Max Planck Institute
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Masterarbeit

Kinetic analysis of *Bacillus sp.* B55 growth on root exudates from *Nicotiana attenuata*

Friedrich-Schiller-Universität Jena

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eingereicht von

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Abstract.

Interactions between plant roots and soil microorganisms in the rhizosphere are critical for plant growth and are mediated by root exudation processes. Precise understanding of how above-ground herbivores influence root exudation and in turn microbial populations however is limited. *Bacillus sp.* B55 isolated from roots of *Nicotiana attenuata* plants impaired in ethylene (ET) perception (*35S-etr1*), has been shown to have dramatic plant growth promotion (PGP) effects on wild type (WT) and its native host plant. In previous experiments herbivory by *Manduca sexta* showed to have a negative effect on B55 rhizosphere colonization. Thus, the main objective of this study was to investigate whether the systemic responses induced by herbivory caused the reduction of *Bacillus sp.* B55 root colonization via changes on the root exudation pattern. This study involved the establishment of a method for tracking *Bacillus sp*. B55 growth on root exudates (RE) *in vitro* by means of optical density (OD) measurements. Different treatments were performed on WT plants (i.e. leaf removal, methyl jasmonate application (MJ), oral secretion applications to wounds (W+OS) and *M. sexta* feeding) and the RE were used as growth substrate for B55. These treatments did not affect B55 growth and no consistent results were found among the different repetitions of the experiment, but in general, RE had a positive effect on B55 growth in comparison to control (no RE added).

A second aspect of my study was to determine if RE from transgenic lines play a role in B55 root colonization. According to previous observations, B55 colonized almost ten fold the endosphere of *35S-etr1* seedlings in comparison to WT. Here I show that, B55 performed better on RE from ET-signaling impaired lines (*ir-aco1* and *35S-etr1)* confirming that their composition might affect the colonization pattern of B55. By means of a motility test and a swarming plate assay, a non-motile and non-chemotactic behavior of B55 was observed, suggesting that B55 might approach the rhizosphere by flagellar-independent movement. My variable results highlight the considerable challenges involved in analyzing root exudates *in vitro.* Nonetheless, the findings of this study represent just the beginning to put together a puzzle composing interactions between herbivores, plants and associated bacteria.

Zusammenfassung.

Wechselwirkungen zwischen Pflanzenwurzeln und Mikroorganismen sind entscheidend für das Pflanzenwachstum und werden hauptsächlich durch Wurzelexsudationsprozesse vermittelt. Unser Verständnis wie Herbivoren die Wurzelexsudation und damit die mikrobiellen Populationen beeinflussen ist jedoch begrenzt.

Der Bakterienstamm *Bacillus* sp. B55 wurde aus Ethen-unempfindlichen *Nicotiana attenuata* Pflanzen (*35S-etr1*) isoliert. *In vitro* und Feldexperimente zeigten, dass dieser Stamm das Wachstum von Wildtyp (WT) und im besonderen Maße das der *35S-etr1* Pflanzen positiv beeinflusste. Jedoch wirkte sich der Blattfraß durch Raupen des Nachtschwärmers *Manduca sexta* negativ auf die Kolonisation der Wurzeln durch B55 aus.

Hauptziel dieser Arbeit war es, zu erforschen, ob die, durch *M. sexta* Fraß verminderte Wurzelkolonisation durch Fraß-induzierte Veränderungen im Wurzelexsudat-Profil hervorgerufen wurden. Die Studie beinhaltete die Etablierung einer *in vitro* Hochdurchsatz-Methode, die es mir ermöglichte, das Wachstum von *Bacillus* sp. B55 auf *N. attenuata* Wurzelausscheidungen durch optische Dichte-Messungen zu verfolgen. Dabei dienten Wurzelausscheidungen unterschiedlich behandelter WT Pflanzen (Kontrolle, Entfernung von Blattmasse, Applikation von Oralsekreten oder Methyl-Jasmonat, sowie Raupenfraß) als bakterielles Wachstumssubtrat. Obwohl *N. attenuata* Wurzelexsudate das Wachstum von B55 generell förderten, konnte ich keine Behandlungs-spezifischen Unterschiede feststellen. Zudem wurde eine hohe Variabilität innerhalb und zwischen Einzelversuchen beobachtet

Ein zweiter Aspekt meiner Arbeit behandelte den Effekt von Wurzelausscheidungen verschiedener transgener *N. attenuata* Linien auf das Wachstum von B55. Vorausgegangene Untersuchungen hatten gezeigt, dass *35S-etr1* Keimlinge im Vergleich zu WT zehnfach stärker kolonisiert werden. Hier demonstriere ich, dass Wurzelexsudate von Pflanzenlinien mit eingeschränkter ET-Signaltransduktion (*ir-aco1* und *35S-etr1*) das Wachstum von B55 stärker fördern die von WT und anderen transgene Linien.

Zudem stellte ich fest, dass es sich bei B55 vermutlich um einen unbeweglichen und nicht-chemotaktischen Bakterienstamm handelt, der sich wahrscheinlich durch passive Mechanismen der Pflanze nähert.

Meine variablen Ergebnisse unterstreichen die Herausforderungen, die mit der Analyse von Wurzelausscheidungen beteiligt sind.

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1. General Introduction

1.1 Root Exudates

The term “rhizosphere” was first introduced by Hiltner in 1904 to describe the soil zone surrounding legume roots with intensive bacterial activity. More recently, this term has been broadened to describe the soil zone immediately adjacent to plant roots that is influenced by root activities (Darrah, 1993).

Living roots exude a wide range of compounds into the rhizosphere soil. These compounds can be classified into two groups according to their utilization as microbial substrates: Low molecular weight compounds (such as sugars, amino acids, organic anions, phenolics and several other secondary metabolites) that can be assimilated by soil microorganisms, and high molecular weight organic exudates (such as proteins, pigments, mucilage and miscellaneous other substances) that require extracellular enzymatic activity to break them down before they can be assimilated (Meharg, 1994).

Since RE are mainly derived from photosynthesis products, they represent a significant carbon (C) cost for plants. Roots can release large amounts of inorganic C which may directly affect the biogeochemistry of the soil (Cheng et al., 1993; Hinsinger, 2001; Hinsinger et al., 2009), but it is the release of organic C that produces the most dramatic changes in the physical, biological and chemical nature of the soil.

Root exudates are believed to have important functions in regulation of plant growth (Bertin et al., 2003; Walker et al., 2003) and regulation of internal plant metabolic processes, such as respiration and nutrient acquisition (Uren, 2007). Root exudates (e.g. phytoalexins) can also be a mechanism of plant defense against soil-borne pathogens and can stimulate or inhibit interactions with other soil organisms (Bais et al., 2004; Bertin et al., 2003; Rengel, 2002).

1.2 Influence of Root Exudates on Soil Microbiota

Root exudates are known as one of the most important factors affecting microbial growth in the rhizosphere (Koo et al., 2005). Some exudates serve as growth substances for soil microorganisms that are present near the roots, while others act as chemo-attractants to attract nearby microorganisms (Bais et al., 2006; Grayston et al., 1996). However, direct studies of the influence of RE on soil microbial communities are technically challenging because of the complexity of the rhizosphere environment and difficulty in sampling (Biedrzycki and Bais, 2009). Hence, these shortcomings have substantially limited the understanding of their chemical composition *in situ*, and by extension, their ecological significance (Phillips et al., 2008).

Positive as well as negative effects of plant roots on microbial growth and microbial decomposition processes have been observed, which are probably related to different soil N and C availabilities, as well as differences in the investigated soil types or soil horizons, RE compositions, plant species and developmental stages (Kuzyakov, 2002). A more direct effect on the non-pathogenic microflora can be expected from secondary metabolites with antimicrobial activities that are secreted as a result of activation of defense responses against pathogenic microorganisms (Doornbos et al., 2012).

1.3 Motility and Chemotaxis to Root Exudates

The ability of an organism to move by itself is called motility. Motility is closely linked to chemotaxis which is the ability to orientate along certain chemical gradients. Procaryotes move by means of un-usual, propeller-like flagella unique to bacteria or by special fibrils that produce a gliding form of motility (Aygan & Arikan, 2007). Chemotaxis plays an important role in microbial population dynamics. Chemotactic bacteria in a non-mixed environment and in the presence of a nutrient gradient have significant growth advantages, as shown experimentally for different bacterial species (Kennedy, 1987; Freter et al., 1978). Modeling of microbial population dynamics indicates that motility and chemotactic ability can be as important for evolutionary competition as cell growth rate (Lauffenburger, 1991; Kelly et al., 1988).

It has been suggested that the chemotactic response of root-associated bacteria to RE may be one of several important traits involved in the establishment of root colonization (Yao & Allen, 2006), although direct evidence has remained scarce.

Most rhizosphere bacteria studied so far are motile. Even though chemotaxis of plant-interacting bacteria towards RE has been often demonstrated *in vitro* (Heinrich and Hess, 1985; Mandimba et al., 1986; Hawes et al. 1988; Caetano-Anolles, 1988), it is not easy to demonstrate the necessity of bacterial motility and chemotaxis for a successful interaction with the host plant. Experimental conditions such as the inoculation procedures or the soil type often determine whether or not bacterial motility and chemotaxis are essential for the establishment of a plant-bacteria interaction (Broek and Vanderleyden, 1995).

1.4 Link Between Above and Belowground Responses

The aboveground and belowground components of ecosystems have traditionally been considered in isolation from one another. There is now increasing recognition of the influence of these components on one other and of the fundamental role played by above-belowground feedbacks in controlling environmental processes. Plants grow in ecosystems and interact with many heterotrophic organisms at several trophic levels (Figure 1). These interactions take place above- and belowground and may reduce or enhance plant growth and consequently community diversity and ecosystem properties (Wardle et al., 2004). Positive and negative feedback mechanisms operate between plant and soil biota through root secreting compounds, which strongly influence rates of nutrient cycling and vegetational change (Badri and Vivanco, 2009).

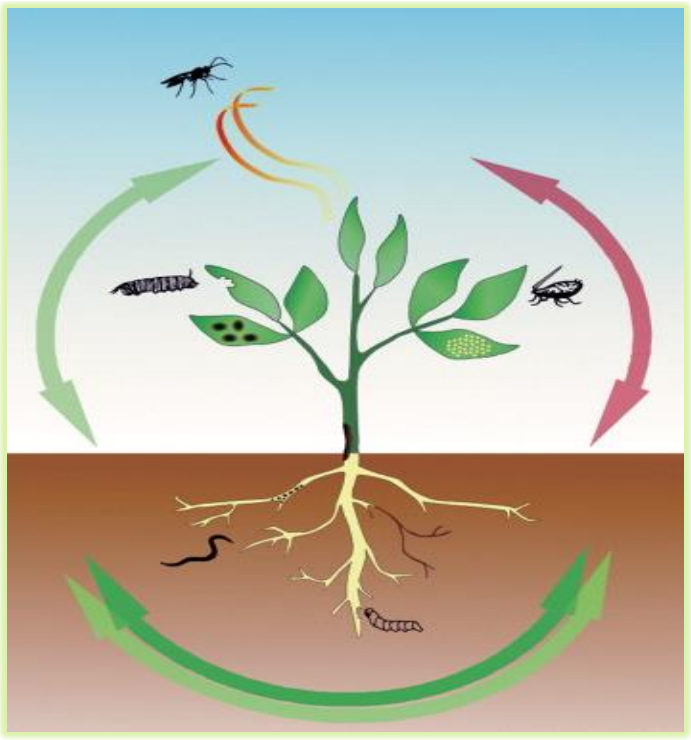


Figure 1. Plant interactions with other organisms above and belowground. (Pozo and Azcon-Aguilar, 2007).

1.5 *Nicotiana attenuata* and its Specialist Herbivore *Manduca sexta*

Nicotiana attenuata is a wild tobacco species (Figure 2A) native to the Great Basin Desert in eastern North America. Its seed germination is regulated by stimulants from burnt wood and inhibitors from litter (Baldwin *et al.*, 1994). As a consequence of the post-fire germination behavior, seeds germinate in nitrogen-rich soils (Lynds & Baldwin, 1998) and hence plants rapidly grow when water availability is high in monoculture-like populations. Because of its germination behavior, intense intraspecific competition, and highly variable herbivore interactions, *N. attenuata* is a particularly useful system to study herbivore resistance responses in nature (Baldwin, 2001).

Manduca sexta L. (Lepidoptera: Sphingidae) (Figure 2B) is a larval specialist herbivore on solanaceous plants including *Nicotiana* species (Madden & Chamberlin, 1945; Lou & Baldwin 2003), and non-nicotine species such as tomato (Chen et al., 2005).

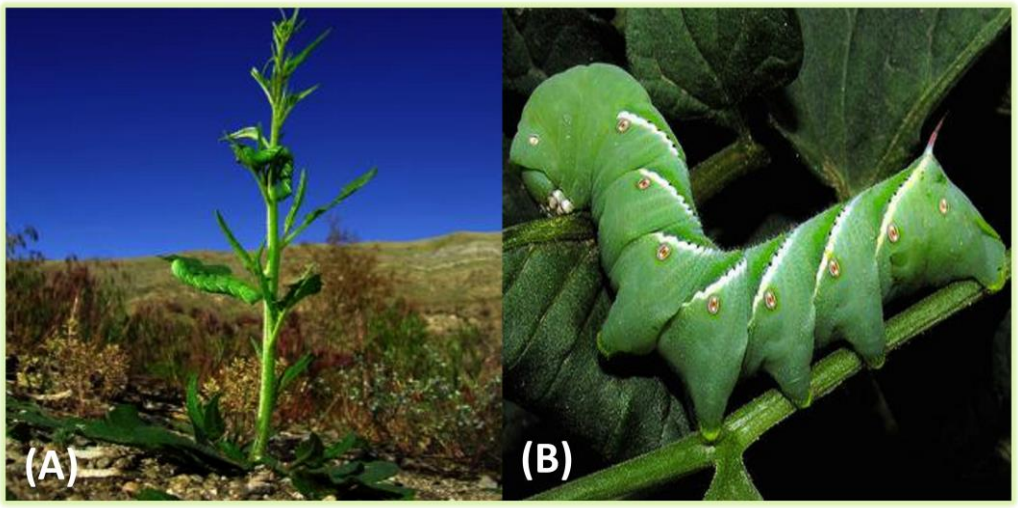


Figure 2. **(A)***Nicotiana attenuata* plant **(B)***Manduca sexta* caterpillar (Photo courtesy: Danny kessler. MPI Chemical Ecology).

Recent studies, suggest that *N. attenuata* response upon *M. sexta* infestation is highly specific. This specificity is achieved when *M. sexta* oral secretions (OS) enter the wounds during feeding. Fatty Acid-amino acid Conjugates (FACs) from OS bind to hypothetical receptors in the cell membrane and activate unknown short-distance signals, which in turn activate Mitogen-Activated Protein Kinases (MAPKs) that encompass Salicylic acid Induced Protein Kinases (SIPK) and a Wound Induced Protein Kinase (WIPK) which phosphorylate transcription factors able to activate phytohormone signaling such as jasmonic acid (JA), salicylic acid (SA) and jasmonic acid-isoleucine (JA-Ile) (Wu et al., 2008). Chewing insects, i.e *M. sexta* induce mainly JA-dependent genes (Ali & Agrawal, 2012).

1.6 Impact of Plant Defense on the Rhizosphere Microbiota

Plant defenses have the potential to affect bacterial populations in the rhizosphere by either recruiting beneficial bacteria or actively repressing pathogen proliferation (Berger et al., 2007; Bolton, 2009).

Plants use an array of metabolites to defend themselves against harmful organisms and to attract others that are beneficial. For example, it has been widely documented that roots secrete secondary metabolites which act as messengers to attract *Rhizobium* and arbuscular mycorrhizal fungi (Kent-Peters and Long, 1988; Besserer et al., 2006).

Several studies have probed the ecological significance of changes in root exudation patterns on rhizosphere microbial community. For example, the root herbivore *Heterodera trifolii* causes changes in the root exudation pattern of white clover, stimulating the biomass and activity of soil microbes (Yeates, 1999). These positive effects subsequently create a positive feed-back that ultimately benefits plant growth (Ayres et al., 2004).

