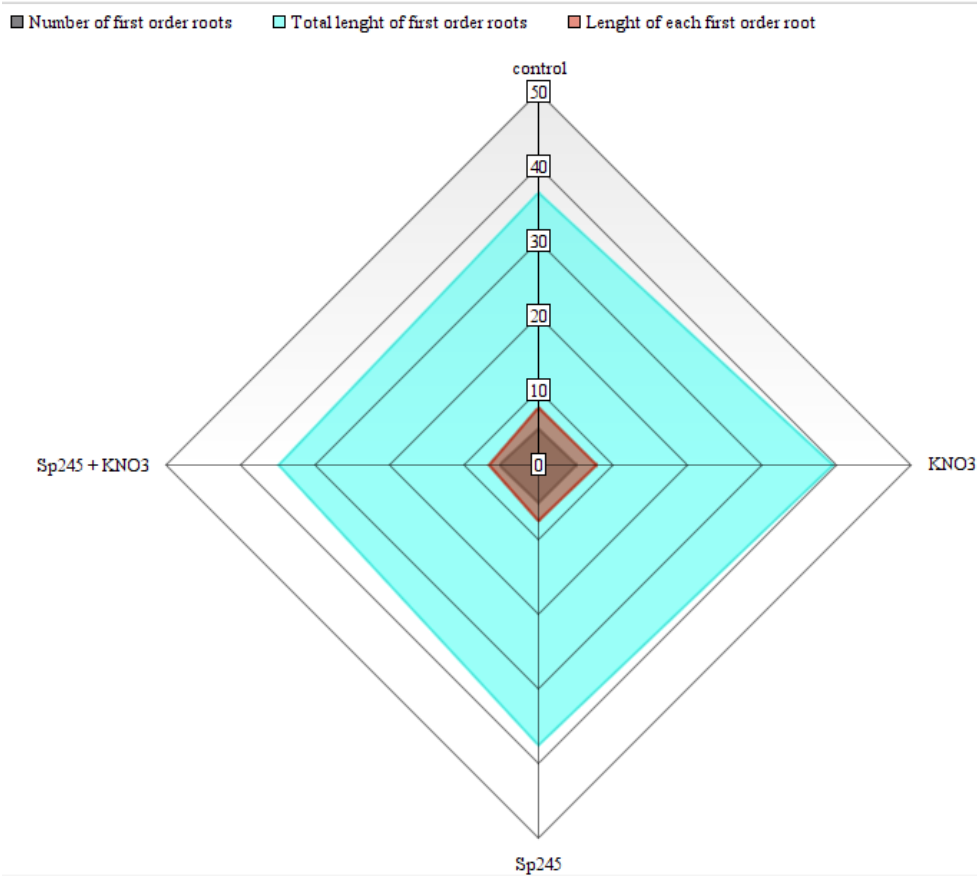
#### 3.4.1 – Effects on the root structure

The number of SORs per plant increased in the plants supplemented with  $\text{KNO}_3$  relatively to those of the control (Fig 3.26; Tukey's test,  $P \leq 0.05$ ). But the number of FORs or their length did not change in response to  $\text{KNO}_3$  (Figs 3.27 and 4.28; ANOVA,  $P > 0.05$ ).



**Figure 3.26** – In *A. brasilense* Sp245 or ARG2 the denitrification pathway leads to the production NO, which will lead to a higher increase in the number of SORs of wheat. (a) 4 different treatments: control, 1 mM of  $\text{KNO}_3$ , inoculated with *A. brasilense* Sp245, inoculated with *A. brasilense* Sp245 plus 1 mM of  $\text{KNO}_3$  (ANOVA,  $F=31.79$ ,  $P \leq 0.001$ ), (b) 4 different treatments: control, 1 mM of  $\text{KNO}_3$ , inoculated with *A. brasilense* ARG2, inoculated with *A. brasilense* ARG2 plus 1 mM of  $\text{KNO}_3$  (ANOVA,  $F=39.91$ ,  $P \leq 0.001$ ). Different small letters at the top of each bar indicated significant differences (Tukey's test,  $P \leq 0.05$ ). Each bar represents mean of  $n=15 \pm \text{SE}$ .

In plants inoculated with *A. brasilense* and supplemented with  $\text{KNO}_3$  (Figs 3.27 and 3.28), FORs number or length did not differ from the control (ANOVA,  $P>0.05$ ). But the number of SORs per plant was higher relatively to those of the control, the non-inoculated treatment supplemented with  $\text{KNO}_3$  and the inoculated treatments with no medium supplementation (Fig 3.26; Tukey's test,  $P\leq 0.05$ ). These results show that inoculation with *A. brasilense* in presence of  $\text{KNO}_3$  leads to a higher increase in the number of SORs relatively to the inoculation alone



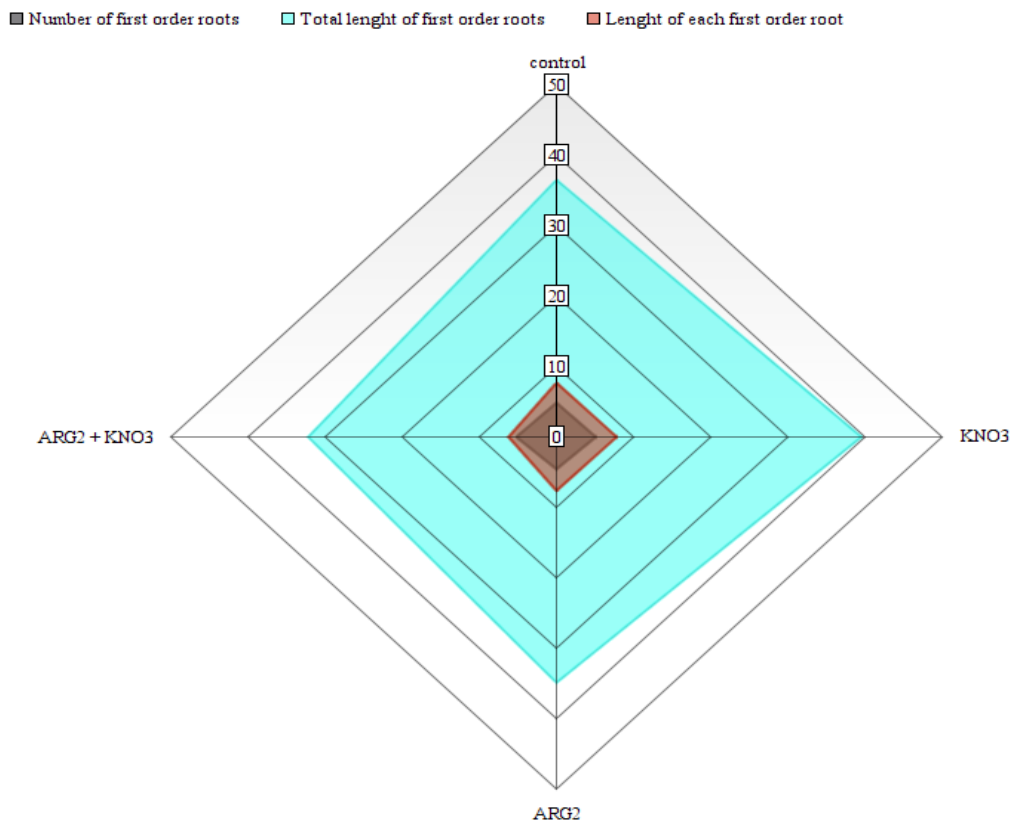
**Figure 3.27** – Although in *A. brasilense* Sp245 the denitrification pathway leads to the production  $\text{NO}$ , it did not influence the FORs of wheat. 4 different treatments: control, 1 mM of  $\text{KNO}_3$ , inoculated with *A. brasilense* Sp245, inoculated with *A. brasilense* Sp245 plus 1 mM of  $\text{KNO}_3$ . Number of FORs (ANOVA,  $F=3.26$ ,  $P>0.05$ ), total length of FORs (cm. plant<sup>-1</sup>; ANOVA,  $F=1.43$ ,  $P>0.05$ ), length of each FOR ((cm.(root.plant)<sup>-1</sup>); ANOVA,  $F=2.41$ ,  $P>0.05$ ).  $n=15$

No differences were found between the 2 strains in response to the supplementation with  $\text{KNO}_3$  (2-way ANOVA,  $P>0.05$ ).

SORs stimulation by  $\text{NO}_3^-$  is known (Molina-Favero et al., 2008, Sun et al., 2017). And the effect is due to  $\text{NO}_3^-$ , itself, since the addition of cPTIO (a  $\text{NO}$  scavenger) did not prevent the formation of SORs induced by  $\text{NO}_3^-$  (Molina-Favero et al., 2008).

The increased number of SORs in plants inoculated with *A. brasilense* and grown in presence of  $\text{KNO}_3$  were observed in tomato under similar experimental conditions (Molina-Favero et al., 2008). Molina-Favero et al. (2008) showed that when seeds were inoculated with *A. brasilense* Sp245 or *A. brasilense* Faj164 (Sp245  $\text{Nap}^-$  mutant) without  $\text{NO}_3^-$  there were no differences in the number of SORs formed, but when  $\text{NO}_3^-$  was present the plants which seeds were inoculated with the wild type strain had more SORs relatively to those inoculated with the mutant strain. In *A. brasilense* Sp245 the *Nap* gene encodes a nitrate reductase which is responsible for the reduction of  $\text{NO}_3^-$  to  $\text{NO}_2^-$  during aerobic denitrification

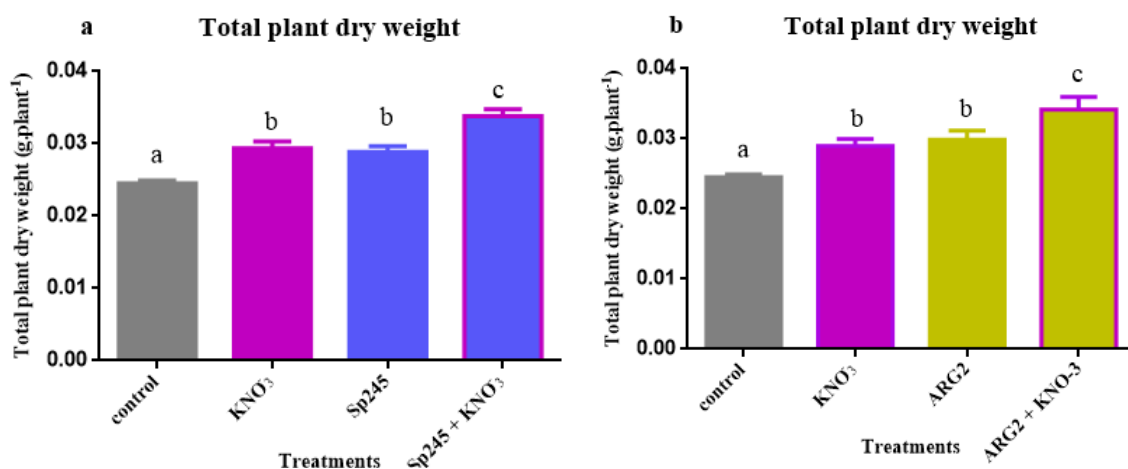
(Molina-Favero et al., 2008). This highlights denitrification as an essential feature of the PGP effects of *A. brasilense*.



**Figure 3.28** – Although in *A. brasilense* ARG2 the denitrification pathway leads to the production NO, it did not influence the FORs of wheat. 4 different treatments: control, 1 mM of KNO<sub>3</sub>, inoculated with *A. brasilense* ARG2, inoculated with *A. brasilense* ARG2 plus 1 mM of KNO<sub>3</sub>. Number of FORs (ANOVA, F=3.26, P>0.05), total length of FORs (cm.plant<sup>-1</sup>; ANOVA, F=1.43, P>0.05), length of each FOR ((cm.(root.plant<sup>-1</sup>); ANOVA, F=2.41, P>0.05). n=15

### 3.4.2 – Effects on the biomass production

Plant biomass increased in plants supplemented with KNO<sub>3</sub> or inoculated with *A. brasilense* (Tukey's test, P<0.05), without changes in the root to shoot ratio (ANOVA, P>0.05). But the biomass increment was higher when plants were simultaneously inoculated and supplemented with KNO<sub>3</sub> (Fig 3.29; Tables 3.9 and 3.10; Tukey's test, P<0.05).



**Figure 3.29** – Inoculation with *A. brasilense* Sp245 or ARG2 and supplementation with KNO<sub>3</sub> leads to higher increases in the wheat biomass production. (a) 4 different treatments: control, 1 mM of KNO<sub>3</sub>, inoculated with *A. brasilense* Sp245, inoculated with *A. brasilense* Sp245 plus 1 mM of KNO<sub>3</sub> (ANOVA, F=21.19, P<0.001), (b) 4 different treatments: control, 1 mM of KNO<sub>3</sub>, inoculated with *A. brasilense* ARG2, inoculated with *A. brasilense* ARG2 plus 1 mM of KNO<sub>3</sub> (ANOVA, F=22.81, P<0.001). Different small letters at the top of each bar indicated significant differences (Tukey's test, P<0.05). Each bar represents mean of n=15 ± SE.

**Table 3.9** – Inoculation with *A. brasilense* Sp245 and supplementation with KNO<sub>3</sub> leads to higher increases in the wheat biomass production. (a) 4 different treatments: control, 1 mM of KNO<sub>3</sub>, inoculated with *A. brasilense* Sp245, inoculated with *A. brasilense* Sp245 plus 1 mM of KNO<sub>3</sub>. Plant shoot dry weight in g per plant (ANOVA, F=21.19, P<0.001), plant roots dry weight in g per plant (ANOVA, F=23.45, P<0.05), dry plant root to shoot weight ratio (ANOVA, F=0.17, P>0.05) Different small letters in the front of each value indicated significant differences (Tukey's test, P<0.05 Each value represents mean of n=15 ±SE.

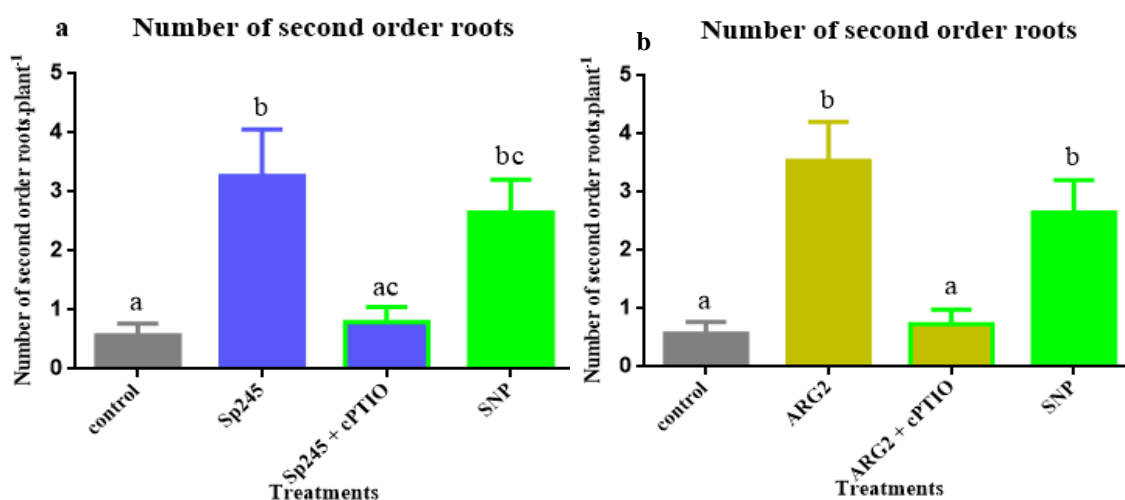
Treatment	Shoot dry weight (g.plant <sup>-1</sup> )	Roots dry weight (g.plant <sup>-1</sup> )	Root to shoot ratio
Control	0.014 ± 0.0003 <sup>a</sup>	0.011 ± 0.0002 <sup>a</sup>	0.80 ± 0.018
KNO <sub>3</sub>	0.016 ± 0.0006 <sup>b</sup>	0.013 ± 0.0004 <sup>b</sup>	0.79 ± 0.023
Sp245	0.016 ± 0.0006 <sup>b</sup>	0.012 ± 0.0003 <sup>b</sup>	0.77 ± 0.020
Sp245 + KNO <sub>3</sub>	0.019 ± 0.0006 <sup>c</sup>	0.015 ± 0.0005 <sup>c</sup>	0.77 ± 0.020

**Table 3.10** – Inoculation with *A. brasilense* ARG2 and supplementation with KNO<sub>3</sub> leads to higher increases in the wheat biomass production. (a) 4 different treatments: control, 1 mM of KNO<sub>3</sub>, inoculated with *A. brasilense* ARG2, inoculated with *A. brasilense* ARG2 plus 1 mM of KNO<sub>3</sub>. Plant shoot dry weight in g per plant (ANOVA, F=18.11, P<0.001), plant roots dry weight in g per plant (ANOVA, F=18.92, P<0.05), dry plant root to shoot weight ratio (ANOVA, F=0.24, P>0.05) Different small letters in the front of each value indicated significant differences (Tukey's test, P<0.05 Each value represents mean of n=15 ±SE.