Rudrappa et al. (2008) provided as well, biochemical evidence that L-malic acid secreted from roots of *Arabidopsis thaliana* which had been induced by the foliar pathogen *Pseudomonas syringae* pv *tomato* (Pst DC3000), recruits the beneficial rhizobacterium *Bacillus subtilis* FB17. Additionally, Kniskern et al. (2007) using Arabidopsis as model plant observed that plants with elevated SA-regulated defense responses were less well colonized by bacterial endophytes and harbored lower species' diversity than plants of the Col ecotype (wild type). And finally, in *N. attenuata,* Long et al. (2010) reported for the first time that plant ET signaling influences the recruitment of root bacterial endophytes.

Taken together, these examples show that the signal, which travels from shoots to roots upon herbivore attack or pathogen infection, might affect the native microbial community in the rhizosphere in terms of diversity or abundance.

1.7 Plant Growth Promoting Rhizobacteria

Plant Growth Promoting Rhizobacteria (PGPR) are reported to influence the growth, yield and nutrient uptake by an array of mechanisms. Direct mechanisms include the interference of the plant's hormone homeostasis (e.g. by production of phytohormones or enzymes which decrease the ET levels), improvement of the plant nutrient status (liberation of phosphates and micronutrients from insoluble sources; non-symbiotic nitrogen fixation) and stimulation of disease-resistance mechanisms (induced systemic resistance). Indirect effects originate for example when PGPR act like biocontrol agents reducing diseases, when they stimulate other beneficial symbioses, or when they protect the plant by degrading xenobiotics in inhibitory contaminated soils (Jacobsen, 1997).

Strains from the genus *Bacillus,* have been shown to enhance the growth of agricultural crops, wild plants, trees, microalgae, and model plants, through different mechanisms of plant-growth promotion (Bashan et al., 2000; Enebak et al., 1998; Hernandez et al., 2009; Kloepper et al., 2004a, b; Ryu et al., 2005; Vessey, 2003). The genus *Bacillus* has great potential uses in agriculture. Its members are able to produce antimicrobial metabolites to control plant pathogens, to fix nitrogen, to form endospores to resist desiccation, heat, and UV irradiation, and survive in adverse conditions. For instance, when *B. subtilis* is present in the immediate vicinity of plant roots, is able to maintain stable contact with higher plants and promote their growth (Saharan & Nehra, 2011).
Some plant colonization studies revealed *Bacillus* colonization patterns on plants, like is the case of *Bacillus pumilus* SE34, a PGPR, which colonized tomato roots, stems, and leaves (Yan et al., 2003) or *Bacillus mojavensis* AB1, another PGPR, which colonized leaves and twigs of coffee plants (Nair et al., 2002). Nevertheless, the colonization of *Bacillus sp*. B55 in *N. attenuata* seems to be restricted to the root (Dorothea Meldau, personal communication, August 29, 2012).

1.8 *Bacillus sp.* B55

Bacillus sp. B55 was isolated by Long et al. (2010) from roots of a *35S-etr1* plant grown in native soils collected in Utah, USA. This isogenic transformed line is deficient in ET perception and in consequence tends to overproduce ET (Von Dahl *et al.*, 2007). The absence of ET perception was shown to weaken the ability of transgenic Tetr tobacco plants to withstand common, generally non-pathogenic, opportunistic soil-borne fungal organisms (Knoester *et al.*, 1998; Geraats *et al.*, 2002).

Meldau et al. (2012) tested the plant growth promoting traits of *Bacillus sp*. B55 and found that *in vitro* cultures produced Aminocyclopropane-1-carboxylate deaminase (an enzyme that decreases ET production by the cleavage of the ET precursor, ACC) and Indole-3-acetic acid (IAA), an auxin analog. Furthermore, qualitative enzyme tests revealed that B55 is able to solubilize phosphate. This native PGPB not only improved *N. attenuata* WT's growth under *in vitro*, glasshouse and field conditions, but it also "rescued" the phenotype of *35S-etr1*

plants increasing the survival and growth of this plants under field conditions (Meldau et al., 2012).

Meldau, (2011 unpublished data) observed that *Bacillus sp.* B55 colonization in WT plants was significantly reduced after leaf herbivory by *M. sexta.* By then it was hypothesized that most likely herbivore-induced changes in RE composition could have an effect on the rhizosphere colonization by this strain. In addition, Meldau et al. (2012) observed also that the endosphere of *35S-etr1* seedlings was almost ten-fold higher colonized by B55 in comparison with WT seedlings. Thus, taking together these two observations, in this study RE from herbivore-induced WT plants and transgenic lines impaired in ET, JA and SA production or perception, were collected and used as a substrate for growing B55 *in vitro*. Growth kinetic parameters were determined for a better understanding of B55 adaptation to the RE and a chemotaxis assay was performed with the aim of investigating whether RE function as attractants or repellants to B55.

2. Materials and Methods

2.1 Plant Material and Growth Conditions

2.1.1 Growth of Wild Type *N. attenuata* Plants

2.1.1.1 Germination Procedure

Seeds of a 31x selfed *Nicotiana attenuata* (*Solanaceae*) inbred line were used for this project.

For germination, the seeds were sterilized for 5 min in 0.1% (w/v) dichloroisocyanuric acid followed by 1 h incubation in 5 mL of “Liquid Smoke” (House of Herbs, Passaic NJ, USA) amended with 50 µL of 0.1 M gibberellic acid. Seeds were thoroughly washed and placed onto 1X strength of GB5 medium (Gamborg’s B5 media, Duchefa, Haarlem, The Netherlands) and maintained in a Percival growth chamber with the following regime set: 13/11h

day/night cycle, 155 µmol m^{-2} s^{-1}, 30/28◦C. After 12 days seedlings were transferred to TEKU pots (Poeppelmann, Lohne, Germany) containing sand (0.7–1.2 mm grain size, Raiffeisen, Germany) before they were transferred to individual 1 L pots containing lecaton (Easy Green, Eschborn, Germany and Fibo ExClay, Lahmstedt, Germany) and sand. Plants were grown in the glasshouse at 26-28 ºC under 16 h of light supplied by Philips Sun-T Agro 400- or 600-W sodium lights. The fertilization was made with 0.6 g $Ca(NO_3)_2 \cdot 4H_2O$ and 0.3 g Flory Basisdünger 1 (Euflor,Munich,Germany) per liter.

2.1.1.2 Plant Treatments

All experiments were performed on plants in the rosette stage. Plants were either not treated (**Control**); wounded using a fabric pattern wheel to punch two rows of holes on each side of the mid vein and applying 20 µL of 1:5 oral secretion of *M.sexta* (**W+OS**) or subjected to 10 µL **MJ** application every 48 h on one leaf. Also, to simulate plant material loss due to herbivory, leaf material was removed by cutting off two half leaves every other day (**CUT**). To observe the effects of *M. sexta* caterpillars, freshly hatched neonates were placed on the leaves (***M. sexta***). Experiments and sampling times are depicted in detail in table 1.

Table 1. Experiments performed with WT plants of *N. attenuata* in the greenhouse.

Experiment	Plant Treatments	Biological replicates	Accumulation time after first treatment (hours)
Freeze dried RE	Control; W+OS; MJ; *M. sexta*; CUT	6	0-72 h 72-75 h
Fresh RE 1	Control; W+OS; MJ; CUT	10	0-72 h 72-75 h
Fresh RE 2	Control; W+OS; MJ; *M. sexta*; CUT	9	0-3 h 3-6 h 6-48 h 48-72 h

Fresh RE 3	Control; Control with sand covered(to avoid *M. sexta* frass contact with the sand); W+OS; *M.sexta* 4 and 8 larvae covered and uncovered (Figure 4)	10	0-72 h 72-75 h 75-144 h

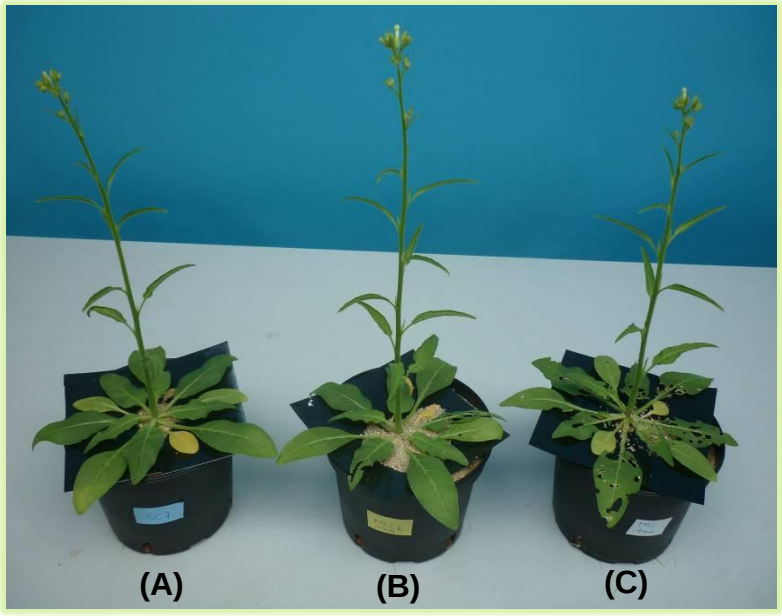


Figure 4. Plants of *N. attenuata* with the sand covered by a plastic layer on top of the pot. **(A)** Control covered plant **(B)** Plant infested by 4 larvae of *M. sexta* with the sand covered **(C)** Plant infested by 8 larvae of *M. sexta* with the sand covered.

2.1.2 Growth of Transgenic Lines of *N. attenuata*

2.1.2.1 Germination Procedure

Seeds of six transgenic lines listed in Table 2 were germinated as described previously (section 2.1.1.1).

Table 2. *N. attenuata* transgenic lines used in this study.

Genotype	Line code	Gene silenced and phenotype	Reference
ir-coi1	A-04-249-A-1	Coronatine insensitive 1, impaired in JA perception	(Paschold *et al*., 2008)
ir-aco1	A-03-321-10	ACC oxidase 1, impaired in ET production	(von Dahl *et al*., 2007)
nahG	A-07-489	Salicylate hydroxylase, impaired in SA production	(Gilardoni *et al*., 2011)

***ir-jar4-1 and ir-jar4-2; ir-jar6-1 and ir-jar6-2* (JAR4*JAR6)**	A-05-380-6	JA resistance protein, impaired in JA-amino acids conjugates production	(Wang *et al*., 2007)
35S-etr1	A-03-328-8	ET receptor mutant, impaired in ET perception	(von Dahl *et al*. 2007)
Ev	A-03-9-1-1	Empty vector (control)	(Zavala *et al*. 2004)

*All lines used were T_3 generation harboring a single T-DNA insertion and silenced or overexpressed of the target gene (35S-etr1 and nahG**)** and fully characterized in the associated reference publication.*

2.1.2.1 Growth Conditions

Twelve plants of each transgenic line were grown in the greenhouse under the same conditions mentioned in point 2.1.1.2 until they reached the rosette stage. For *in vitro* RE production, nine 15-day-old seedlings from each transgenic line were transferred to ½ strength GB5 broth using 6-well culture plates (Greiner Bio-One GmbH, Frickenhausen, Germany). One well contained 3 seedlings of one line floating in 5 mL broth (3 biological replicates per transgenic line with 3 seedlings per well). Plates were incubated in a Percival (13/11 h day/night cycle,155 $\mu molm^{-2}$ s^{-1}, 30/28°C) on an orbital shaker at 90 rpm for 6 d. Afterward, seedlings were placed into new culture plates containing 1/8 strength GB5 broth and incubated under the same conditions for 3 more days before RE were collected.

2.2 Root Exudates Collection

2.2.1 Plants Grown in the Greenhouse

Two days before collecting the RE, the 1L pots were washed with tap water in order to get rid of the fertilizer. Subsequently, the plants were placed on trays and watered by hand during the time of the experiment to avoid the accumulation of fertilizer salts which might affect B55 growth. For collecting the RE, each pot was rinsed with tap water homogenously and the first 50 mL of RE running off the pot were collected in falcon tubes (Figure 5). Afterward, RE were

filtered through 0.20 µm pore size filter and stored at -80 ºC for further analysis. After collecting the RE the FM of the roots was recorded.

Figure 5. Setup for the collection of RE. The pot was placed on top of a glass funnel and with a 25 mL dispenser tap water was distributed over the sand. RE were collected in a 50 mL beaker and transferred to 50 mL falcon tubes.

2.2.2 Plants Grown in GB5 Broth

Seedlings were withdrawn from the broth using tweezers under sterile conditions and the seedling weight was recorded. Then, 1/8X GB5 broth was collected in a 15 mL falcon tube and stored at -80 ºC for further analysis.

2.3 Growth of *Bacillus sp.* B55 on Root Exudates

2.3.1 Bacteria Strains and Media

Bacillus sp. B55, *Escherichia coli* strain and *Corynebacterium glutamicum* strain were cultured in LB (Luria-Bertani) and stored in 10 % glycerol (v/v) at -80 ºC. Cells from stock cultures were inoculated onto half-strength YPDA (yeast peptone dextrose agar) for *Bacillus sp.* B55 and onto LB for the control strains.

2.3.2 *Bacillus sp.* B55 Growth Curve

The growth pattern of *Bacillus sp.* B55 was studied by culturing the strain in 1 % tryptone broth (BD Becton, Dickinson and Company, Franklin Lakes, NJ USA) amended with 0.5 % NaCl. *Bacillus sp*. B55 cultures were shook at 30 ºC, 200 rpm and optical density (OD) measurements and viable cell number counts were tracked every 25 min for 6.25 h. The viable cell number was determined by spreading serial dilutions onto 1 % tryptone agar and the optical density of the culture was measured at 600 nm wavelength. In this way was possible to make a correlation between the OD_{600nm} and viable cell counting.

2.3.3 Microtiter Plate Assay for Bacterial Growth

In order to assess *Bacillus sp.* B55 growth on RE from WT plants and transgenic lines, a new method using a microplate reader (Tecan Infinite® 200 Pro series, Maennedorf, Switzerland) was developed. Pre-experiments had shown that B55 did not growth with RE only. Thus, it was necessary to add an exogenous nitrogen source to perform bacterial growth curves. Therefore, tryptone in a very low concentration was used (0.037 %). From an overnight B55 strain streaked onto half-strength YPDA, a bacterial suspension with an OD_{600nm} 1.0 was prepared. Serial dilutions in 0.85 % NaCl up to 10^{-3} were made and the latest dilution ($1.2 * 10^8$ CFU/mL) was used as inoculum. A flat bottom microtiter plate (Greiner Bio-One GmbH, Frickenhausen, Germany) was inoculated with 20 µL or 140 µL of Freeze dried RE and Fresh RE, respectively; 30 µL 0.25 % tryptone and 30 µL bacterial suspension per well and volume was balanced by adding sterile distilled water (Figure 6). For every biological replicate, 4 technical replicates were performed. To prevent evaporation of the media, the outer rows of the microtiter plate were filled with 200 µL sterile water and the plate was incubated on the Tecan microplate reader for 1000 min at 30 ºC 200 rpm with an interval time of 10 min among readings.

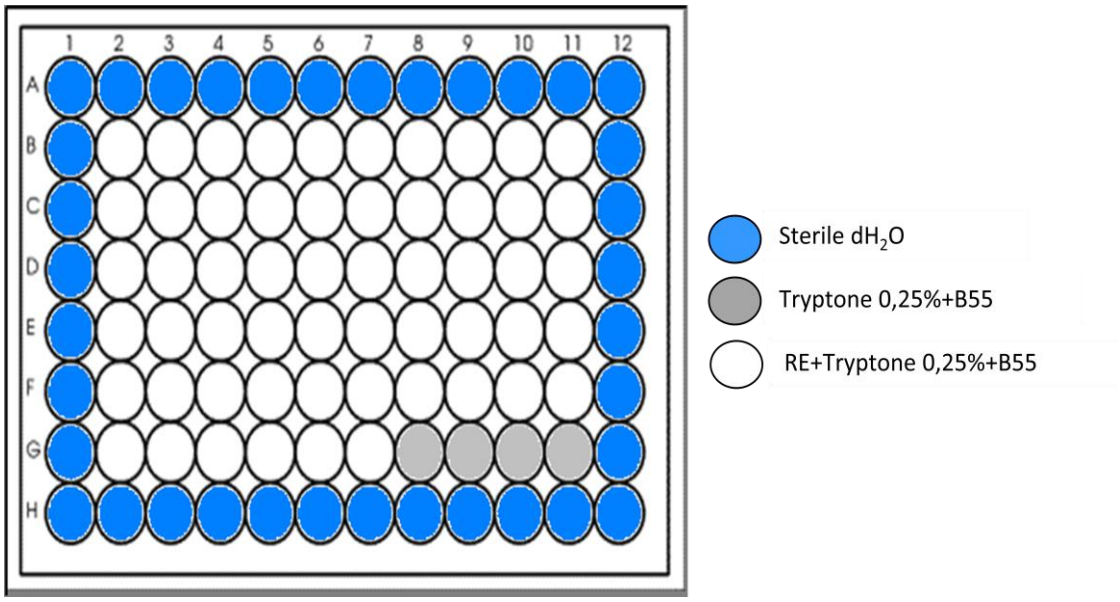


Figure 6. 96-well microtiter plate scheme followed for bacterial growth curves.