Treatment	Shoot dry weight (g.plant <sup>-1</sup> )	Roots dry weight (g.plant <sup>-1</sup> )	Root to shoot ratio
Control	0.014 ± 0.0003 <sup>a</sup>	0.011 ± 0.0002 <sup>a</sup>	0.80 ± 0.018
KNO <sub>3</sub>	0.016 ± 0.0006 <sup>b</sup>	0.013 ± 0.0004 <sup>b</sup>	0.79 ± 0.023
ARG2	0.017 ± 0.0008 <sup>b</sup>	0.013 ± 0.0006 <sup>b</sup>	0.77 ± 0.031
ARG2 + KNO <sub>3</sub>	0.020 ± 0.0010 <sup>c</sup>	0.015 ± 0.0009 <sup>c</sup>	0.76 ± 0.030

### 3.5 – Does the *A. brasilense* PGP effects vary with the wheat variety?

One of the problems most reported for biofertilizers is the effect of bacterial strains and plant varieties in the outputs of the interaction (Malhotra & Srivastava, 2009). It is generally accepted that *A. brasilense* bacteria have big PGP effects, but are these effects dependent on the plant varieties? We compared the

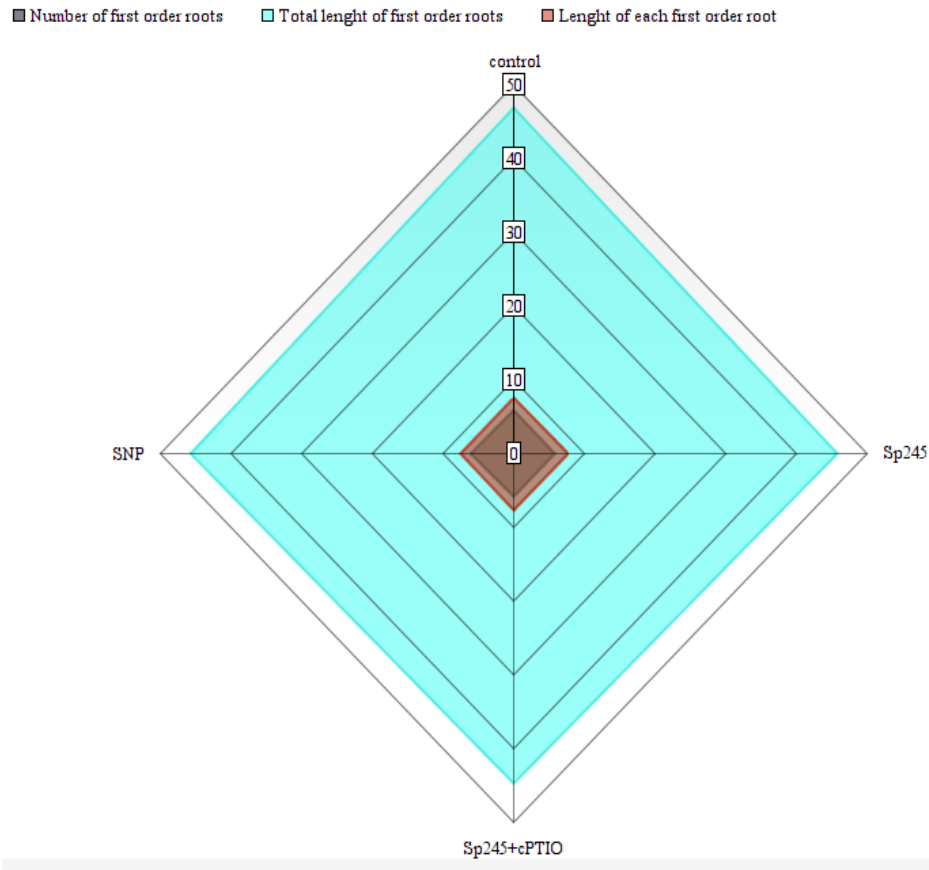


**Figure 3.30** – Effects of the inoculation with *A. brasilense* Sp245 or ARG2 on number of SORs of Preto Amarelo wheat are due to the production of NO by the bacterium. (a) 4 different treatments: control, inoculated with *A. brasilense* Sp245, inoculated with *A. brasilense* Sp245 plus 10 μM of cPTIO, 10 μM of SNP (ANOVA, F=6.52, P<0.001). (b) 4 different treatments: control, inoculated with *A. brasilense* ARG2, inoculated with *A. brasilense* ARG2 plus 10 μM of cPTIO, 10 μM of SNP (ANOVA, F=9.45, P<0.001). Different small letters at the top of each bar indicated significant differences (Tukey's test, P<0.05). Each bar represents mean of n=15 ± SE.

response of 2 wheat varieties (Marialva and Preto Amarelo) to 2 strains of *A. brasilense* (Sp245 and ARG2).

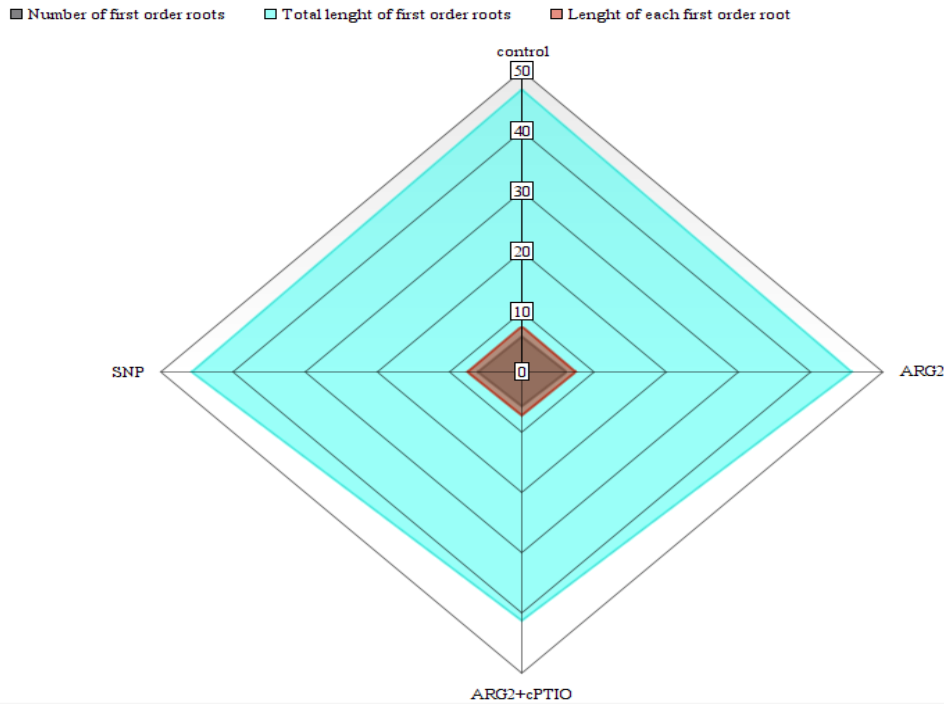
### 3.5.1 – Effects on the root structure

Inoculation of the Preto Amarelo wheat with *A. brasilense* also lead to an increased number of SORs (Fig 3.30; Tuckey’s Test,  $P \leq 0.05$ ), without influencing the number or length of the FORs (Figs 3.31 and 3.32; ANOVA,  $P > 0.05$ ).

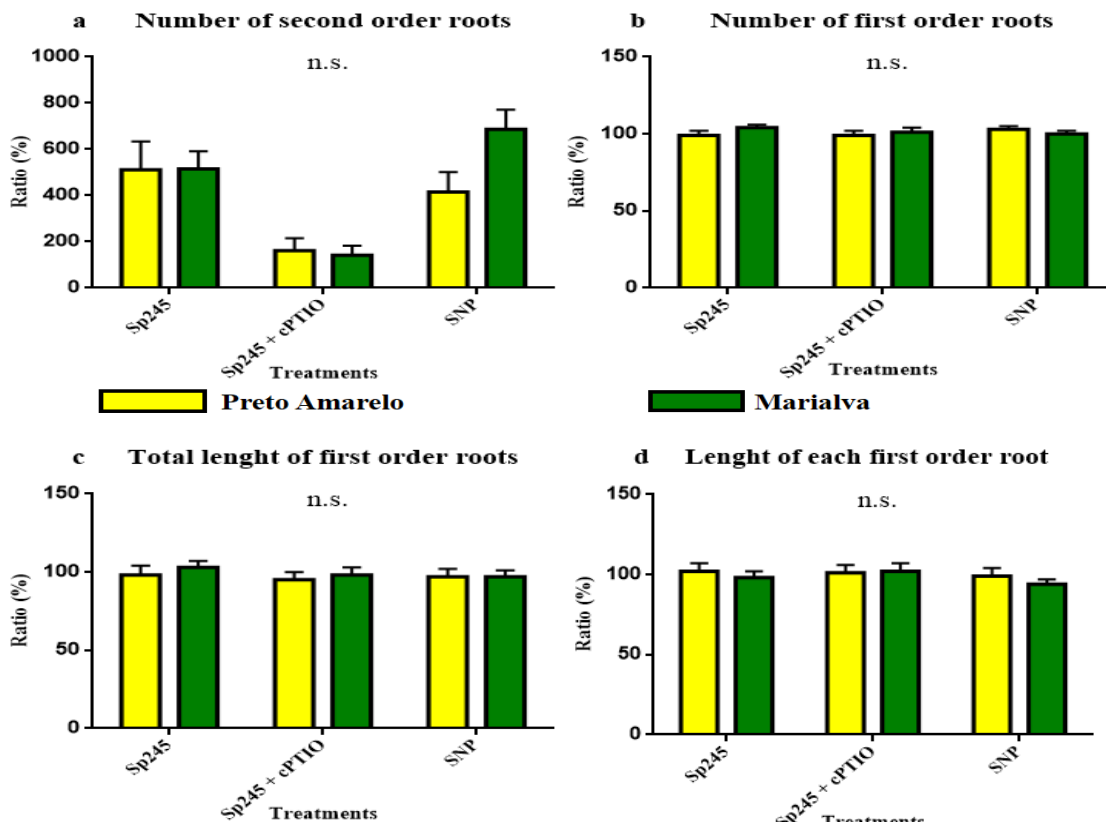


**Figure 3.31** – Inoculation with *A. brasilense* Sp245 did not influence the FORs of the Preto Amarelo wheat. 4 different treatments: control, inoculated with *A. brasilense* Sp245, inoculated with *A. brasilense* Sp245 plus 10  $\mu\text{M}$  of cPTIO, 10  $\mu\text{M}$  of SNP. Number of FORs (ANOVA,  $F=0.49$ ,  $P>0.05$ ), total length of FORs ( $\text{cm. plant}^{-1}$ ; ANOVA,  $F=0.13$ ,  $P>0.05$ ), length of each FOR ( $(\text{cm.}(\text{root.plant})^{-1})$ ; ANOVA,  $F=0.05$ ,  $P>0.05$ ).  $n=15$