To measure the adaptation and utilization of the RE by *Bacillus sp.* B55, kinetic parameters like lag-phase duration in hours (LPD), the rate of growth (GR) and OD_{600nm} after 1000 min were investigated. The no-asymptote Baranyi model was fitted to data using DMFit and the mean of LPD and maximum GR were generated (Institute of Food Research, Norwich Research Park, Norwich, United Kingdom; http://www.ifr.bbsrc.ac.uk).

2.4 Motility Assay

A motility assay was performed by inoculating one isolated CFU (colony forming unit) in motility test medium with triphenyltetrazolium chloride (TTC) (beef extract 3 g, pancreatic digest of casein 10 g, NaCl 5 g, 5 mL 1 % TTC and agar 4 g/L) (Kelly & Fulton, 1953) using a straight needle. As organisms grow, they incorporate and reduce TTC, creating a diffuse red color. *E. coli* was used as positive control and *C. glutamicum* as negative control for motility. The tubes were incubated at 37 ºC for *E. coli* and at 30 ºC for *C. glutamicum* and *Bacillus sp.* B55 for 48 h.

2.5 Chemotaxis Assay

2.5.1 Swarming Plate Assay

According to the literature *E. coli* is a model organism for the study of bacterial chemotaxis. Chemotaxis was assayed by the ability of cells to form swarm rings on “swarm plates” containing 0.3 % agar, 1 % tryptone and 0.5 % NaCl (Wolfe & Berg, 1989). Two µL of liquid culture from *E. coli*, *Bacillus sp.* B55 and *C. glutamicum* in LB broth with an OD_{600nm} of 0.4 were inoculated at the center of the swarm plate. The plates were incubated at 30 ºC for 72 h before pictures were taken.

2.5.2 Capillary Assay

The capillary assay was based on previous work conducted by Park et al. (2011) with some modifications. For instance the OD_{600nm} of B55 suspension for inoculating the microtiter plate was increased up to ~0.5 for this study because in previous experiments with a lower OD there was no growth on fresh tryptone. Two overnight cultures one with *E. coli* (positive control) and the other with *Bacillus sp*. B55 were carried out in 125 mL 1 % tryptone, 0.5 % NaCl broth at 37 ºC and 200 rpm for *E. coli* and in 125 mL minimal broth (per liter: Na_2HPO_4 $2H_2O$ 11.2 g; KH_2PO_4 2.4 g; $(NH_4)_2SO_4$ 2 g; $MgSO_4$ $7H_2O$ 50 mg; $MnCl_2$ $4H_2O$ 4 mg and $FeSO_4$ $7H_2O$ 2.8 mg) at 30 ºC and 200 rpm for *Bacillus sp*. B55. Then, overnight cultures were diluted 1:50 (3 mL pre-culture: 147 mL broth) and shook under the same conditions until the OD_{600nm} reached 0.4. Cells were washed twice by centrifugation at 3000 rpm for 5 min in motility medium (0.1 mM EDTA, 0.1 mM L-methionine, 10mM potassium phosphate, pH 7.0) before they were resuspended in the same medium to an OD_{600nm} ~0.012 for *E. coli* and ~0.5 for *Bacillus sp.* B55. Three hundred µL of these diluted cell solutions were distributed into each well of a 96-well microtiter plate just before the chemotaxis assay was started.

To build the capillary system, 96 narrow needles (inner diameter = 0.4 x 25 mm 27 Gx1” Braun Medical, Prague, Czech Republic) were firmly attached to the ends of a 96-well pipetting device (Vaccu-Pette/96™, Scienceware, NJ, USA).

By slowly withdrawing the attached syringe pump to 5 mL, the needles were filled with motility medium (negative control), tryptone 0.25 % in motility medium (positive control for *Bacillus sp.* B55), 10^{-2} M L-aspartate (positive control for *E. coli*) or RE from control WT plants (test chemo-attractant). The capillary device was placed approximately 3.5 mm above the bottom of each well containing the cell solutions and to prevent the capillaries to touch the bottom of the well, a plastic device was built (Figure 7).

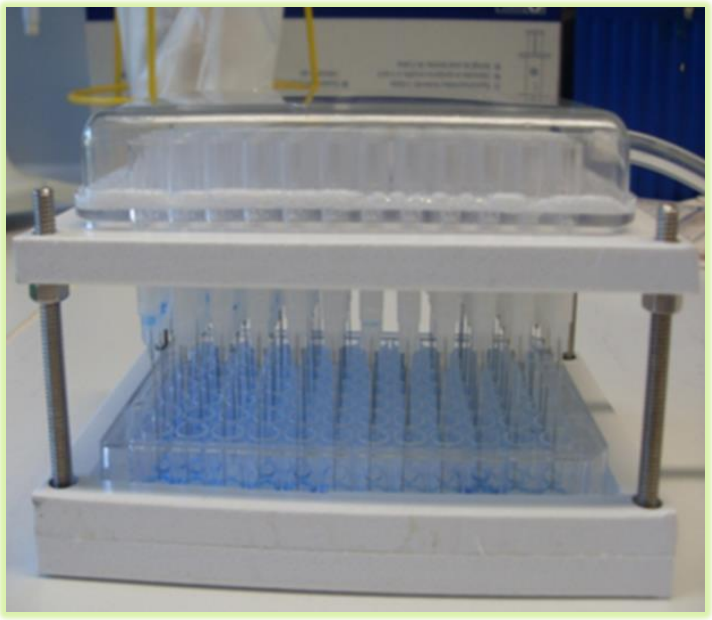


Figure 7. Capillary system. The upper part of the Vaccu-Pette with the attached needles was held by a plastic device to prevent the tip of the needle to touch the bottom of each well.

The capillary system was incubated at 30ºC for 40 min and then the content of the needles was emptied into a new 96-well microtiter plate containing 200 µl 0.25 % tryptone per well. Overnight OD_{600nm} measurements were performed in the Tecan every 50 min with multiple reads per well. The chemotactic response was referred as positive, when the bacteria from the capillaries containing RE were able to grow in the fresh tryptone broth after flushing them out of the capillaries and negative when no growth was observed.

2.6 Statistical Analysis

Statistical analysis was performed using SigmaPlot 12.0. Data are expressed as mean ± standard error of the mean (SEM). Two-way ANOVAs were performed to find differences between treatments in the bacterial growth on RE and to detect temporal changes for each parameter (LPD, GR and OD_{600nm} at 1000 min). Normality was tested by means of Shapiro-Wilk and the equal variance was rejected with a P value of 0.050.

3. Results

3.1 *Bacillus sp.* B55 Growth Curve

In tryptone broth, B55 cells reached the early exponential stage after 2 h. The generation time of B55 strain in tryptone broth at 30 ºC and 200 rpm was between 37 and 46 min. Therefore, B55 is considered a fast-growing bacterium.

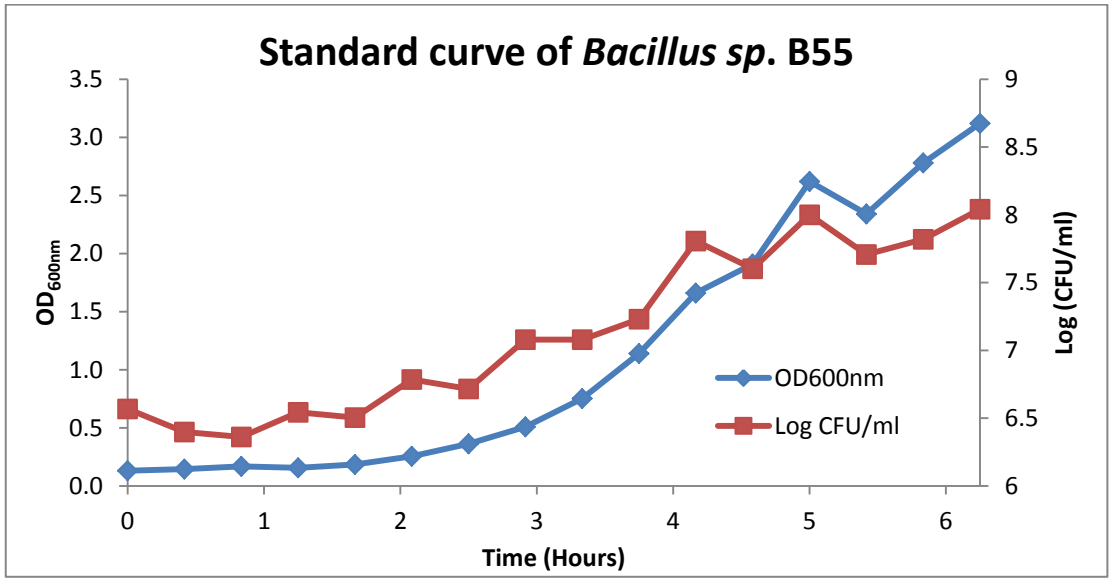


Figure 8. The growth curve of *Bacillus sp.* B55 in tryptone broth at 30 ºC and 200 rpm with an initial concentration of about $3.70x10^6$ CFU/mL (OD_{600nm} = 0.131).

3.2 Microtiter Plate Assay for *Bacillus sp.* B55 growth on WT Root Exudates

Using microtiter plates to cultivate B55 facilitated the simultaneous measurement of a high number of samples. Thus, it was an advantage to standardize this assay in order to assess several RE in a quicker way than cell counting. The results of the growth curves corresponded to a classical bacterial growth curve with lag, exponential and steady phase. In most of the growth curves, the death phase was not reached (Figure 9).

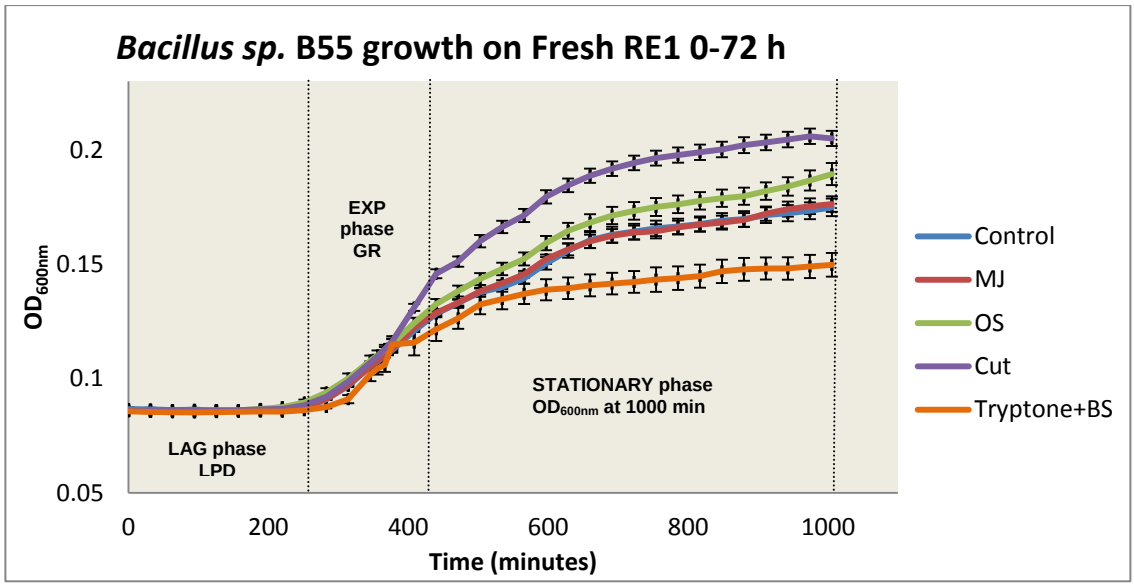


Figure 9. Example of *Bacillus sp.* B55 growth curve on RE from Fresh collected RE2 recorded with the Tecan microplate reader. Tryptone+BS: tryptone+B55 bacterial suspension diluted up to 10^{-3}.

With some RE, the plot of the OD_{600nm} versus time resulted in a diauxic growth curve, which showed two or even more distinct phases of active growth (Figure 10). This diauxic phenomenon was present only when B55 grew on Freeze Dried RE.

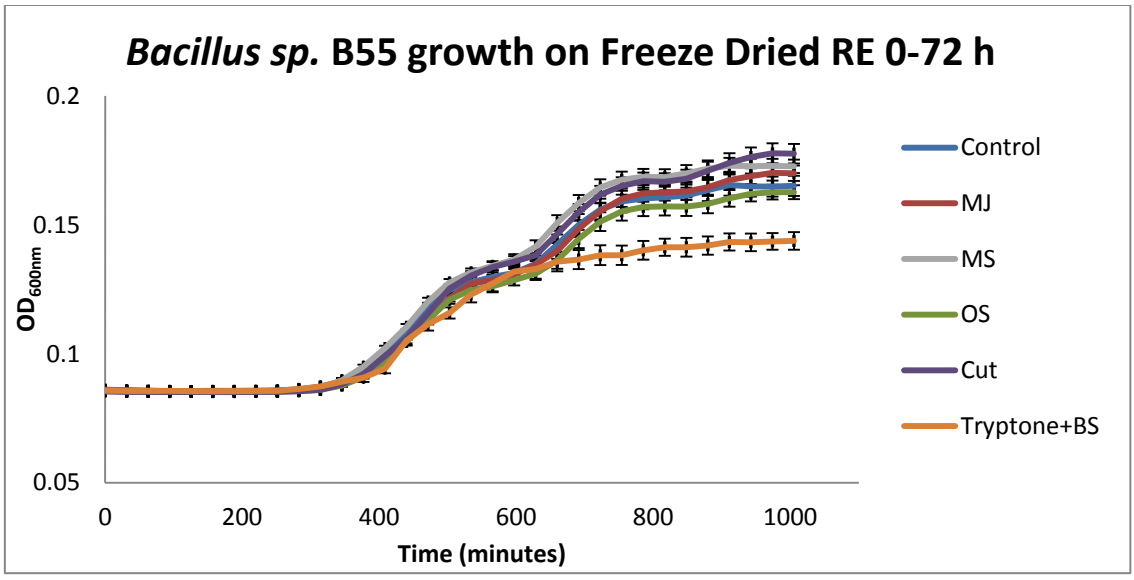
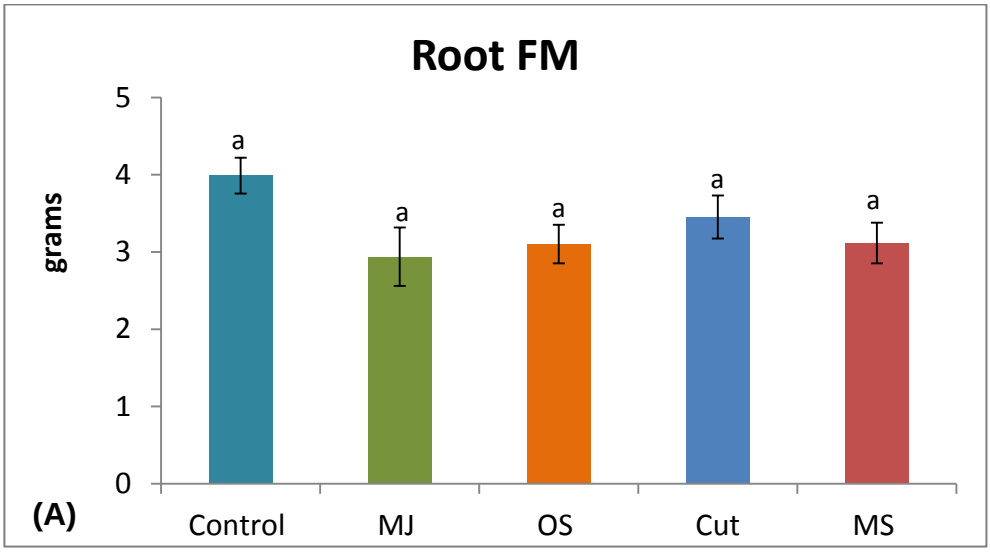


Figure 10. Diauxic growth curve of *Bacillus sp.* B55 on RE from Freeze Dried 0-72 h. Tryptone+BS: tryptone+B55 bacterial suspension diluted up to 10^{-3}.