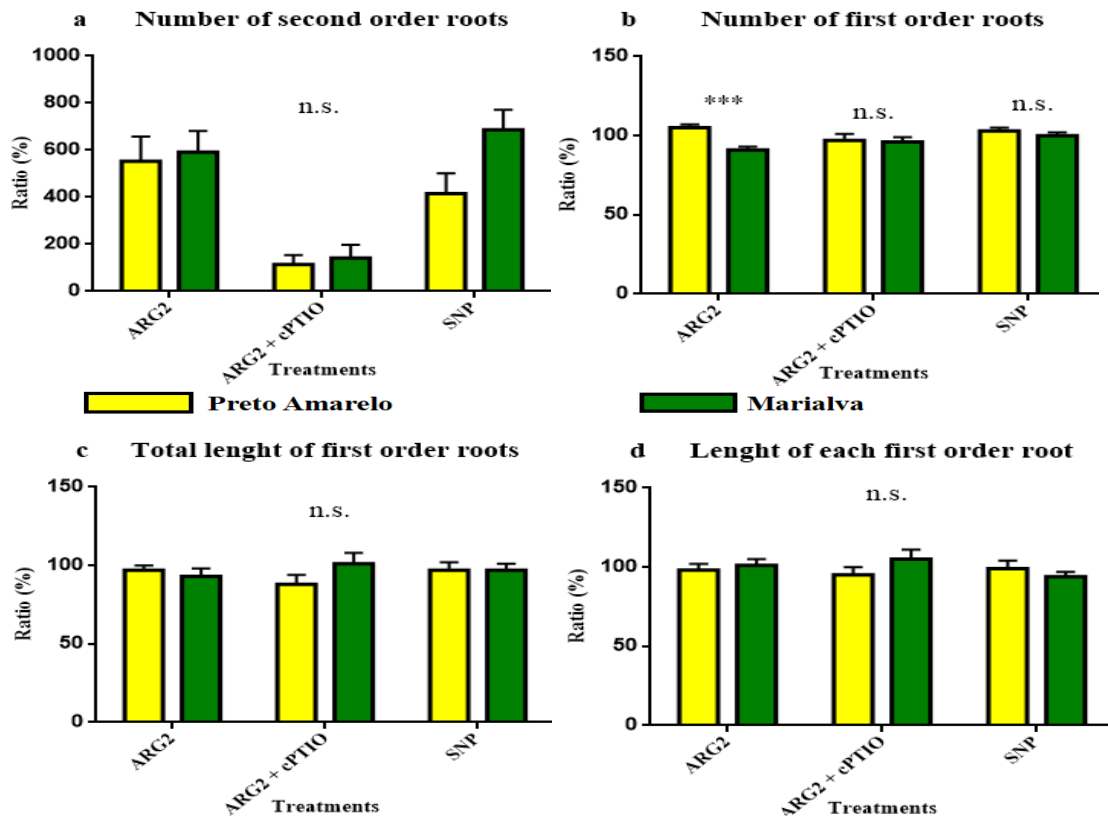
Although very different, no differences were found in the number or length of the FORs and SORs of the wheat varieties in response to inoculation with *A. brasilense* (Figs 3.33 and 3.34; 2-way ANOVA,  $P>0.05$ ). Results also reinforced the importance of NO in the interaction between the plant and *A. brasilense*, since: 1 – the presence of the NO scavenger (cPTIO) completely reverted the effect of inoculation (with Sp245 or ARG2); and 2 - the addition of the NO donor (SNP) stimulated the number of SORs.



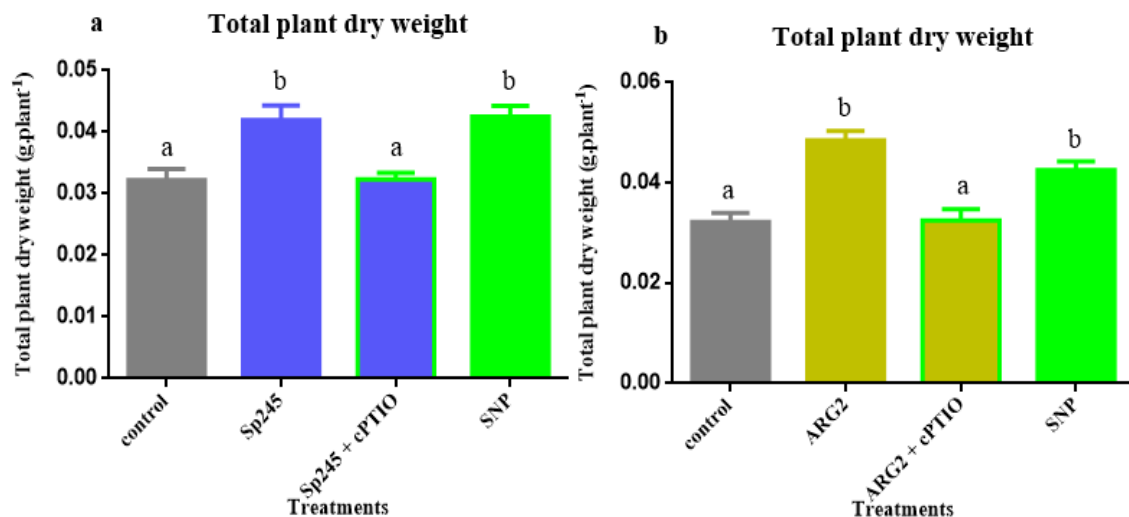
**Figure 3.32** – Inoculation with *A. brasilense* ARG2 did not influence the FORs of the Preto Amarelo wheat. 4 different treatments: control, inoculated with *A. brasilense* ARG2, inoculated with *A. brasilense* ARG2 plus 10  $\mu$ M of cPTIO, 10  $\mu$ M of SNP. Number of FORs (ANOVA,  $F=0.49$ ,  $P>0.05$ ), total length of FORs (cm. plant<sup>-1</sup>; ANOVA,  $F=0.13$ ,  $P>0.05$ ), length of each FOR ((cm.(root.plant)<sup>-1</sup>); ANOVA,  $F=0.05$ ,  $P>0.05$ ). n=15



**Figure 3.33** – Comparison of the effects of the inoculation with *A. brasilense* Sp245 and the application of NO on the roots structure, between the 2 wheat varieties. Preto Amarelo (yellow bars) and Marialva (green bars). For each variety, each treatment was relativized relatively to the control treatment. 4 different treatments: control, inoculated with *A. brasilense* Sp245, inoculated with *A. brasilense* Sp245 plus 10  $\mu$ M of cPTIO, 10  $\mu$ M of SNP. (a) number of SORs (2-way ANOVA,  $F=1.52$ ), (b) number of FORs (2-way ANOVA,  $F=0.24$ ), (c) total length of FORs (2-way ANOVA,  $F=0.3$ ), (d) mean length of FORs (2-way ANOVA,  $F=0.51$ ). n.s. treatments show no significant differences by 2-way ANOVA ( $P>0.05$ ). Each bar represents mean of  $n=15 \pm SE$ .



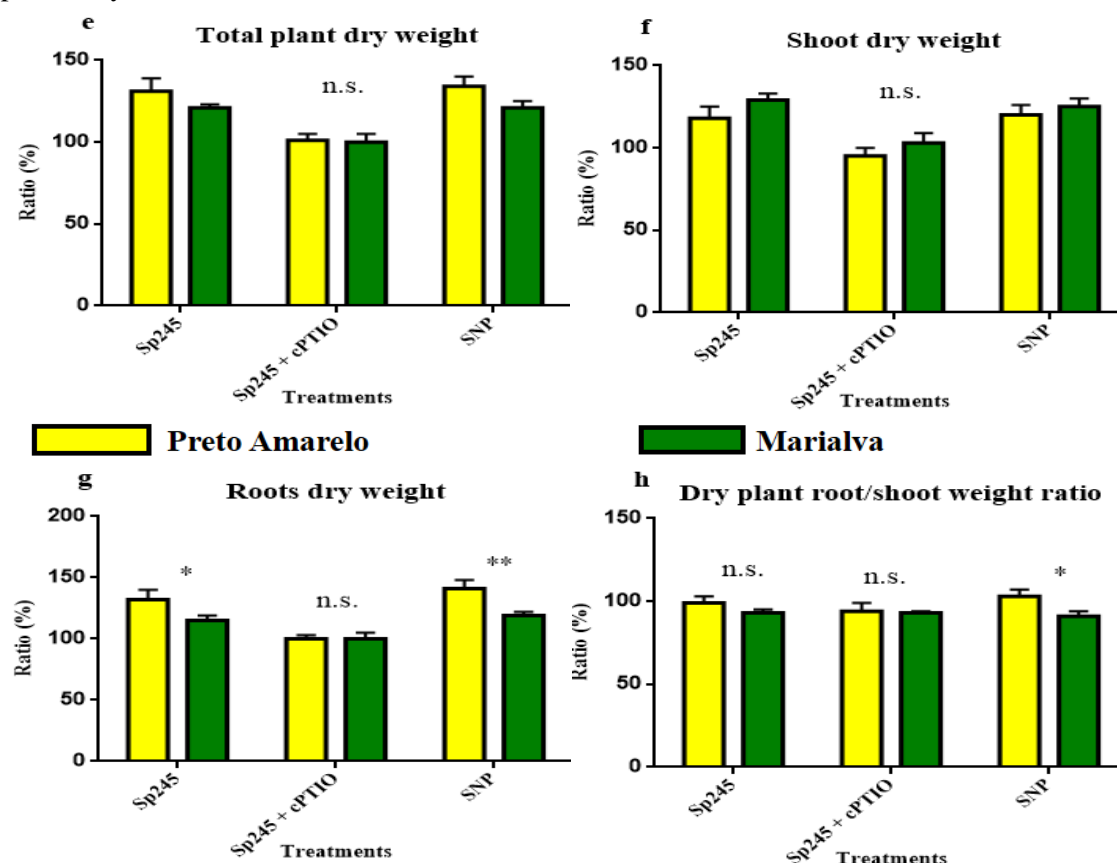
**Figure 3.34** – Comparison of the effects of the inoculation with *A. brasilense* ARG2 and the application of NO on the roots structure, between the 2 wheat varieties. Preto Amarelo (yellow bars) and Marialva (green bars). For each variety, each treatment was relativized relatively to the control treatment. 4 different treatments: control, inoculated with *A. brasilense* ARG2, inoculated with *A. brasilense* Sp245 plus 10  $\mu$ M of cPTIO, 10  $\mu$ M of SNP. (a) number of SORs (2-way ANOVA,  $F=2.78$ ), (b) number of FORs (2-way ANOVA,  $F=6.53$ ,  $P\leq 0.05$ ), (c) total length of FORs (2-way ANOVA,  $F=0.36$ ), (d) mean length of FORs (2-way ANOVA,  $F=0.42$ ). n.s. treatments show no significant differences by 2-way ANOVA ( $P>0.05$ ). n.s. means are not significantly by Student's t test at  $P\leq 0.05$ . \*\*\* Means are statistically different by Student's t test at  $P\leq 0.001$ . Each bar represents mean of  $n=15\pm SE$ .



**Figure 3.35** – Effects of the inoculation with *A. brasilense* Sp245 or ARG2 on Preto Amarelo wheat biomass accumulation are due to the production of NO by the bacterium. (a) 4 different treatments: control, inoculated with *A. brasilense* Sp245, inoculated with *A. brasilense* Sp245 plus 10  $\mu$ M of cPTIO, 10  $\mu$ M of SNP (ANOVA,  $F=9.3$ ,  $P\leq 0.001$ ). (b) 4 different treatments: control, inoculated with *A. brasilense* ARG2, inoculated with *A. brasilense* ARG2 plus 10  $\mu$ M of cPTIO, 10  $\mu$ M of SNP (ANOVA,  $F=16.3$ ,  $P\leq 0.001$ ). Different small letters at the top of each bar indicated significant differences (Tukey's test,  $P\leq 0.05$ ). Each bar represents mean of  $n=15\pm SE$ .

### 3.5.2 – Effects on the biomass production

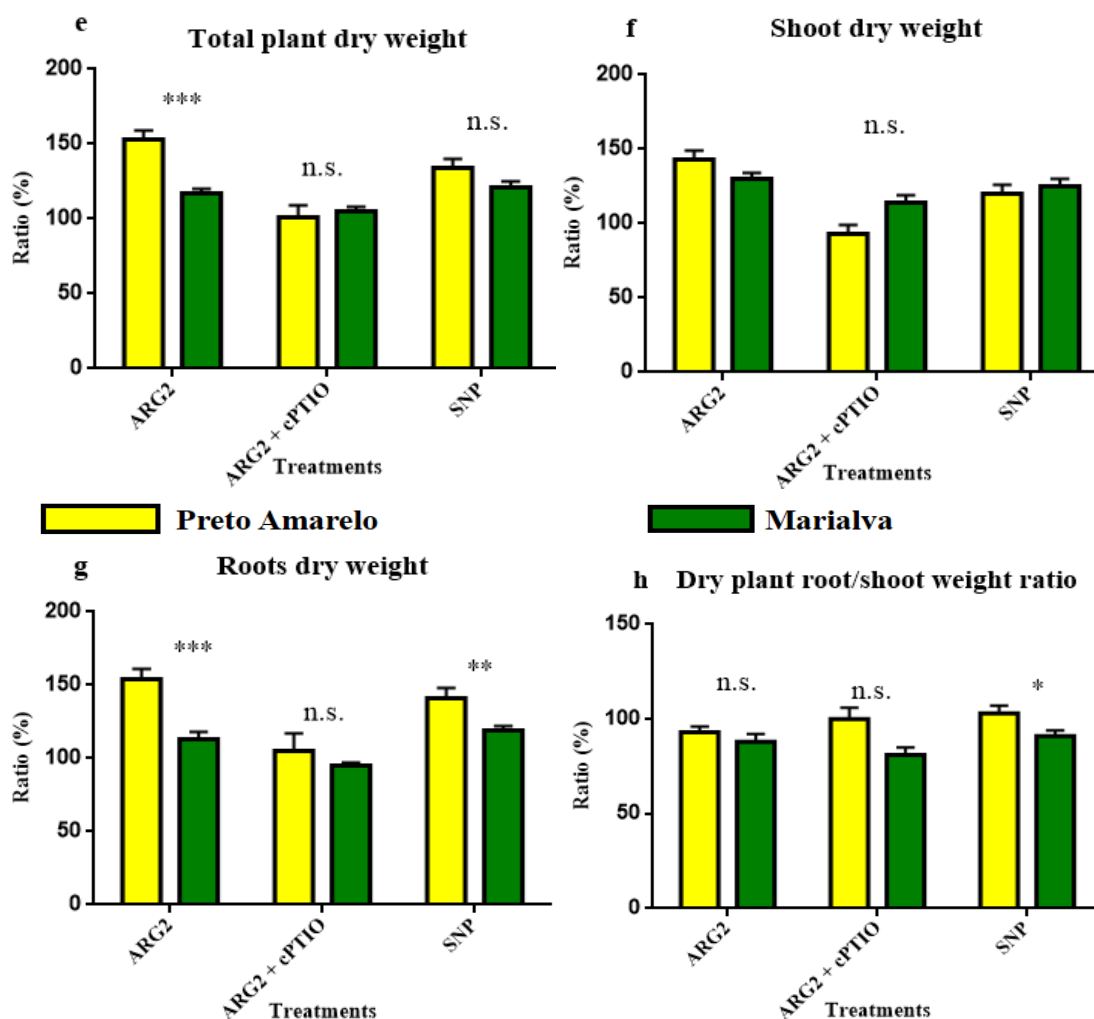
The inoculation of each bacterial strain lead to an increase in plant biomass (Fig 3.35; Table 3.11 and 3.12; Tuckey's Test,  $P \leq 0.05$ ) in the Preto Amarelo variety, that was higher in the root level in the variety Preto Amarelo relatively to those of the Marialva (Figs 3.36 and 3.37). With special attention to the interaction of the strain ARG2 with the Preto Amarelo variety which biomass accumulation was higher in comparison to those of the Marialva variety (t-test,  $P \leq 0.05$ ) in the plants which seeds had been previously inoculated with ARG2 strain.



**Figure 3.36** – Comparison of the effects of the inoculation with *A. brasilense* Sp245 and the application of NO on the biomass production, between the 2 wheat varieties. Preto Amarelo (yellow bars) and Marialva (green bars). For each variety, each treatment was relativized relatively to the control treatment. 4 different treatments: control, inoculated with *A. brasilense* Sp245, inoculated with *A. brasilense* Sp245 plus 10  $\mu\text{M}$  of cPTIO, 10  $\mu\text{M}$  of SNP. (e) total plant dry weight in g per plant (2-way ANOVA,  $F=3.16$ ) (f) plant shoot dry weight in g per plant (2-way ANOVA,  $F=2.49$ ), (g) plant roots dry weight in g per plant (2-way ANOVA,  $F=8.64$ ,  $P \leq 0.001$ ), (h) dry plant root to shoot weight ratio (2-way ANOVA,  $F=4.13$ ,  $P \leq 0.05$ ). n.s. treatments show no significant differences by 2-way ANOVA ( $P > 0.05$ ). n.s. means are not significantly by Student's t test at  $P \leq 0.05$ . \* Means are statistically different by Student's t test at  $P \leq 0.05$ . \*\* Means are statistically different by Student's t test at  $P \leq 0.01$ . Each bar represents mean of  $n=15 \pm \text{SE}$ .