3.2.1 Freeze Dried RE

Root exudates were lyophilized and diluted with sterile distilled water according to the FM with the aim of having a homogenous concentration of the exudates. Figure 11 shows that there were no statistically significant differences for the root FM, B55 GR and LPD after leaves were induced. Nevertheless, the OD_{600nm} at 1000 min, showed a difference when the leaves were cut (CUT vs. OS P = 0.019). Furthermore, statistically significant differences were found between time points for the OD_{600nm} at the steady phase (P = 0.002).



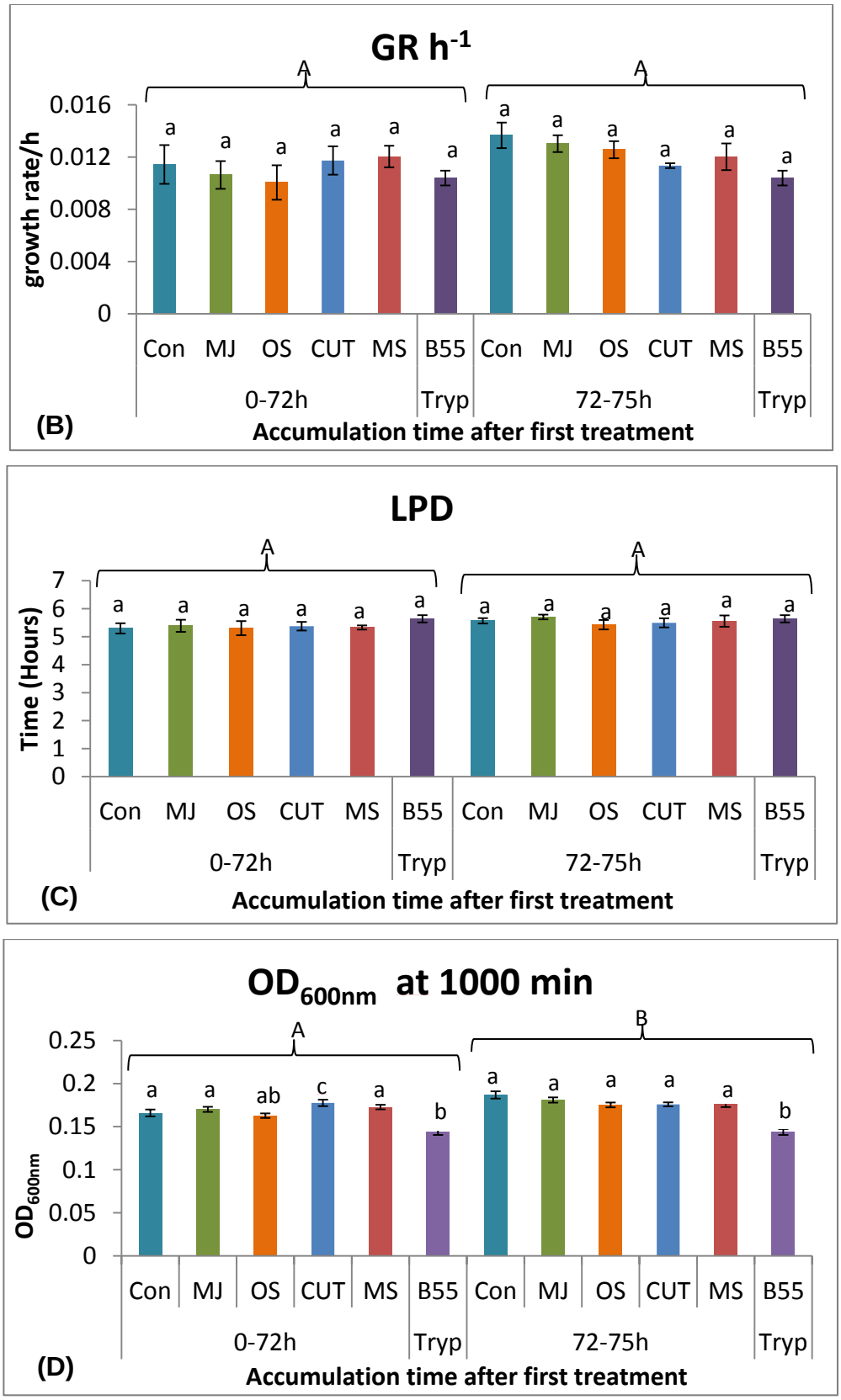


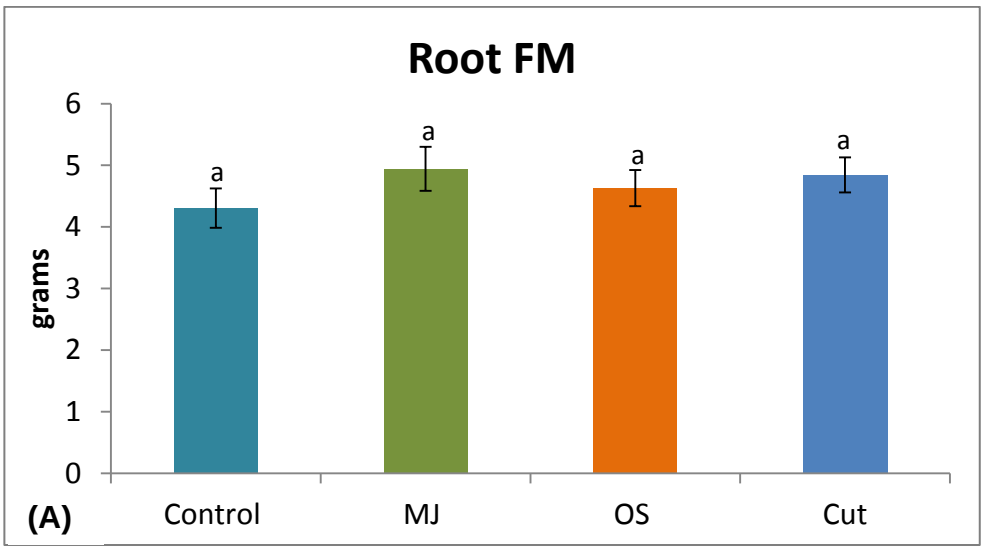
Figure 11. Weight corrected kinetic parameters **(A)** Root FM **(B)** *Bacillus sp.* B55 GR during exponential phase. **(C)** *Bacillus sp.* B55 LPD and **(D)** *Bacillus sp.* B55 OD_{600nm} at 1000 min during the stationary phase. (Two way ANOVA test: uppercase letters refer to differences between time points and lowercase letters to differences between treatments; $P \leq 0.05$).

In addition, when B55 was grown on tryptone without RE as sole nutrient source, the OD_{600nm} at 1000 min was lower comparing to the growth on RE (Figure 11D).

3.2.2 Fresh RE1

Based on the principle that the amount of RE has been reported to range from 2 % root dry weight to several tens of percent under non-sterile conditions (Prikryl and Vancura, 1980), every kinetic parameter analyzed during B55 growth on RE was corrected with the root FM. The FM corrected and uncorrected data are shown to observe the effects that root FM may have on B55 kinetics.

Due to the high concentration of salts after the process of lyophilization, the three remaining experiments with RE (Fresh RE1, 2 and 3) were kept fresh (non-lyophilized) for the analysis. There were no statistically significant differences in the root FM, the FM corrected GR and in the FM corrected OD_{600nm} at 1000 min (Figures 12A, 12B and 12C). For the LPD, B55 got adapted faster to the RE from 0-72 h than to 72-75 h (P = 0.001).



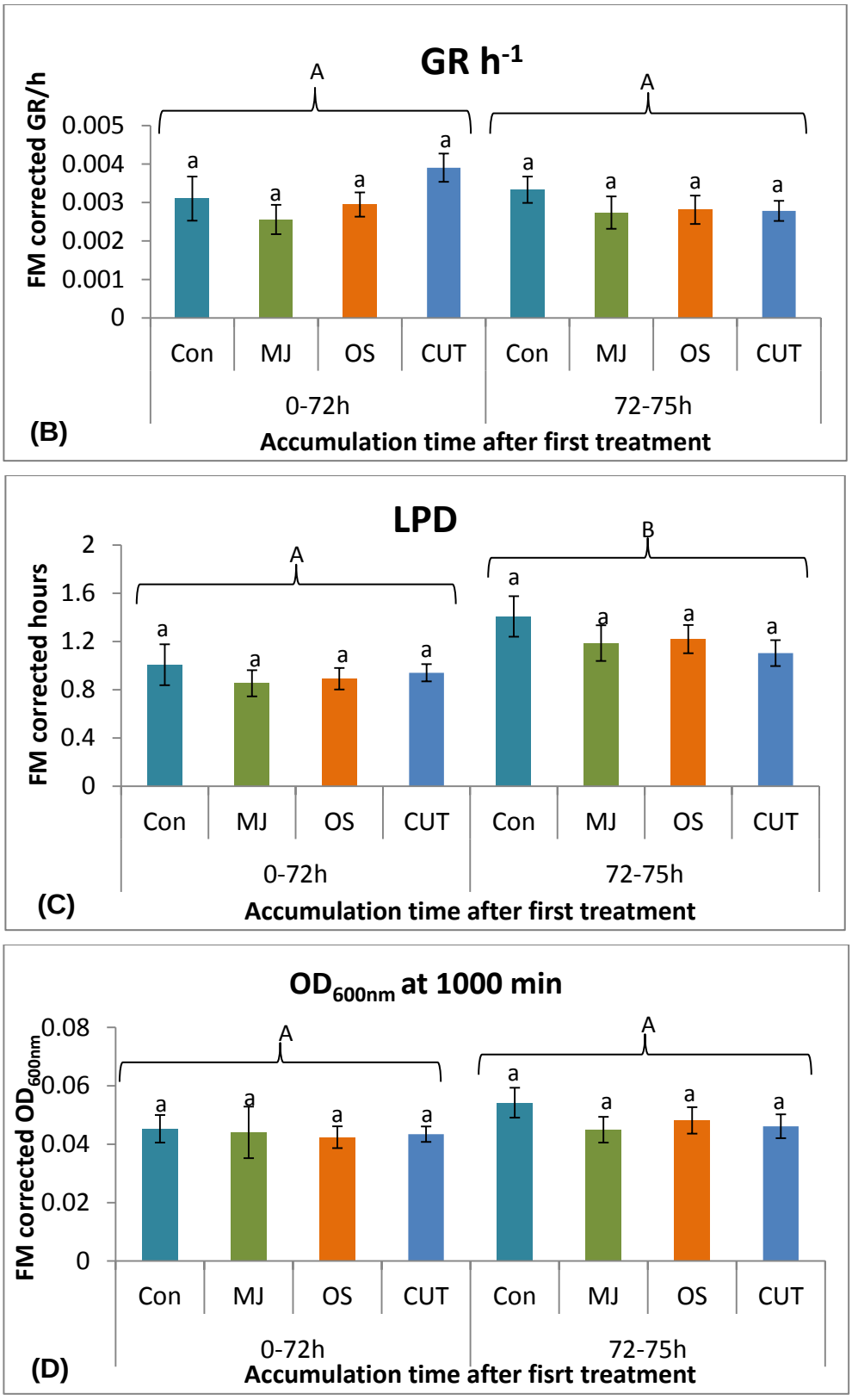
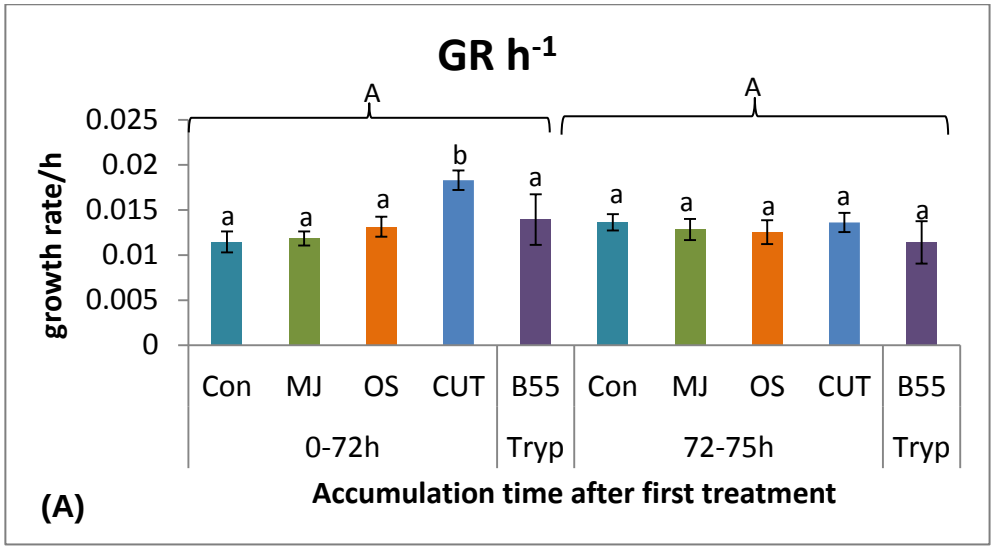
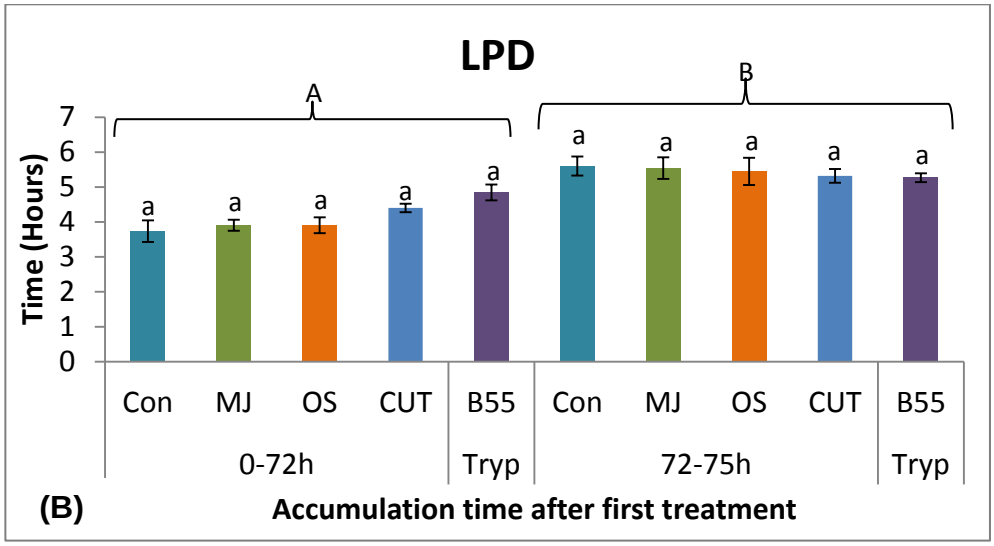


Figure 12. FM corrected kinetic parameters **(A)** Root FM **(B)** *Bacillus sp.* B55 GR during exponential phase. **(C)** *Bacillus sp.* B55 LPD and **(D)** *Bacillus sp.* B55 OD_{600nm} at 1000 min during the stationary phase. (Two way ANOVA test: uppercase letters refer to differences between time points and lowercase letters to differences between treatments; $P \leq 0.05$).

The uncorrected kinetic parameters showed that B55 GR was affected by exudates from CUT treatment having the highest value at 0-72 h (P = 0.040), as well as for the OD_{600nm} at 1000 min (P < 0.001) (Figure 13A and 13C, respectively). Statistically significant differences between time points were found for LPD and OD_{600nm} at 1000 min.