**Table 3.11** – Effects of the inoculation with *A. brasilense* Sp245 on Preto Amarelo wheat biomass accumulation are due to the production of NO by the bacterium. 4 different treatments: control, inoculated with *A. brasilense* Sp245, inoculated with *A. brasilense* Sp245 plus 10  $\mu\text{M}$  of cPTIO, 10  $\mu\text{M}$  of SNP. Plant shoot dry weight in g per plant, (ANOVA,  $F=3.98$ ,  $P \leq 0.05$ ), plant roots dry weight in g per plant (ANOVA,  $F=14.39$ ,  $P \leq 0.001$ ), dry plant root to shoot weight ratio (ANOVA,  $F=0.41$ ,  $P > 0.05$ ). Different small letters in the front of each value indicated significant differences (Tukey's test,  $P \leq 0.05$ ). Each value represents mean of  $n=15 \pm \text{SE}$ .

Treatment	Shoot dry weight (g.plant <sup>-1</sup> )	Roots dry weight (g.plant <sup>-1</sup> )	Root to shoot ratio
Control	0.022 $\pm$ 0.0014 <sup>a</sup>	0.012 $\pm$ 0.0007 <sup>a</sup>	0.60 $\pm$ 0.068
Sp245	0.026 $\pm$ 0.0014 <sup>b</sup>	0.015 $\pm$ 0.0008 <sup>b</sup>	0.60 $\pm$ 0.023
Sp245 + cPTIO	0.021 $\pm$ 0.0012 <sup>a</sup>	0.012 $\pm$ 0.0013 <sup>a</sup>	0.57 $\pm$ 0.029
SNP	0.026 $\pm$ 0.0013 <sup>b</sup>	0.016 $\pm$ 0.0007 <sup>b</sup>	0.64 $\pm$ 0.026



**Figure 3.37** – Comparison of the effects of the inoculation with *A. brasilense* ARG2 and the application of NO on the biomass production, between the 2 wheat varieties. Preto Amarelo (yellow bars) and Marialva (green bars). For each variety, each treatment was relativized relatively to the control treatment. 4 different treatments: control, inoculated with *A. brasilense* ARG2, inoculated with *A. brasilense* ARG2 plus 10  $\mu$ M of cPTIO, 10  $\mu$ M of SNP. (e) total plant dry weight in g per plant (2-way ANOVA,  $F=11.16$ ,  $P\leq 0.01$ ), (f) plant shoot dry weight in g per plant (2-way ANOVA,  $F=0.74$ ), (g) plant roots dry weight in g per plant (2-way ANOVA,  $F=17.87$ ,  $P\leq 0.001$ ), ((h) dry plant roots/shoot weight ratio (2-way ANOVA,  $F=10.78$ ,  $P\leq 0.01$ ). n.s. treatments show no significant differences by 2-way ANOVA ( $P>0.05$ ). n.s. means are not significantly by Student's t test at  $P\leq 0.05$ . \*\*\* Means are statistically different by Student's t test at  $P\leq 0.001$ . Each bar represents mean of  $n=15 \pm SE$ .

**Table 3.12** – Effects of the inoculation with *A. brasilense* ARG2 on Preto Amarelo wheat biomass accumulation are due to the production of NO by the bacterium. 4 different treatments: control, inoculated with *A. brasilense* ARG2, inoculated with *A. brasilense* ARG2 plus 10  $\mu$ M of cPTIO, 10  $\mu$ M of SNP. Plant shoot dry weight in g per plant, (ANOVA,  $F=13.51$ ,  $P\leq 0.05$ ), plant roots dry weight in g per plant (ANOVA,  $F=10.31$ ,  $P\leq 0.001$ ), dry plant root to shoot weight ratio (ANOVA,  $F=0.63$ ,  $P>0.05$ ). Different small letters in the front of each value indicated significant differences (Tukey's test,  $P\leq 0.05$ ). Each value represents mean of  $n=15 \pm SE$ .

Treatment	Shoot dry weight (g.plant <sup>-1</sup> )	Roots dry weight (g.plant <sup>-1</sup> )	Root to shoot ratio
Control	0.022 $\pm$ 0.0014 <sup>ac</sup>	0.012 $\pm$ 0.0007 <sup>a</sup>	0.60 $\pm$ 0.068
ARG2	0.031 $\pm$ 0.0013 <sup>b</sup>	0.017 $\pm$ 0.0007 <sup>b</sup>	0.56 $\pm$ 0.019
ARG2 + cPTIO	0.020 $\pm$ 0.0012 <sup>a</sup>	0.012 $\pm$ 0.0013 <sup>a</sup>	0.65 $\pm$ 0.069
SNP	0.026 $\pm$ 0.0013 <sup>c</sup>	0.016 $\pm$ 0.0007 <sup>b</sup>	0.64 $\pm$ 0.026

## Chapter 4 - Integrated analyses, conclusions and future perspectives

The aim of this project was to understand the role of IAA and NO produced by *A. brasilense* in its PGP effects on wheat (*Triticum durum*). In order to pursue this objective, and due to the relevance of IAA and NO on the initiation of formation of SORs, we used the number of SORs as an indicator of the plant-bacteria interaction. Since the IAA and NO produced by *A. brasilense* did not affect the FORs structure, but were essential to increase the number of SORs, and the wheat biomass production through stimulation of plant photosynthetic activity.

The interaction between wheat and *A. brasilense* was interpreted as being positive to the plant because the maintenance of the plant FORs structure ensures that roots, keep the ability to forage water and maintain productivity under relatively dry conditions (Lilley & Kirkegaard, 2011; Uga et al., 2013). A higher number of seedling SORs, leads to an increased plant root density, and potentially to increased nutrient and water uptake, the so-called “sponge effect” (Richardson et al., 2009). Which may contribute to minimize the environmental agriculture footprint by reducing the water and fertilizers waste. This is crucial to achieve a sustainable intensification of agriculture, which is a form of production wherein yields are increased without adverse environmental impact and cultivation of more land (The Royal Society, 2009). Both wheat varieties tested, Marialva and Preto Amarelo, had their growth increased by inoculation with the *A. brasilense* strains. Since these varieties are very distinct, it suggests that the effects of *A. brasilense* are broad and not limited to some wheat genotypes. However the variety Preto Amarelo (more ancestral) tended to be more responsive to *A. brasilense* inoculation.

However, to benefit from this plant-bacteria interaction from the farming point of view it is necessary to determine the doses in which the bacteria should be used. We found no differences in the PGP effects on wheat seedlings when *A. brasilense* was applied at concentrations ranging from  $10^6$  to  $10^9$  CFU.seed<sup>-1</sup>. But it has to be considered that the wheat seeds were subjected to an intensive decontamination process that may have helped the hegemony of the inoculants. In any case it seems that  $10^6$  CFU.seed<sup>-1</sup> was enough to produce the minimal IAA and NO concentrations responsible for the maximum effect on the number of SORs.

Results show that IAA and NO are essential to mediate the PGP effects of *A. brasilense*. However, they also indicate that the plant is able to internally adjust its phytohormone balance (specially that between IAA and NO) in response to distinct IAA and NO external concentrations.

The results also seem to reinforce the recent idea (Bashan & De-Bashan, 2010) that denitrification is not a negative feature of PGPR, but indeed a positive one, since despite reducing the amount of N available for plant growth (W. Zimmer et al., 1984), it leads to the production of NO (Molina-Favero et al., 2008).

Due to the PGP effects that the bacteria has on the plant, it is obvious that the plant benefits from the interaction. But why are the bacteria using N and carbon to produce NO and IAA? We can argue that the synthesis of phytohormones, specially IAA and NO, by the bacterial strains would serve as a compensation for the plants root exudates that provide nutrients for those microbial populations (Raaijmakers et al., 2009). This would make a perfect story, but nature is not like this, not so simple, not so straight forward.

It is possible that bacteria use IAA as part of their colonization strategy by stimulating proliferation of plant tissues and thus enhancing the colonization surface and root exudation of nutrients for bacterial growth (Spaepen et al., 2007).

But IAA production among prokaryotes is not restricted to those inhabiting plant environments. It can be found among thermoacidophilic archaeon isolated from a hot spring (Wakagi et al., 2002) and even among human pathogens, as *Salmonella typhimurium* (Smith & Macfarlane, 1996), raising the question whether IAA production might play other roles.

A possible role was proposed by Patten et al., (2013), relating the catabolism of Trp with the N metabolism. The 4 major IAA biosynthetic pathways are dependent of Trp (Duca et al., 2014), with the release of an amine group. But from these processes of transamination of aromatic and branched-chain amino acids, results carbonyl intermediates that often cannot be used as carbon sources, and are toxic when accumulated in the cells (O'Brien et al., 2005). As the affinity of bacteria for Trp is high (Marlow & Kosuge, 1972), when the concentration of Trp is high, great amounts of Trp are imported and it will be used for the N metabolism, when preferred N sources are exhausted, which will produce great amounts of those carbonyl intermediates. The production of IAA, followed by exportation from the bacterial cells, may provide a mechanism to detoxify the bacterial cells from those toxic intermediates. This is congruent with the observation that the major accumulation of IAA occurs in the stationary phase of growth (Ona et al., 2005), probably when preferred N sources have been exhausted. In agreement with this, it was reported a higher IAA accumulation in cultures of *A. brasilense* SM strain, when grown in 50 % less ammonium sulphate (Malhotra & Srivastava, 2009).

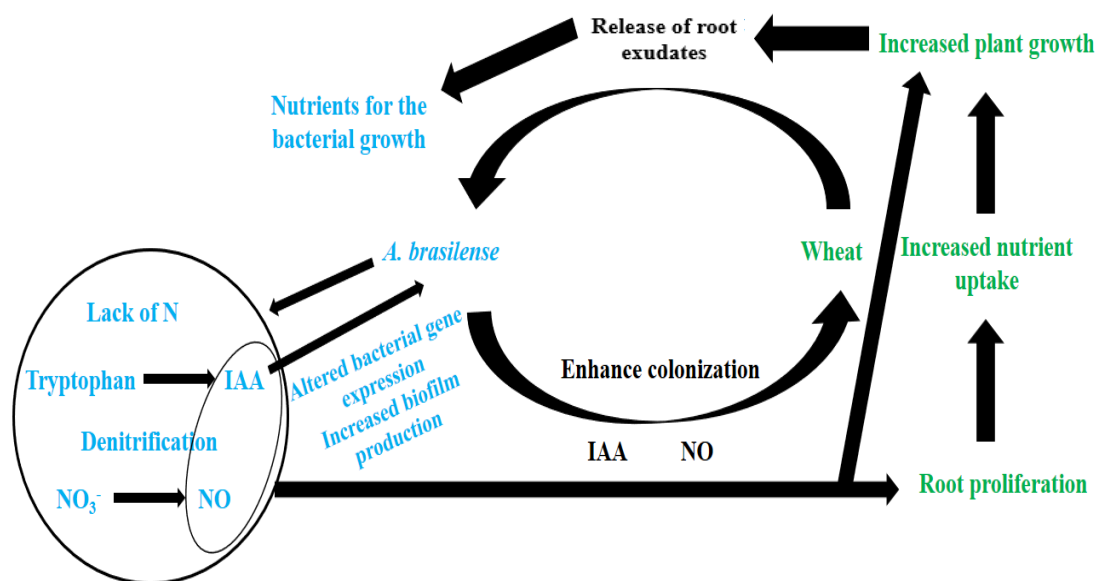
Regarding the production of NO by the bacterial strains, it was already discussed that the denitrification pathway is the major source of NO in *A. brasilense* (Molina-Favero et al., 2008). But denitrification consumes  $\text{NO}_3^-$ , which can be used as N source by the bacteria. So, to produce NO, the bacterial cells are depending on nutrients that are essential for their growth. In the plant-bacteria interactions, the production of NO could be a kind of “bargaining chip”, that the bacteria supply to the plant in exchange for root exudates. But there are reports of NO producing prokaryotes, which do not inhabit the plant environment, like *Bacillus anthracis* (Sudhamsu & Crane, 2009). This raised the same question which was placed relatively to the IAA. This is, if the pathways involved in the production of NO might have another role in bacteria, besides being involved in the plant-bacterial interactions, even in the bacteria which are involved in those interactions as is the case of *A. brasilense*.

The denitrification pathway can be active in both, anaerobic and aerobic conditions. Under low  $\text{O}_2$  levels or anaerobic conditions, it allows bacteria to generate the energy necessary for their growth (Zumft, 1997). When denitrification occurs under high  $\text{O}_2$  levels, it allows the bacterial cells to maintain an optimal redox balance by dissipation of the reducing equivalent stress, accumulated during aerobic growth (Steenhoudt et al., 2001a; Steenhoudt et al., 2001b). In agreement with this, it was reported that, the more reduced the C-source available for the bacteria is (butyrate or caproate), the higher the periplasmic nitrate reductase activity (Robertson & Kuenen, 1988), which, catalyses the reduction of  $\text{NO}_3^-$  to  $\text{NO}_2^-$  in aerobic conditions (Molina-Favero et al., 2008).

As a disclaimer, with this we are not saying that IAA and NO are not important for the bacteria-plant interactions, quite the opposite. A recent report has highlighted that IAA and NO act as signalling molecules in bacteria-plant interactions, as they modulate the gene expression of both (Koul et al., 2015a). What we are saying is that IAA and NO are not a simple “bargaining chip” that the bacteria give to the plant as “payment” for the nutrients that the plant exudates through their roots, not a simple *quid pro quo*, in the Anglo-Saxon meaning of the expression. A report has highlighted the importance of IAA as a signal molecule for the *A. brasilense* to adapt itself to the plant rhizosphere, specially by upregulating the expression of the T6SS, which is involved in bacterium-eukaryotic host interactions (Puyvelde et al., 2011). NO was also shown to influence the bacterial gene expression (Koul et al., 2015a) and to promote biofilm formation in *A. brasilense* (Arruebarrena et al., 2013). Biofilm formation

is of special importance in the plant-bacteria interactions as biofilm can increase the resistance to certain environmental stresses and antimicrobial tolerance, in the plant and associate bacteria (Danhorn & Fuqua, 2007).

It is likely that the pathways which lead to the production of IAA and NO have multiple roles, as in a single bacterium there are several different pathways for their production and the production of both phytohormones is widespread among prokaryotes, in the more diverse environments. From an evolutionary perspective, IAA might serve as “toxic waste dump” for the toxic carbonyl intermediates, which result from the catabolism of Trp for the N metabolism. While NO might be produced as a by-product of the denitrification pathways to generate energy under low O<sub>2</sub> levels or to dissipate the reducing stress, during aerobic growth. For bacteria which interact with plants, the produced IAA and NO will stimulate the proliferation of plant tissues and nutrient exudation (Spaepen et al., 2007), through the auxins mediated acid-growth, which involves acidification of the plants cells, leading to altered growth (Roosjen et al., 2018). The 2 phytohormones will also affect the bacteria, as IAA changes the arsenal of transport and cell surface proteins (Puyvelde et al., 2011), while NO will increase the formation of biofilm on the surface of the plant roots (Arruebarrena et al., 2013). It is also possible that the plant itself secretes IAA in order to induce the described changes in the bacteria cells. All these effects combined, allow a more enhanced colonization of the plants roots and bacterial growth, while also having positive effects on the host plant growth and development. A simplified schematic representation of these processes and the relation between wheat and *A. brasilense*, is outlined in the Fig 4.1. With the passage of time that relationship between the plants and the PGPR became stronger and developed in to a symbiotic mutualistic relationship. Despite not being essential, both of the partners benefit a great deal from it.



**Figure 4.1** – A extended view of the symbiotic relationship between *A. brasilense* and wheat plants. In conditions of low N availability, tryptophan is used for the N metabolism, which leads to the production of IAA. Under anaerobic conditions NO<sub>3</sub><sup>-</sup> is reduced to produce energy for the cells, while in aerobic conditions is reduced to dissipate the reducing stress. In both cases, it is through the denitrification pathways and results in the production of NO. The produced phytohormones, for one side, will change the bacterial gene expression, adapting it to the rhizosphere and increasing the production of biofilm, which enhances the colonization of the wheat roots. For the other side they will affect the wheat plant growth directly, by increasing it, while increasing the root proliferation, which increases nutrient uptake and consequently more plant growth. This root proliferation leads to an increase exudation of nutrients for the bacterial growth. Abbreviations: IAA, indole-3-acetic acid; N, nitrogen; NO, nitric oxide, NO<sub>3</sub><sup>-</sup>, nitrate.

The overall results from this work, show that the production of NO and IAA, are essential for the *A. brasilense* PGP effects on wheat. But the bacteria did not produce those phytohormones just to “help” the plant, they were produced as a result of the activity of several metabolic pathways which are activated in response to environmental, nutritional or energy stresses. One of those pathways is denitrification, which was once considered as negative for agriculture, but might actually be essential for the relation between PGPR and plants, especially for the PGP effects of the firsts in the seconds (Molina-Favero et al., 2008).

As in this work only the early stage of the vegetative growth was analysed, to strengthen these results, the realization of greenhouse and field experiments, in which the full life cycle of the plants will be analysed, is essential. And although we are fully aware that in this work we barely scratched the surface of the universe that are the interactions between plants and PGPR, we hope that this work can be one of the infinite number of steps that we, mankind, need to take towards a more sustainable tomorrow.

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## **Appendices**

### **Glossary**

Symbiotic<sup>1</sup> – intimate association and long term biological association between at least two species, it can be mutualistic, communalistic or parasitic (Saffo, 1993).

Mutualistic<sup>2</sup> – a form of symbiose in which the participant species benefits from the activity of the other (Bronstein, 1994).

Rhizosphere<sup>3</sup> – narrow zone of soil directly surrounding the root system and directly influenced by root secretions and associated soil microorganisms (Estabrook & Yoder, 1998; Harsh et al., 2001).

Biofertilizers<sup>4</sup> – general term: a product that contains living microorganisms, which exert direct or indirect beneficial effects on plant growth and crop yield through different mechanisms; more specific term: those effects are trough N<sub>2</sub> fixation and phosphorus solubilization (Malusá & Vassilev, 2014).

Phytostimulators<sup>5</sup> – the biofertilizers from the general term which enhance plant growth by producing phytohormones (Malusá & Vassilev, 2014).

Phytohormones<sup>6</sup> – organic substances that, at very low concentration (pM-nM) promote plant physiological responses (Van Overbeek, 1944).

First order roots<sup>7</sup> – are embryonic roots which arise directly from the seed embryo (Atkinson et al., 2014).

Second order roots<sup>8</sup> – are post-embryonic roots which arise and outgrowth from the existing FORs (Atkinson et al., 2014).