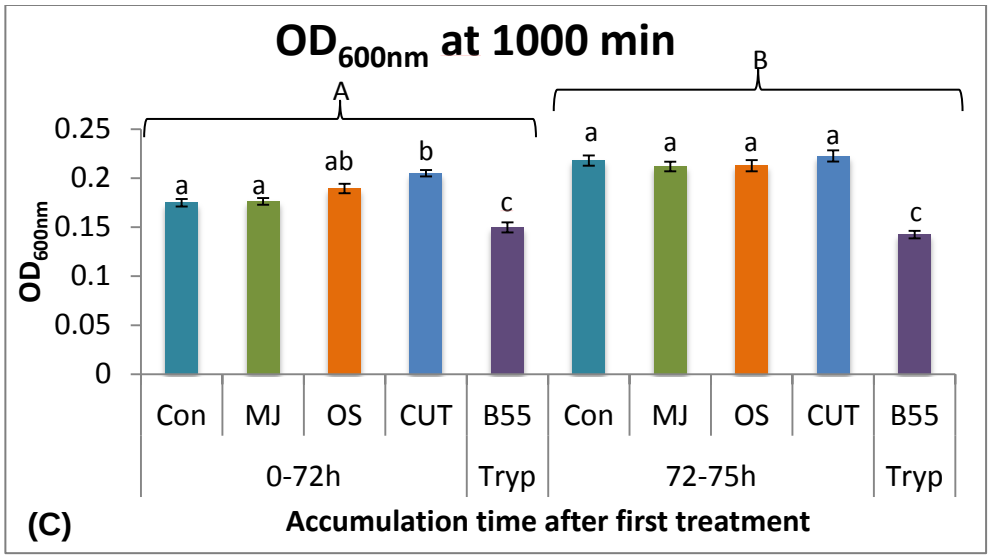
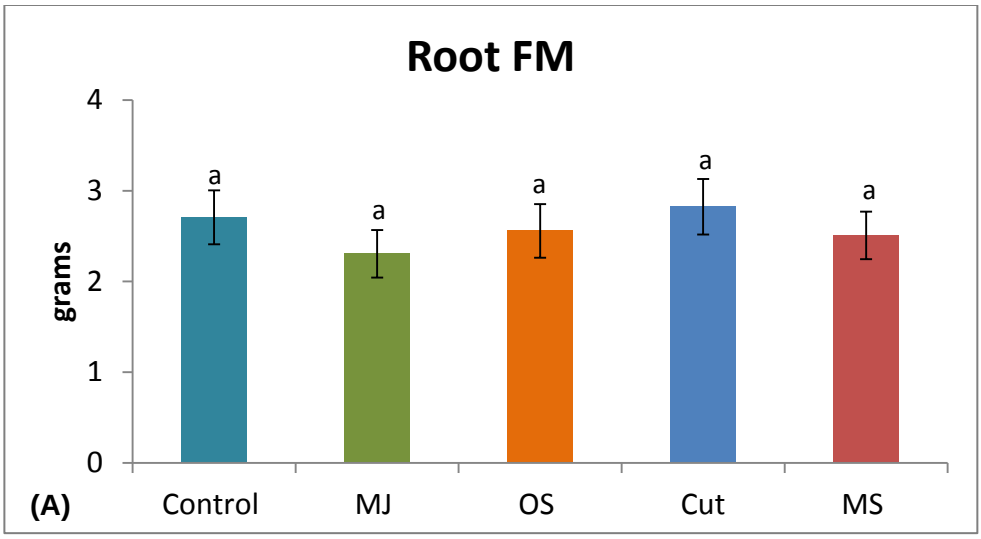


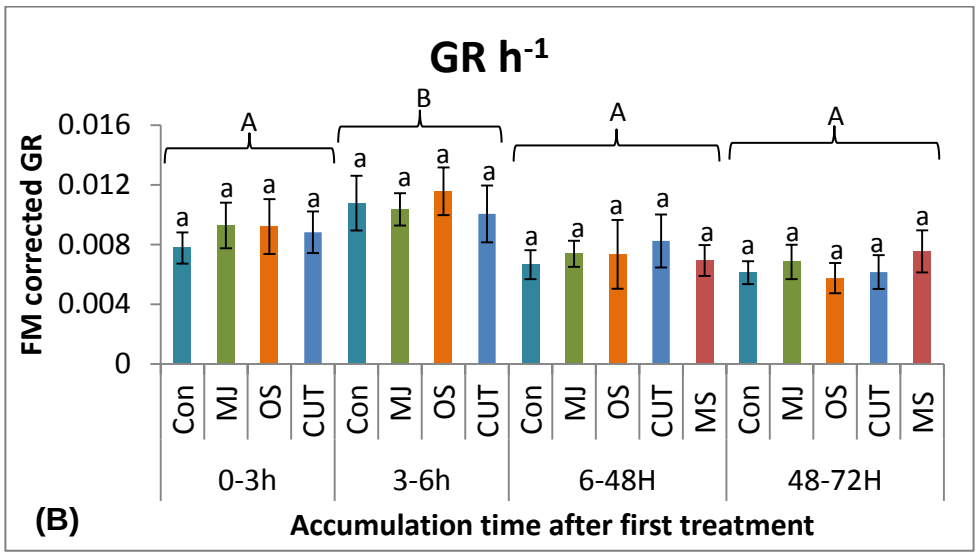
Figure 13. Uncorrected kinetic parameters **(A)** *Bacillus sp.* B55 GR during exponential phase. **(B)** *Bacillus sp.* B55 LPD and **(C)** *Bacillus sp.* B55 OD_{600nm} at 1000 min during the stationary phase. (Two way ANOVA test: uppercase letters refer to differences between time points and lowercase letters to differences between treatments; $P \leq 0.05$).

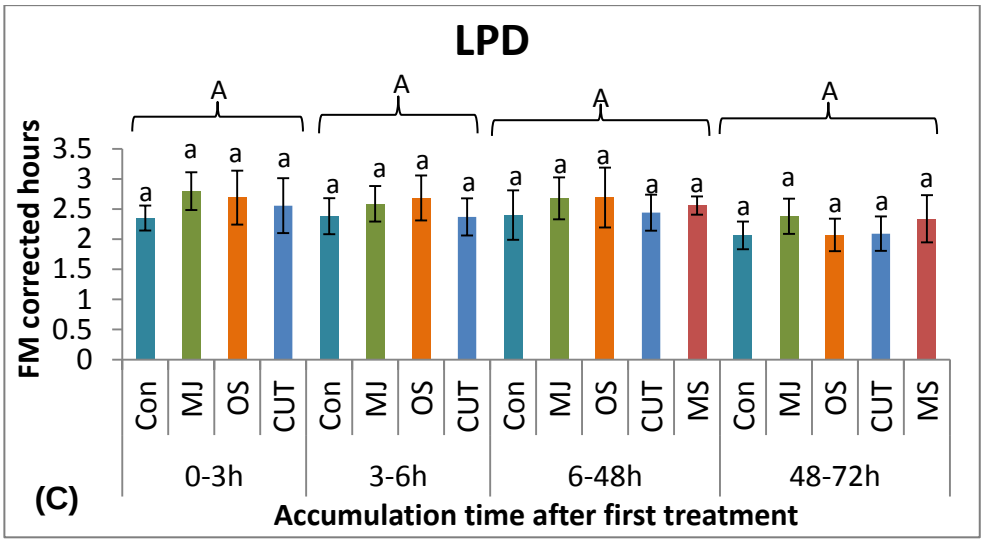
3.2.3 Fresh RE2

With the sample set "Fresh RE2" there was the possibility to test B55 growth on very fresh RE (3 h after induction) up to 3-day-old RE. As it is already known, after elicitation there is a JA accumulation in *N. attenuata* with an initial JA burst that attains maximum values 45-60 min after OS elicitation and is quickly metabolized within 2 h and then slowly returning to undetectable levels (Paschold et al., 2007). Thus, the sampling time 0-3 h after the first treatment, was useful to detect an impact of this JA burst on the RE and in turn on B55 growth.

As can be observed in figure 14, different treatments did not have any effect on root FM neither on B55 growth kinetic parameters. Nonetheless, sampling time did have an effect on B55 FM corrected GR h^{-1} ($P < 0.001$) and OD_{600nm} at 1000 min ($P < 0.001$), where B55 grew better on exudates from 3-6 h.


Root FM
grams
4
3
2
1
0
a
a
a
a
a
(A)
Control
MJ
OS
Cut
MS


GR h-1
FM corrected GR
0.016
0.012
0.008
0.004
0
A
B
A
A
Con
MJ
OS
CUT
MS
0-3h
3-6h
6-48H
48-72H
(B)
Accumulation time after first treatment


LPD
FM corrected hours
3.5
3
2.5
2
1.5
1
0.5
0
A
A
A
A
Con
MJ
OS
CUT
MS
0-3h
3-6h
6-48h
48-72h
(C)
Accumulation time after first treatment

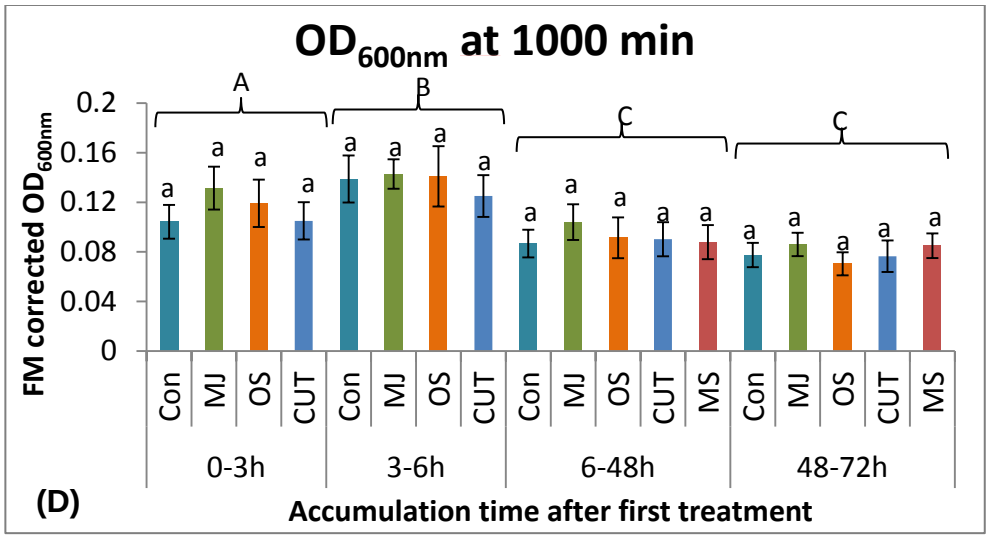
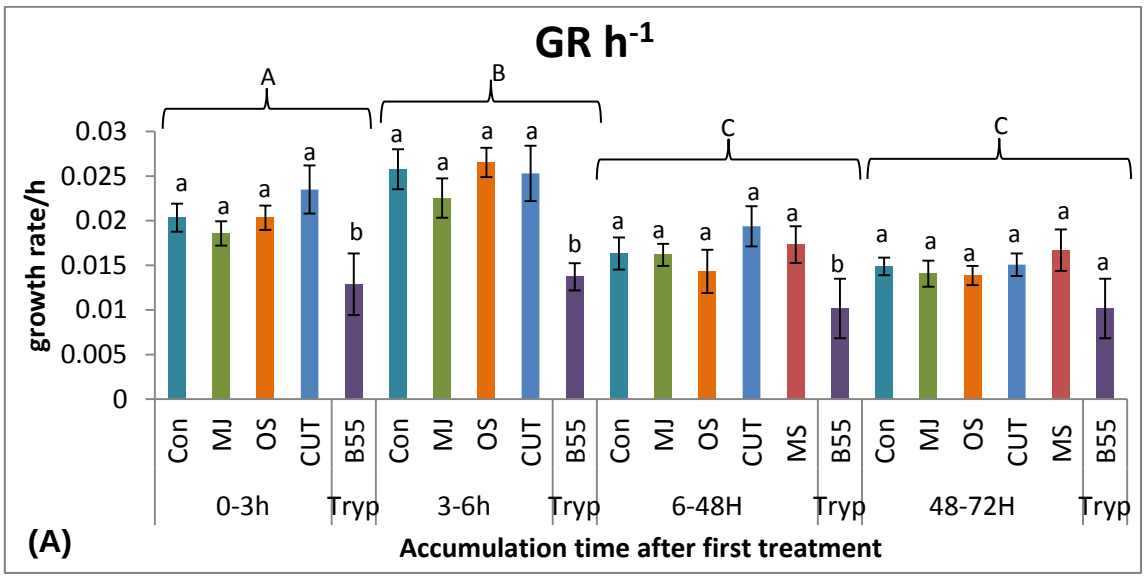


Figure 14. FM corrected kinetic parameters **(A)** Root FM **(B)** *Bacillus sp.* B55 GR during exponential phase. **(C)** *Bacillus sp.* B55 LPD and **(D)** *Bacillus sp.* B55 OD_{600nm} at 1000 min during the stationary phase. (Two way ANOVA test: uppercase letters refer to differences between time points and lowercase letters to differences between treatments; $P \leq 0.05$).

Concerning the uncorrected kinetic parameters (Figure 15) the results of the statistical analysis for B55 GR was similar to the FM corrected GR. The LPD was shorter for the 48-72 h RE ($P < 0.001$) comparing to the FM corrected LPD, and for the OD_{600nm} at 1000 min the tendency was once more B55 growing the best on the exudates from 3-6 h ($P < 0.001$).



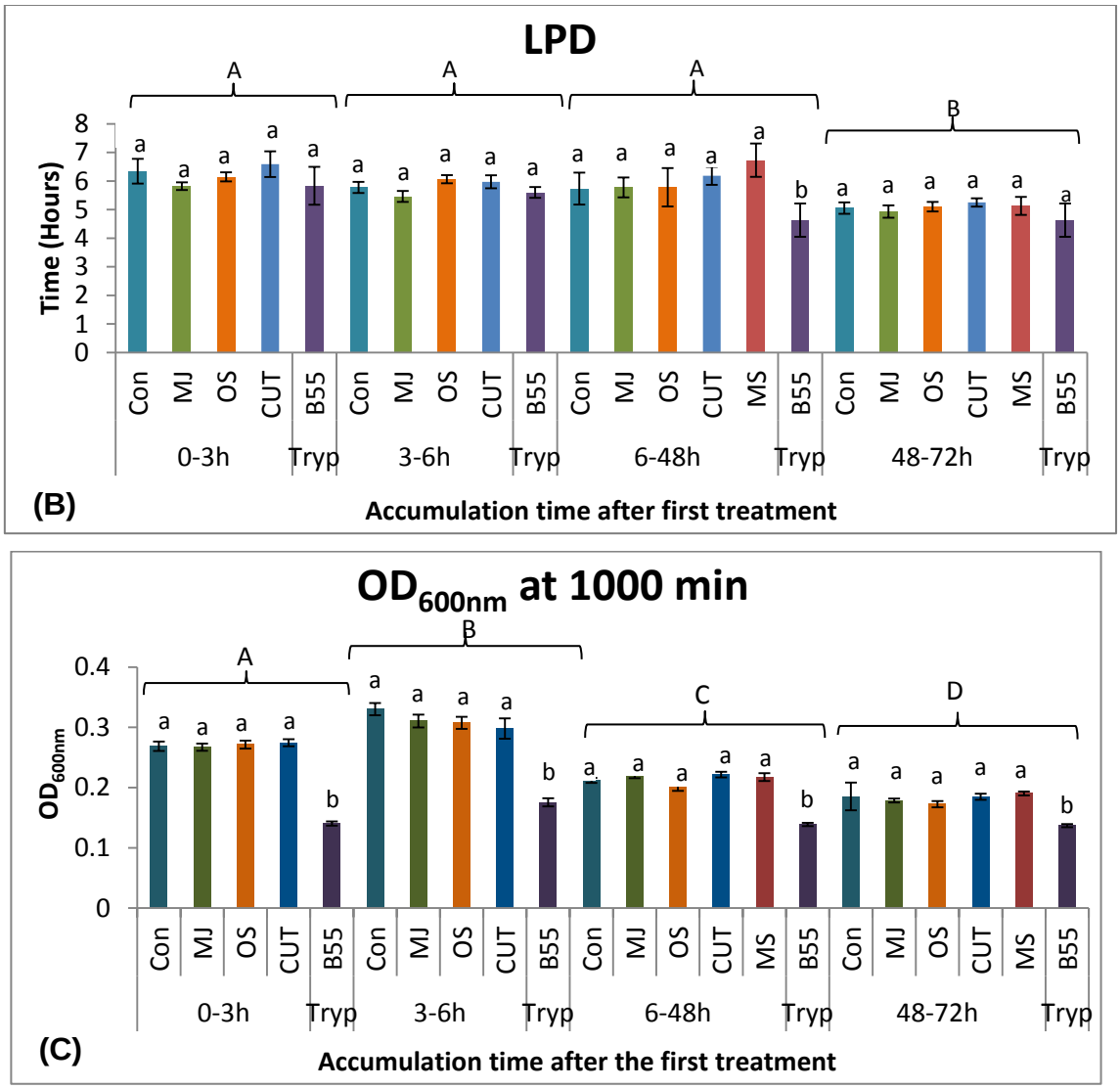


Figure 15. Uncorrected kinetic parameters **(A)** *Bacillus sp.* B55 GR during exponential phase. **(B)** *Bacillus sp.* B55 LPD and **(C)** *Bacillus sp.* B55 OD_{600nm} at 1000 min during the stationary phase. (Two way ANOVA test: uppercase letters refer to differences between time points and lowercase letters to differences between treatments; $P \leq 0.05$).