### **Solutions**

#### **NA medium (1 L)**

- 20 g of Nutrient Broth;
- 15 g of Bacteriological agar;
- Up to 1 L with distilled water;
- Sterilization trough autoclave during 15 min at 121 °C.

#### **NB medium (1 L)**

- 20 g of Nutrient Broth;
- Up to 1 L with distilled water;
- Sterilization by autoclaving during 15 min at 121 °C.

#### **Saline solution 0.85% (1 L)**

- 8.5 g of NaCl;
- Up to 1 L with distilled water;
- Sterilization by autoclaving during 15 min at 121 °C.

#### **Sterile distilled water (1 L)**

- Up to 1 L with distilled water;
- Sterilization by autoclaving during 15 min at 121 °C.

**SNP 1 mM (200 mL)**

- 59.5 mg of SNP;
- Up to 200 mL with distilled water;
- Sterilization through filtration.

**cPTIO 1 mM (31.7 mL)**

- 10 mg of cPTIO;
- Up to 31.7 mL with distilled water;
- Sterilization through filtration.

**NAA 1 mM (100 mL)**

- 18.6 mg of NAA;
- Dissolved in 2 mL of NaOH (1 M)
- Up to 100 mL with distilled water;
- Sterilization by co-autoclaving with the other medium components during 15 min at 121 °C.

**PCIB 1 mM (100 mL)**

- 21.465 mg of PCIB;
- Up to 100 mL with distilled water;
- Sterilization through filtration.

**KNO<sub>3</sub> 100 mM (100 mL)**

- 1.01 g of KNO<sub>3</sub>;
- Up to 100 mL with distilled water;
- Sterilization by co-autoclaving with the other medium components during 15 min at 121 °C

**Media for the petri dish experiments:**

**Plant growth medium without any supplementation (1 L)**

- 15 g of bacteriological agar;
- Up to 1 L with distilled water;
- Sterilization through autoclave during 15 min at 121 °C.

**Plant growth medium supplemented with 1 μM of SNP (1 L)**

- 15 g of bacteriological agar;
- Up to 990 mL with distilled water;
- Sterilization through autoclave during 15 min at 121 °C;
- 1 mL of a sterile SNP solution at 1 mM.

**Plant growth medium supplemented with 10  $\mu$ M of SNP (1 L)**

- 15 g of bacteriological agar;
- Up to 990 mL with distilled water;
- Sterilization trough autoclave during 15 min at 121 °C;
- 10 mL of a sterile SNP solution at 1 mM.

**Plant growth medium supplemented with 100  $\mu$ M of SNP (1 L)**

- 15 g of bacteriological agar;
- Up to 990 mL with distilled water;
- Sterilization trough autoclave during 15 min at 121 °C;
- 100 mL of a sterile SNP solution at 1 mM.

**Plant growth medium supplemented with 10  $\mu$ M of cPTIO (1 L)**

- 15 g of bacteriological agar;
- Up to 990 mL with distilled water;
- Sterilization trough autoclave during 15 min at 121 °C;
- 10 mL of a sterile cPTIO solution at 1 mM.

**Plant growth medium supplemented with 10  $\mu$ M of cPTIO plus 10  $\mu$ M of SNP (1 L)**

- 15 g of bacteriological agar;
- Up to 980 mL with distilled water;
- Sterilization trough autoclave during 15 min at 121 °C;
- 10 mL of a sterile cPTIO solution at 1 mM;
- 10 mL of a sterile SNP solution at 1 mM.

**Plant growth medium supplemented with 10 nM of NAA (1 L)**

- 15 g of bacteriological agar;
- 10  $\mu$ L of a NAA solution at 1 mM;
- Up to 1 L with distilled water;
- Sterilization trough autoclave during 15 min at 121 °C.

**Plant growth medium supplemented with 50 nM of NAA (1 L)**

- 15 g of bacteriological agar;
- 50  $\mu$ L of a sterile NAA solution at 1 mM;
- Up to 1 L with distilled water;
- Sterilization trough autoclave during 15 min at 121 °C.

**Plant growth medium supplemented with 100 nM of NAA (1 L)**

- 15 g of bacteriological agar;
- 100  $\mu$ L of a NAA solution at 1 mM;
- Up to 1 L with distilled water;
- Sterilization through autoclave during 15 min at 121 °C.

**Plant growth medium supplemented with 10  $\mu$ M of PCIB (1 L)**

- 15 g of bacteriological agar;
- Up to 990 mL with distilled water;
- Sterilization through autoclave during 15 min at 121 °C;
- 10 mL of a sterile PCIB solution at 1 mM.

**Plant growth medium supplemented with 10  $\mu$ M of PCIB plus 100 nM of NAA (1 L)**

- 15 g of bacteriological agar;
- 100  $\mu$ L of a NAA solution at 1 mM;
- Up to 990 mL with distilled water;
- Sterilization through autoclave during 15 min at 121 °C;
- 10 mL of a sterile PCIB solution at 1 mM.

**Plant growth medium supplemented with 10  $\mu$ M of PCIB plus 10  $\mu$ M of SNP (1 L)**

- 15 g of bacteriological agar;
- Up to 980 mL with distilled water;
- Sterilization through autoclave during 15 min at 121 °C;
- 10 mL of a sterile PCIB solution at 1 mM.
- 10 mL of a sterile SNP solution at 1 mM.

**Plant growth medium supplemented with 10  $\mu$ M of cPTIO plus 100 nM of NAA (1 L)**

- 15 g of bacteriological agar;
- 100  $\mu$ L of a NAA solution at 1 mM;
- Up to 990 mL with distilled water;
- Sterilization through autoclave during 15 min at 121 °C;
- 10 mL of a sterile cPTIO solution at 1 mM.

**Plant growth medium supplemented with 10  $\mu$ M of SNP plus 100 nM of NAA (1 L)**

- 15 g of bacteriological agar;
- 100  $\mu$ L of a NAA solution at 1 mM;
- Up to 990 mL with distilled water;
- Sterilization through autoclave during 15 min at 121 °C;
- 10 mL of a sterile SNP solution at 1 mM.

**Plant growth medium supplemented with 1 mM of KNO<sub>3</sub> (1 L)**

- 15 g of bacteriological agar;
- 10 mL of a KNO<sub>3</sub> solution at 100 mM;
- Up to 1 L with distilled water;
- Sterilization through autoclave during 15 min at 121 °C.

**Media for the bacterial cells viability**

**NB medium without any supplementation (70 mL)**

- 70 mL of sterile NB medium.

**NB medium supplemented with 10 μM of cPTIO (70 mL)**

- 0.7 mL of a sterile cPTIO solution at 1 mM;
- Up to 70 mL of sterile NB medium.

**NB medium supplemented with 10 μM of PCIB (70 mL)**

- 0.7 mL of a sterile PCIB solution at 1 mM;
- Up to 70 mL of sterile NB medium.

**NB medium supplemented with 10 μM of cPTIO plus 10 μM of PCIB (70 mL)**

- 0.7 mL of a sterile cPTIO solution at 1 mM;
- 0.7 mL of a sterile PCIB solution at 1 mM;
- Up to 70 mL of sterile NB medium.

**Media and solutions for the determination of the IAA production**

**IAA 1000 μg mL<sup>-1</sup> (10 mL)**

- 10 mg of IAA;
- Up to 10 mL of acetone.

**L-tryptophan 10 mg mL<sup>-1</sup> (200 mL)**

- 2 g of L-tryptophan
- Up to 200 mL of distilled water;
- Sterilization through filtration.

**Naringenin 200 μg mL<sup>-1</sup> (10 mL)**

- 2 mg of naringenin;
- Up to 10 mL of distilled water;
- Sterilization through filtration.

**NB medium without any supplementation (377.9 mL)**

- 79.85 mL of distilled sterile water;
- Up to 377.9 mL of sterile NB medium.

**NB medium supplemented with 2 mg mL<sup>-1</sup> of L-tryptophan (377.9 mL)**

- 1.95 mL of distilled sterile water;
- 77.9 mL of a sterile tryptophan solution at 10 mg mL<sup>-1</sup> mM;
- Up to 377.9 mL of sterile NB medium.

**NB medium supplemented with 1 μM mL<sup>-1</sup> of naringenin (377.9 mL)**

- 77.9 mL of distilled sterile water;
- 1.95 mL of a sterile naringenin solution at 200 μg mL<sup>-1</sup>;
- Up to 377.9 mL of sterile NB medium.

**NB medium supplemented with 2 mg mL<sup>-1</sup> of L-tryptophan 1 μg mL<sup>-1</sup> plus of naringenin (377.9 mL)**

- 77.9 mL of a sterile tryptophan solution at 10 mg mL<sup>-1</sup> mM;
- 1.95 mL of a sterile naringenin solution at 200 μg mL<sup>-1</sup>;
- Up to 377.9 mL of sterile NB medium.

**Iron (III) chloride hexahydrate (FeCl<sub>3</sub>.6H<sub>2</sub>O) 0.5 M (20 mL)**

- 2.7 g of FeCl<sub>3</sub>.6H<sub>2</sub>O;
- Up to 20 mL of distilled water.

**Salkowski reagent (1 L)**

- 452.3 mL of distilled water;
- 527.7 mL of perchloric acid (HClO<sub>4</sub>) at 65 %;
- 20 mL Iron (III) chloride hexahydrate.