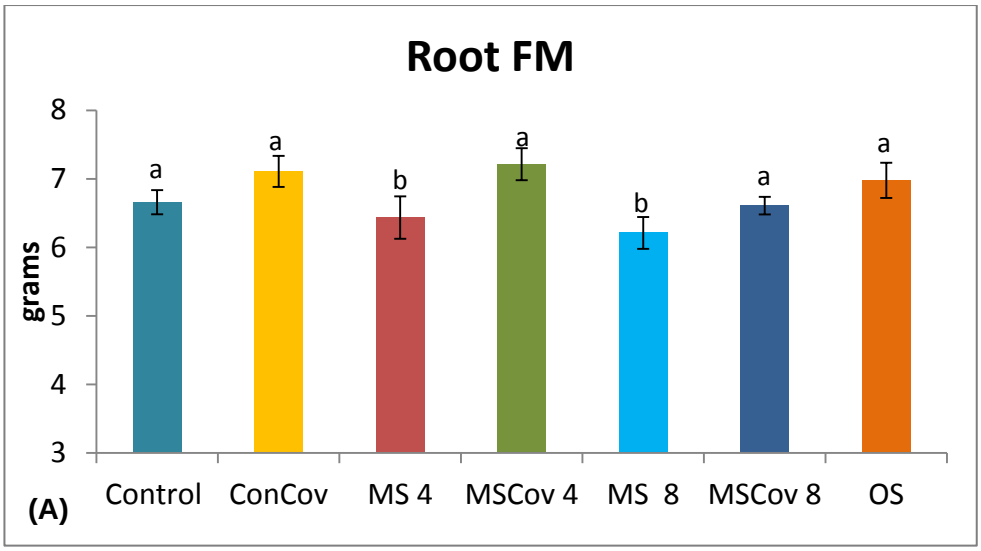
3.2.4 Fresh RE3

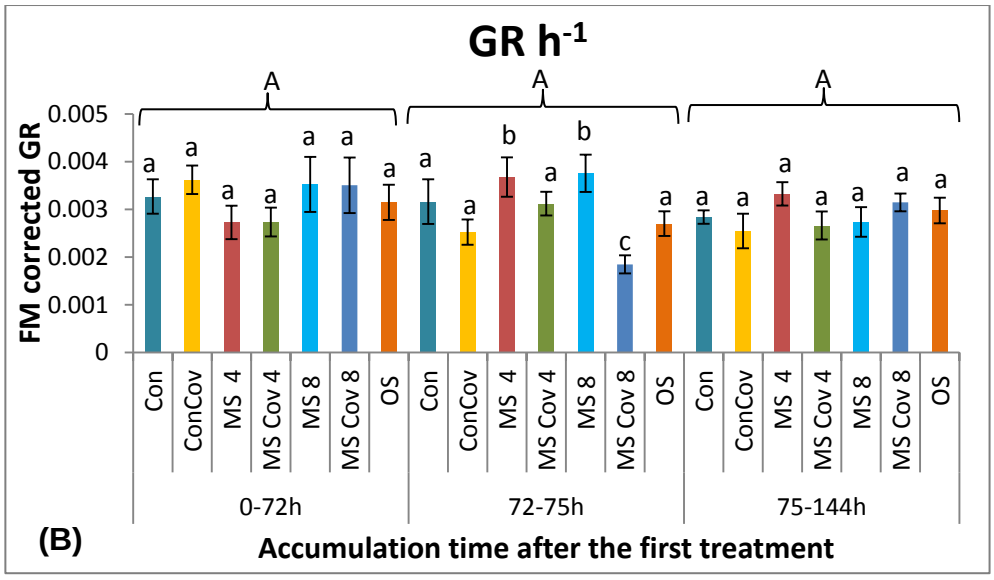
This experiment aimed at testing two variables that were not tested in the previous experiments:

- Two different levels of *M. sexta* infestation
- The effect of *M. sexta* frass on B55 growth

As can be seen in figure 17A, there was a statistically significant difference in the root FM (P = 0.027). Treated plants with 4 and 8 *M. sexta* larvae, had lighter roots than *M. sexta* covered treatments. Although the significant differences in

the root FM and in the FM corrected B55 growth kinetic parameters favored the uncovered treatments. For instance, the FM corrected GR for MS 4 and MS 8 at 72-75 h were higher than in other treatments (Figure 16B) and concerning the OD_{600nm} at 1000nm, MS 4 was higher than MS Cov 4 at 72-144 h (Figure 16D).





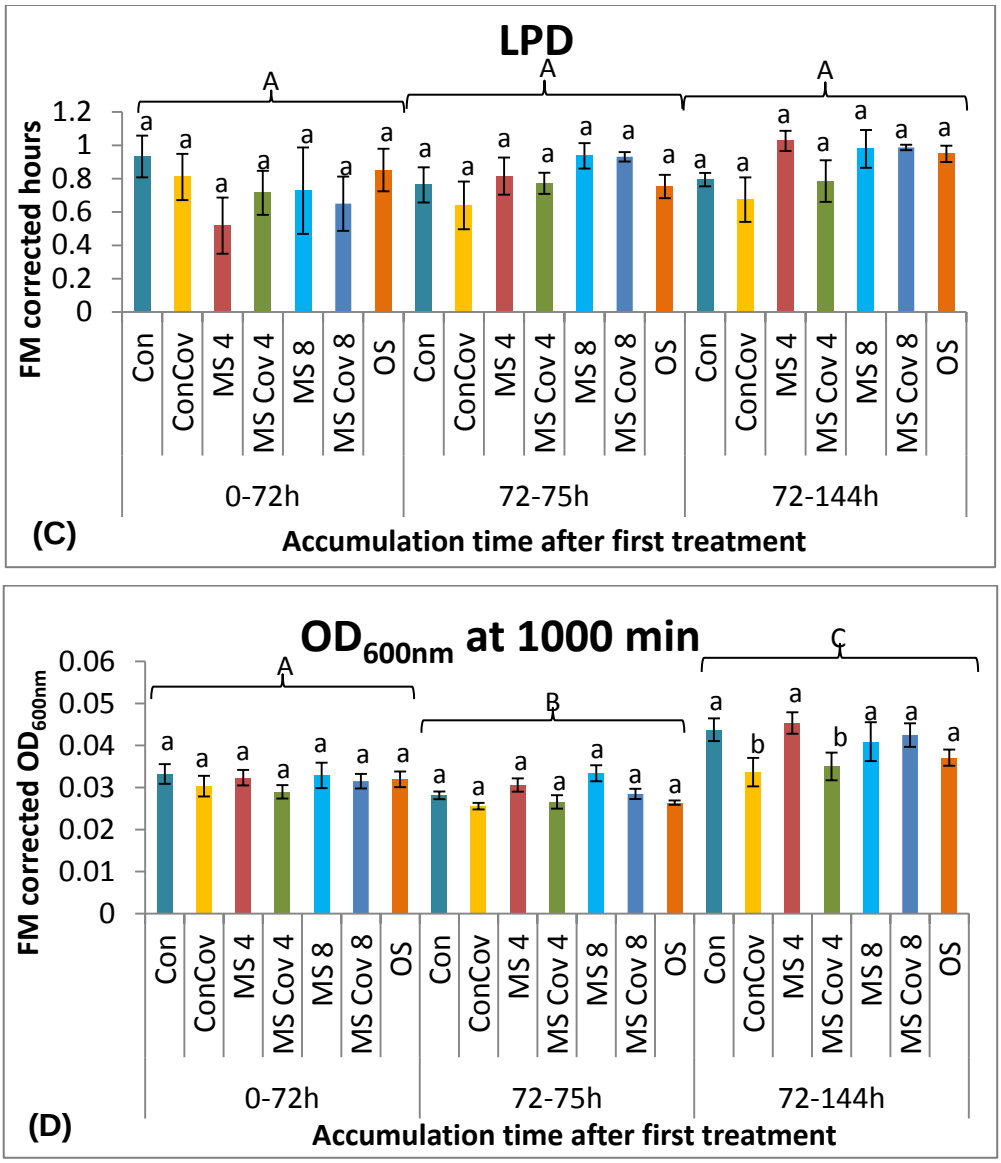


Figure 16. FM corrected kinetic parameters **(A)** Root FM **(B)** *Bacillus sp.* B55 GR during exponential phase. **(C)** *Bacillus sp.* B55 LPD and **(D)** *Bacillus sp.* B55 OD_{600nm} at 1000 min during the stationary phase. (Two way ANOVA test: uppercase letters refer to differences between time points and lowercase letters to differences between treatments; $P \leq 0.05$).

Figure 17 shows the uncorrected kinetic parameters where it can be observed that B55 GR was affected by the induction of leaves with *M. sexta*. MSCov 8 treatment displayed the highest GR ($P < 0.001$) at 0-72 h. However, this effect disappeared in the following time point of the experiment. Concerning the LPD, neither the treatments nor the sampling time did have an effect on B55 LPD and finally for the OD_{600nm} at 1000 min statistically significant differences were found ($P < 0.001$) between time points. As in the "Fresh RE2" experiment, the control with tryptone had a lower GR and OD_{600nm} at 1000 min confirming that RE enhance B55 growth.

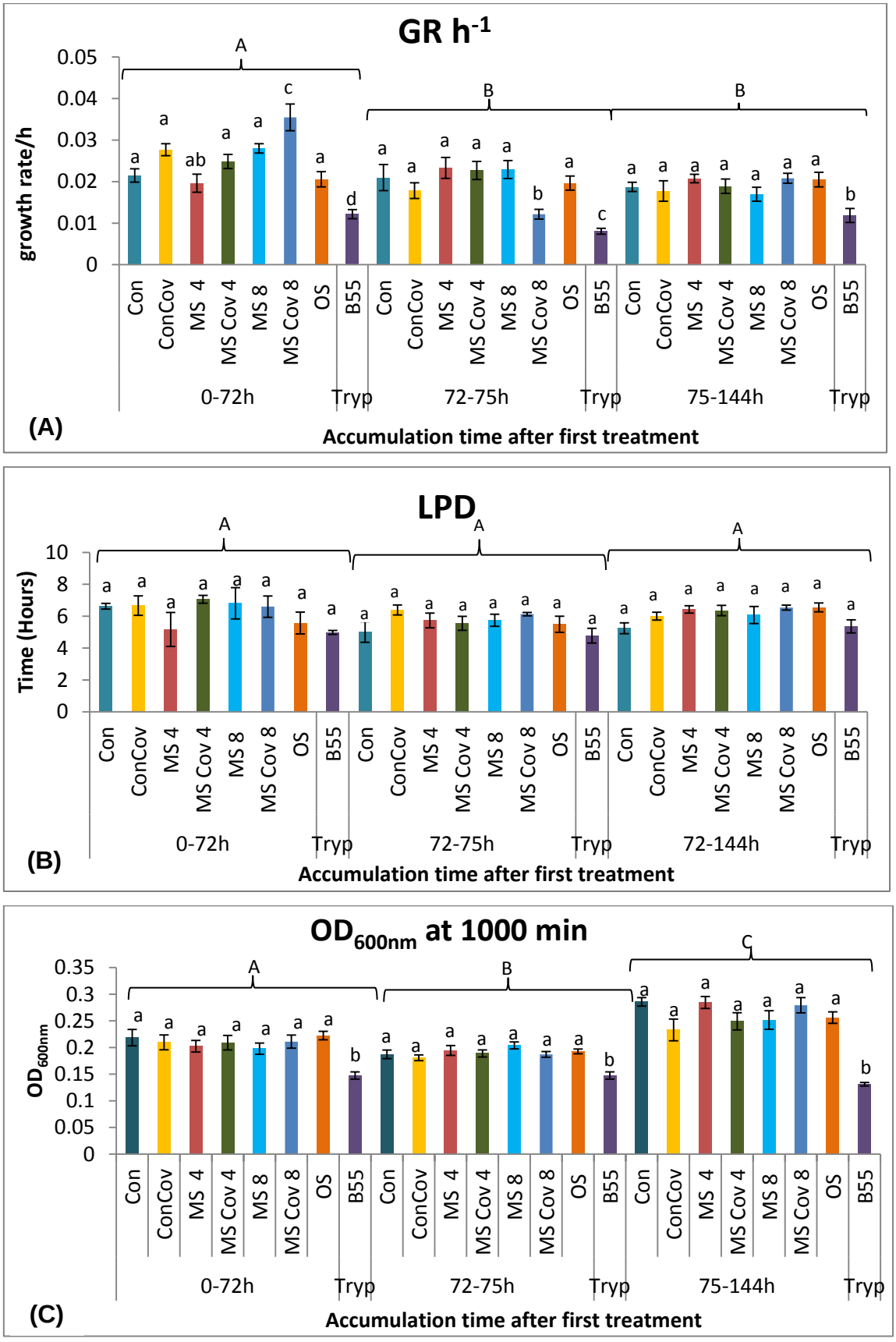
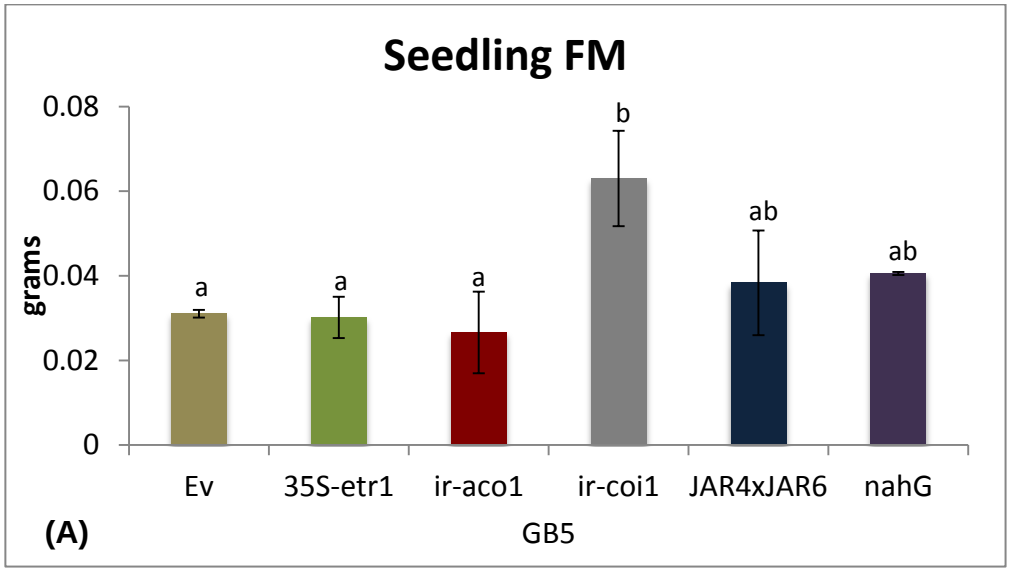


Figure 17. Uncorrected kinetic parameters **(A)** *Bacillus sp.* B55 GR during exponential phase. **(B)** *Bacillus sp.* B55 LPD and **(C)** *Bacillus sp.* B55 OD_{600nm} at 1000 min during

the stationary phase. (Two way ANOVA test: uppercase letters refer to differences between time points and lowercase letters to differences between treatments; $P \leq 0.05$).

3.3 Microtiter Plate Assay for Bacterial Growth on Root Exudates from Transgenic Lines.

As in the previous experiments performed with WT plants, B55 kinetic parameters of RE from transgenic lines were also corrected with the FM of the seedlings or the roots depending on the substrate (GB5 or sand). Figure 17 depicts the statistically significant differences in the FM of seedlings and roots. In GB5 all the seedlings grew homogenously except for *ir-coi1* line which grew outstandingly better than the other transgenic lines. In contrast, for sand grown plants the root FM of *35S-etr1* was extremely low in comparison to the other lines.



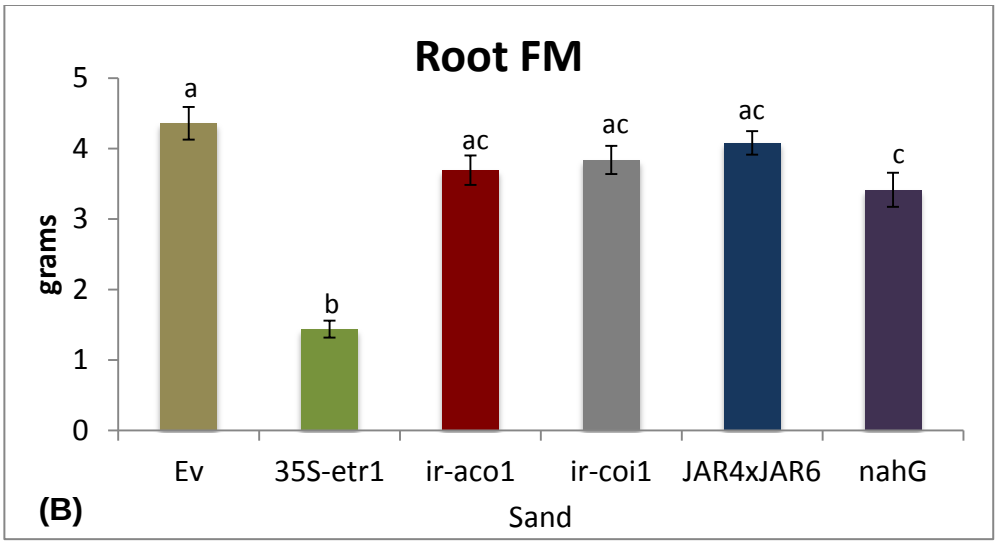
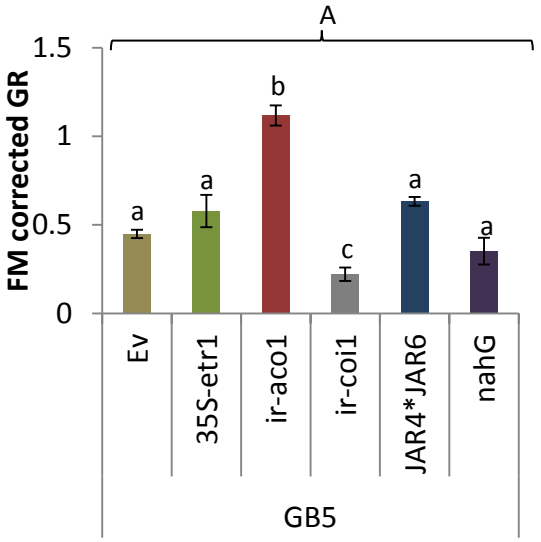
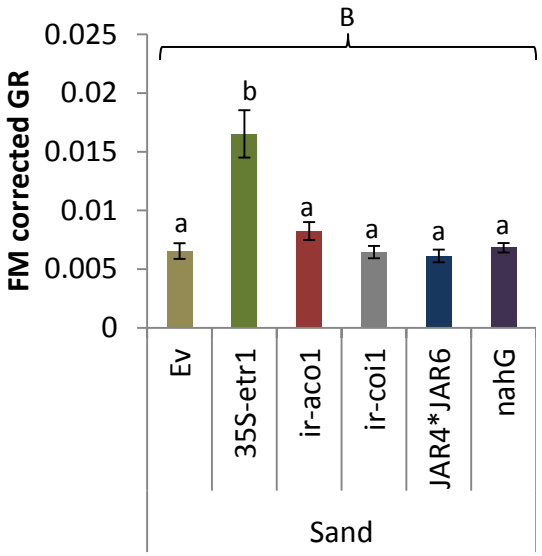


Figure 18. Seedling and root FM of transgenic lines **(A)** FM of seedlings. One biological replicate consisted of 3 pooled seedlings (n = 3x3) **(B)** Root FM of sand grown plants (n = 12).

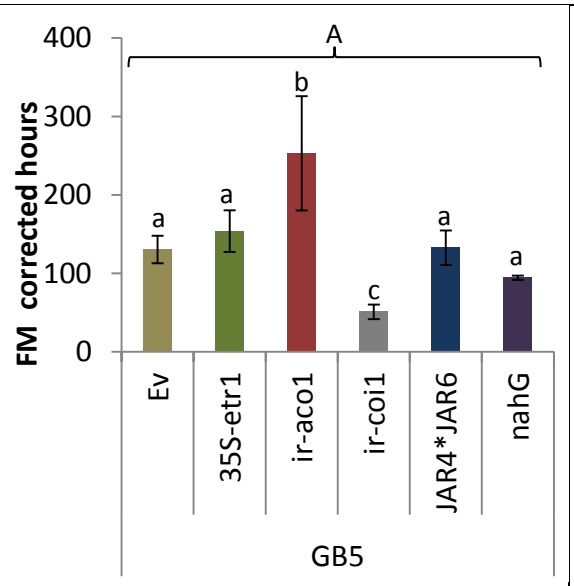
On the other hand, figure 19 shows that B55 growth kinetic parameters with RE from GB5 presented statistically significant differences for the transgenic lines *ir-aco1* and *ir-coi1*. In contrast, when the substrate is sand, *35S-etr1* presented the highest mean values. It is also worthy to mention that statistically significant differences were found in terms of the substrate used to grow the plants. Bearing in mind that seedlings have a much lower FM than roots from plants grown in sand, the original values of the kinetic parameters changed drastically.

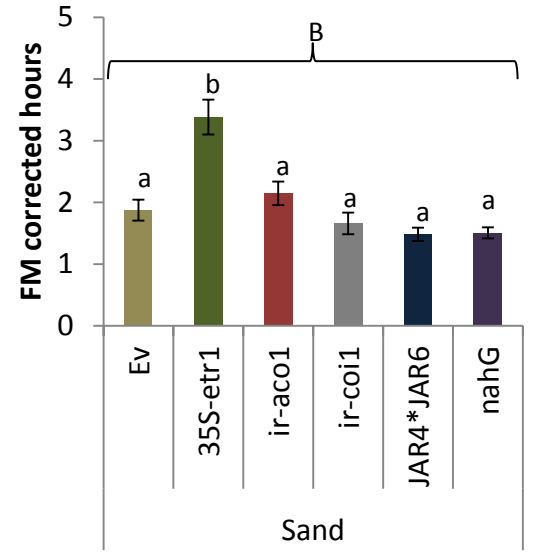
GR h^{-1}





LPD





OD_{600nm} at 1000 min

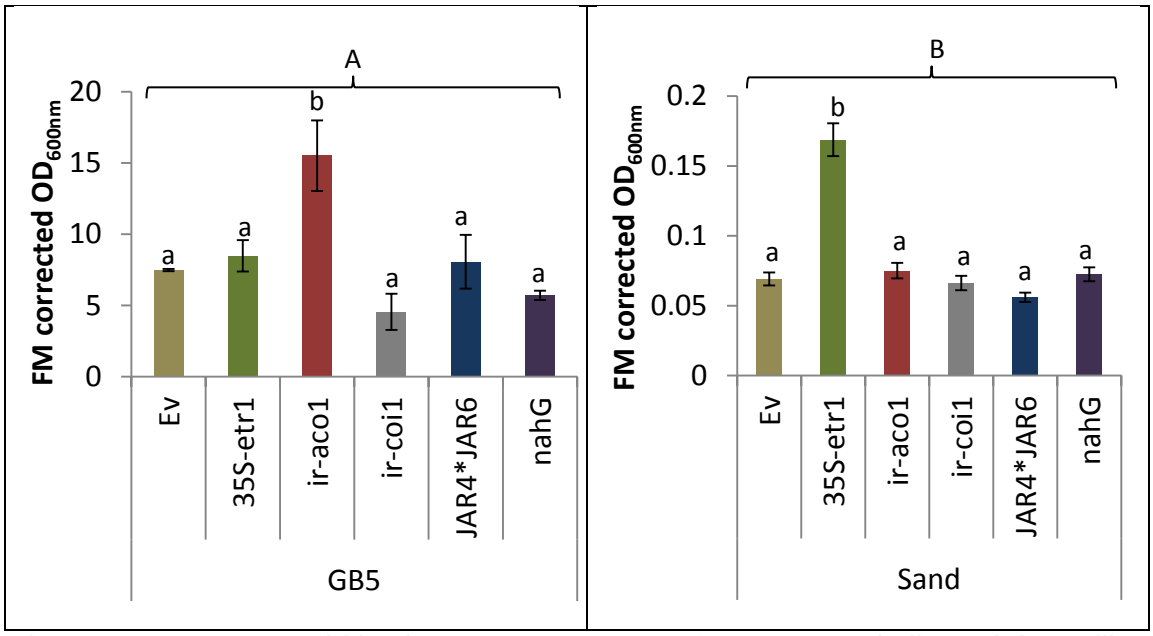
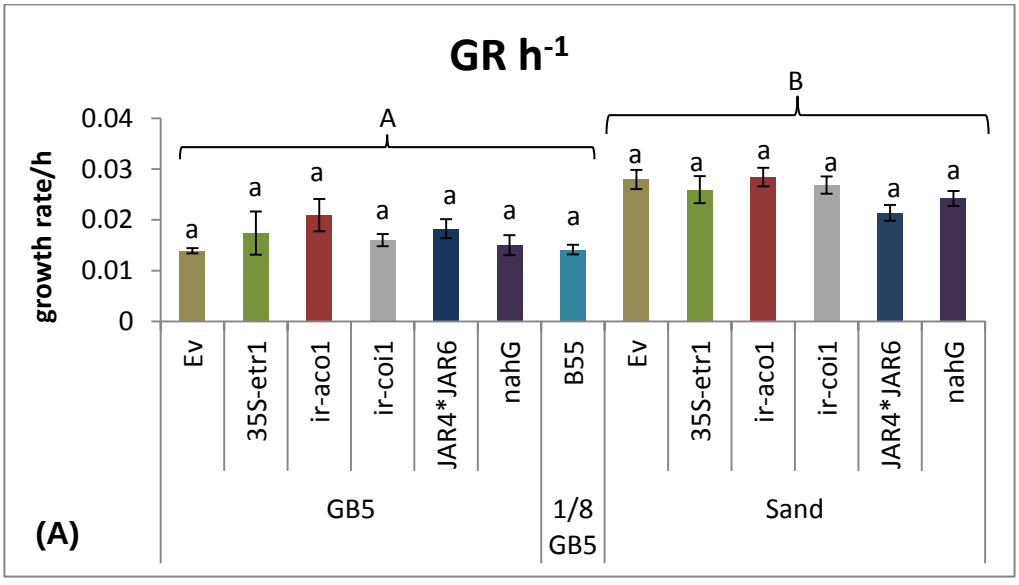


Figure 19. FM corrected kinetic parameters on RE from transgenic lines. **(A)** *Bacillus sp.* B55 GR during exponential phase in GB5 and sand **(B)** *Bacillus sp.* B55 LPD in GB5 and sand **(C)** *Bacillus sp.* B55 OD_{600nm} at 1000 min during the stationary phase in GB5 and sand. (Two way ANOVA test: uppercase letters refer to differences between substrate and lowercase letters to differences between transgenic lines; $P \leq 0.05$).

In figure 20, the uncorrected kinetic parameters are shown. Statistically significant differences between substrates for GR and LPD were found ($P < 0.001$). Concerning the OD_{600nm} at 1000 min, there were statistically significant differences among transgenic lines in sand as substrate ($P = 0.006$). It is also noteworthy that when B55 was grown on 1/8 GB5 without RE, there were not statistically significant differences in comparison with the kinetic parameters from transgenic lines. Thus, B55 was able to grow on GB5 as a sole carbon source.



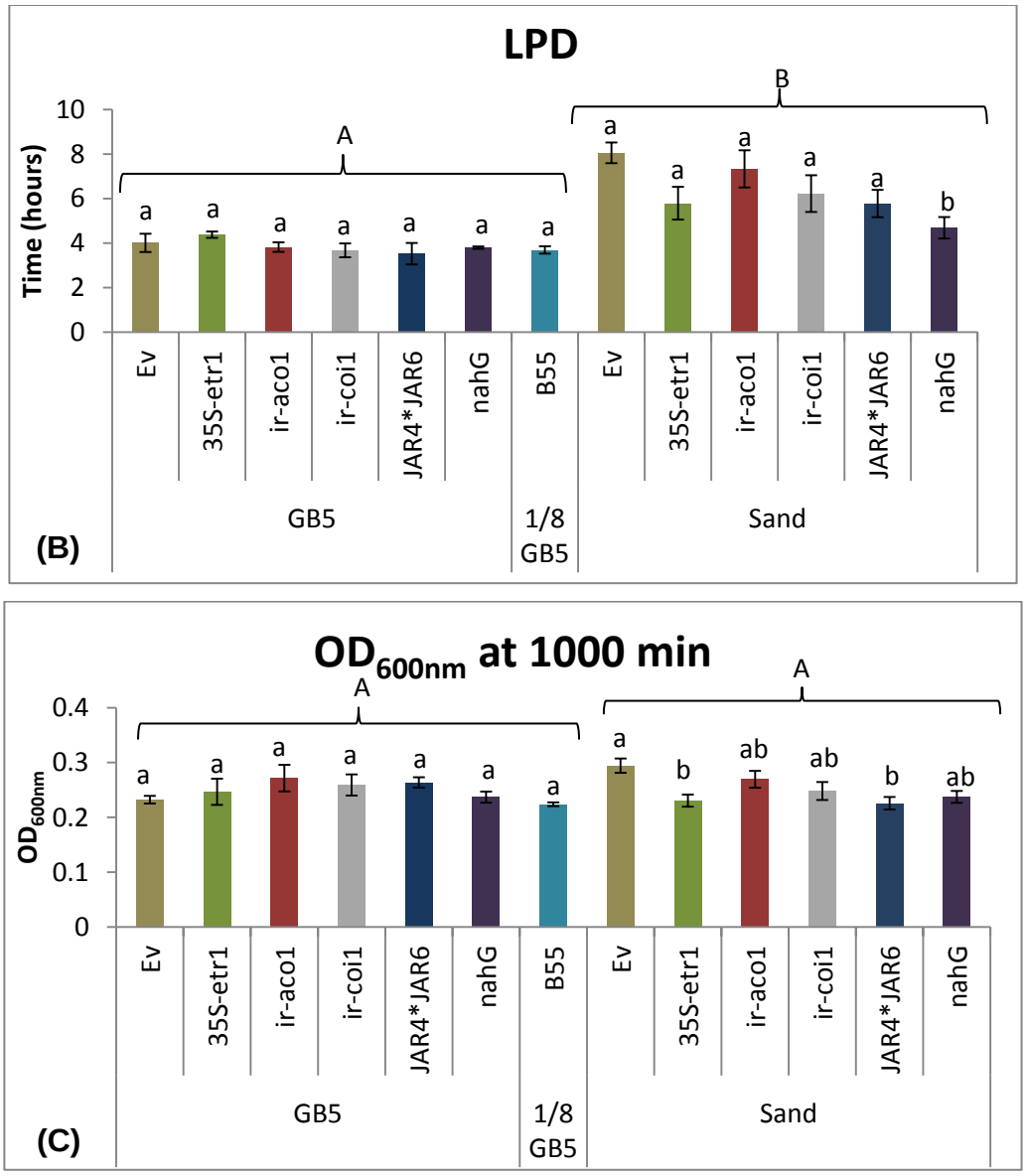


Figure 20. Uncorrected kinetic parameters on RE from transgenic lines. **(A)** *Bacillus sp.* B55 GR during exponential phase in GB5 and sand **(B)** *Bacillus sp.* B55 LPD in GB5 and sand **(C)** *Bacillus sp.* B55 OD_{600nm} at 1000 min during the stationary phase in GB5 and sand. (Two way ANOVA test: uppercase letters refer to differences between substrate and lowercase letters to differences between transgenic lines; $P \leq 0.05$).

3.4 Motility Assay

As it is shown in figure 21, the positive and negative controls were useful to observe if *Bacillus sp.* B55 was motile or not. The clear, sharp red line of bacterial growth present in tubes B and C proves the lack of movement of *Bacillus sp*. B55 and *C. glutamicum.* In contrast, the fuzzy, indistinct red line of

bacterial growth in tube A shows that the motile *E. coli* has moved from its initial inoculation along a single stab line outward in all directions.

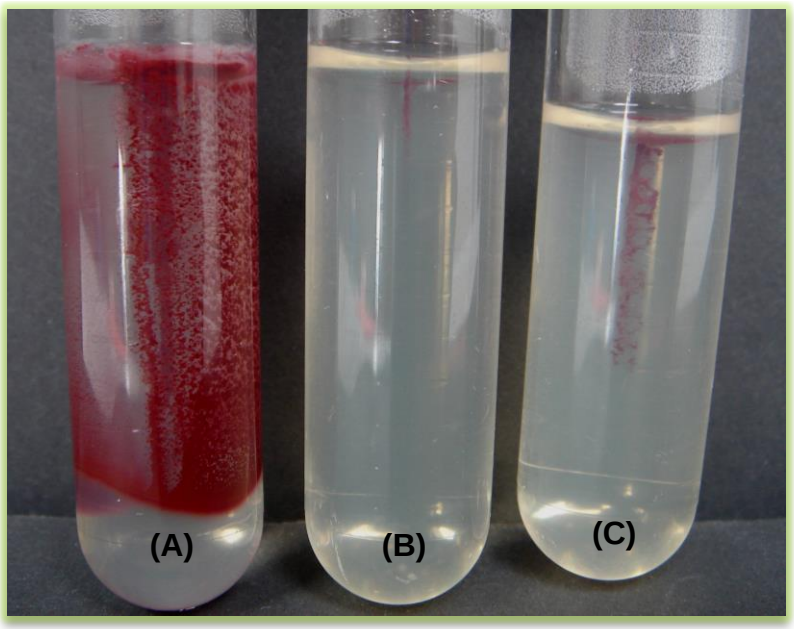


Figure 21. Motility test medium supplemented with TCC salts. The red areas indicate the presence of bacteria upon TCC reduction. **(A)** *E. coli* as positive control for motility **(B)** *Bacillus sp*. B55 **(C)** *C. glutamicum* as negative control for motility.

3.5 Swarming Plate Assay

Figure 22 shows three different pictures of swarm plates. *E. coli* was able to swarm outwards the center of the petri dish unlike *Bacillus sp*. B55 and *C. glutamicum.* By using this qualitative assay *E. coli*, showed a clear motility alone the tryptone plate in comparison to the other two strains. *Bacillus sp.* B55 and *C. glutamicum* did not display swarming and they just were able to grow around the spot of inoculation.

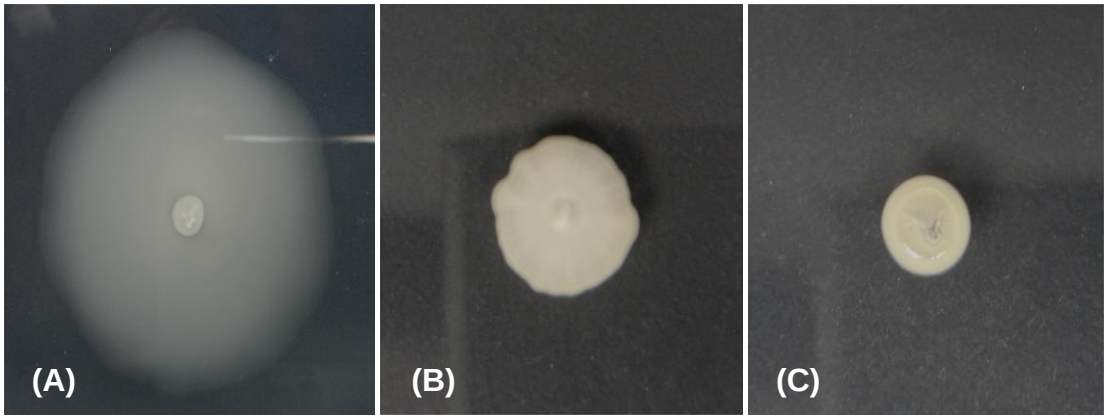


Figure 22. Pictures of swarms produced by cells of **(A)** *E. coli* **(B)** *Bacillus sp.* B55 and **(C)** *C. glutamicum* on tryptone 1% semisolid plates. Cells were inoculated at the center

of tryptone 1% swarm plates containing 0.3% agar and photographed after 72 h incubation.

3.6 Capillary Assay

Although the swarming plate assay is a convenient and standard approach to characterize chemotactic behaviors, it is often difficult to interpret. The main difficulty lies in the fact that swarming behavior of bacteria is accompanied with cell growth (Belas et al., 1991). These conditions make the swarming assay difficult to read because the results are due to chemotaxis or the growth of bacteria or both. Hence we carried out the capillary assay decoupled from the growth of bacteria. Figure 23 shows that *Bacillus sp.* B55 entered the capillaries of the device regardless of the substrate tested.

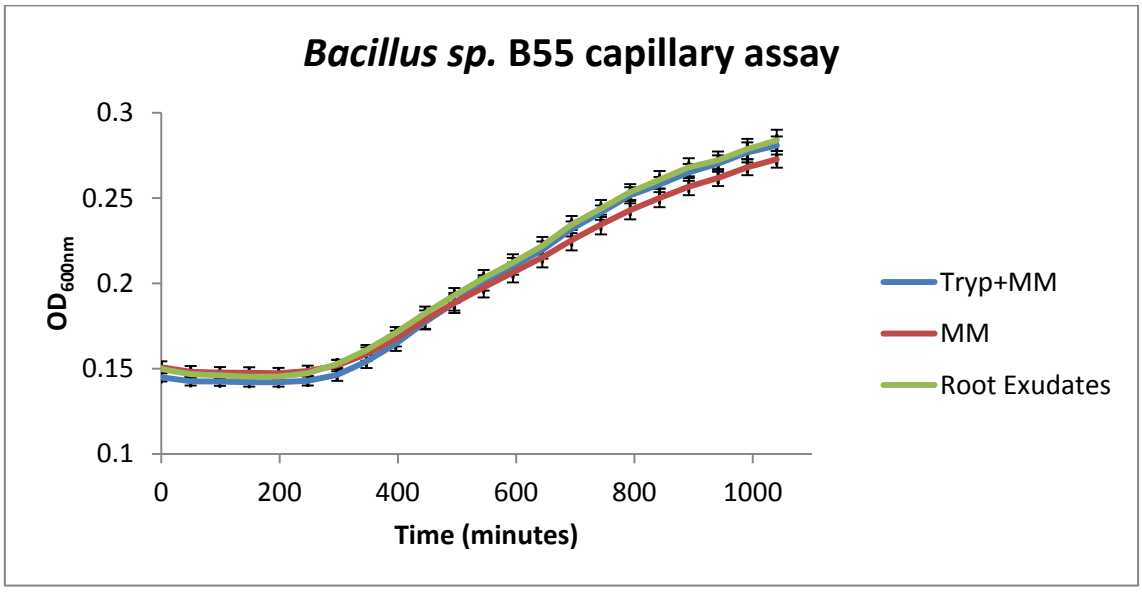


Figure 24. *Bacillus sp.* B55 chemotaxis assay using the capillary device. Tryp+MM: *Bacillus sp.* B55 in tryptone+motility medium and MM: *Bacillus sp.* B55 in motility medium.

Figure 24 shows the capillary assay using *E. coli* (positive control of chemotaxis). According to these results, the capillary assay was not reliable since it did not work either for the positive control. *E. coli* entered all the capillaries including the negative control with MM.

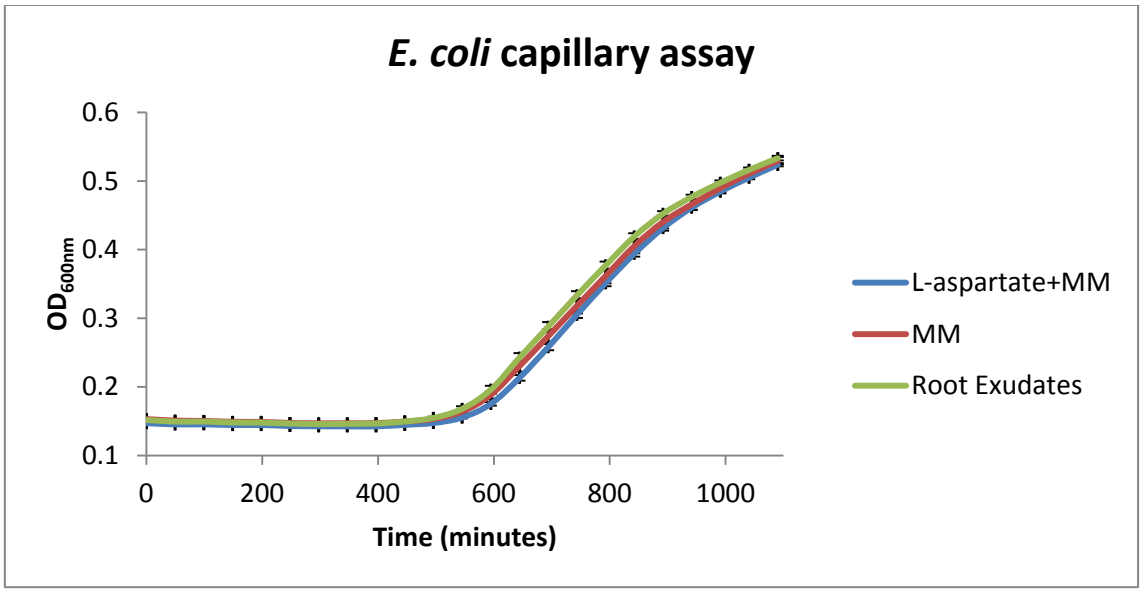


Figure 24. *E. coli* chemotaxis assay using the capillary device. L-aspartate+MM: *E. coli* in L-aspartate 10^{-2} M+ motility medium and MM: *E. coli* in motility medium.

4. General Discussion:

Plant-microbe interactions in the rhizosphere are mediated by signaling compounds and RE are thought to play an important role in determining microbial abundance. Meldau in 2011, (data no published) observed that B55 re-isolation from WT rhizosphere was statistically significant lower when leaves were induced by cutting, *M. sexta* and MJ application (Figure 25). Therefore, it was hypothesized that there was most likely a signal from induced WT plants to repel B55 in the rhizosphere.

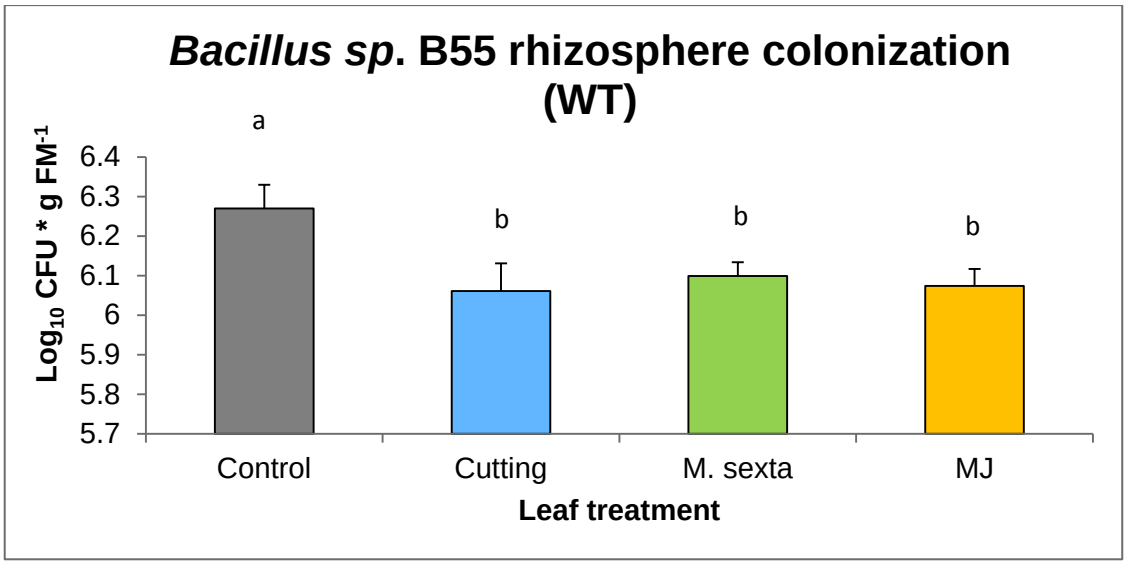


Figure 25. *Bacillus sp*. B55 rhizosphere colonization in WT plants of *N. attenuata.*

My results suggest that herbivory-mediated changes in RE composition are most likely not responsible for the reduced B55 colonization of herbivore-induced plants observed in previous experiments. Microbial kinetic parameters analyses (GR, LPD and OD_{600nm} at 1000 min) did not follow a clear pattern among the experiments and statistically significant differences concerning time points were more frequently found than differences between treatments. For instance, in the set of samples Fresh RE 3 there were significant differences between treatments at 72-75 h, but this effect disappeared at 75-144 h. Hence, other plant-specific factors such as herbivory-induced changes in root architecture and surface structure may participate in B55 root colonization. Furthermore, volatile organic compounds (VOCs) like carbon dioxide, alcohols

and aldehydes which are also part of the RE (Ortiz-Castro et al., 2009) and can attract or repel bacteria from a distance (Minerdi et al., 2008) might have been involved also in B55 reduced root colonization. However, these compounds were not analyzed in this study.

The kinetics of growth indicated fast utilization of the RE by *Bacillus sp.* B55. Assuming that rhizobacterial relative growth rates can range from 0.029 to 0.05 h^{-1} (Watt et al., 2006), the GR from the experiments was similar to this range with values between 0.015 and 0.035 h^{-1} for the uncorrected GR. The enhancement on growth exerted by RE could be observed when kinetic parameters of RE were compared with parameters of tryptone as sole nutrient source (Figures 15A and 17A). Furthermore, B55 can be classified as a "fast grower bacterium" according to the criteria used by De La Fuente et al. (2007), who tested different strains of *Pseudomonas fluorescens* on seed and RE from wheat and made a distinction between strains with lag phases shorter than 7 h ("fast growers") and other strains with lag phases between 16 and 22 h ("slow growers"). Thus, B55 possess one of the major competitive traits for being a PGPB which is a high GR in RE (Lugtenberg and Kamilova, 2009).

On the other hand, the OD_{600nm} at 1000 min showed that "youngest" RE (0-3 h, 3-6 h and 72-75 h accumulation time after the first treatment) might have been more nutritious in comparison to the "oldest" RE (0-72 h and 48-72 h accumulation time after the first treatment). This excess of nutrients might have came from the fertilizer since pots were rinsed once or twice to collect the "youngest" RE before collecting the "oldest". For instance, Figure 15C shows that the OD_{600nm} at 1000 min was higher for the 0-3 h and 3-6 h in comparison to 6-48 h and 48-72 h. It can be expected that with prolonged accumulation time, microbial populations grow assimilating the available C sources and reducing RE quantity and quality for B55. Another possibility is that "oldest" RE may contain antimicrobial plant secondary metabolites (Flores, 1999; Dixon, 2001), which might have reduced B55 growth.

Concerning the experiments performed using transgenic lines, the FM corrected kinetic parameters showed that B55 performed best on RE from *ir-aco1*

seedlings grown in GB5 (Figure 19). Even though a chemical characterization of the RE was not performed in this study, most likely ET deficient plants might have a higher flavonoid concentration in the roots as it was reported by Buer et al. (2006) who observed that ET signaling modulates flavonoid accumulation in *A. thaliana* roots. Similarly, RE from *35S-etr1* plants grown in sand were more nutritious for B55 (Figure 19) comparing to the other transgenic lines. Although, the root FM of these plants was the lowest, it seems that the quality of these RE has a positive effect on B55 growth. Long et al. (2010) also reported that *35S-etr1* line produces few root hairs and lateral roots, as it was observed in this study, but they also observed a different community composition and colonization pattern by root endophytes in this transgenic line. Likewise, Meldau et al. (2012) who worked with B55 observed that this strain colonized almost ten-fold the endosphere of *35S-etr1* seedlings in comparison to WT. From these results they hypothesized that uncharacterized changes in root metabolomics and exudates could explain the higher endophytic colonization. Thus, I demonstrated here, that RE from ET-deficient plants play an important role as substrates for B55 growth. However, the direct effect of these RE on other endophytes remains to be tested.

In contrast, as is shown in Figure 18A, *ir-coi1* seedlings grew outstandingly better on GB5 compared to the other transgenic lines. One would expect that seedlings whose FM was higher, might have a greater effect on B55 growth since bigger roots produce a higher quantity of RE. Nonetheless, B55 did not perform better on RE from *ir-coi1* seedlings and this might be due to the possibility that JA signaling does not have any effect on the root exudation pattern of *Nicotiana attenuata.* This finding does not go in accordance with Badri et al. (2008) who reported that JA and SA can mediate changes in the composition of RE in *A. thaliana.* Therefore, it is possible to say that this signaling-mediated changes on RE are also plant species-dependant.

During this study two different approaches for collecting the RE were used. The first and the closest to the natural conditions, was the RE collection from plants grown in sand and the second and most widely used on root exudation studies, was the RE collection from seedlings grown in liquid culture. Obtaining RE is

tedious and in most studies they are collected in axenic liquid culture, which lack from all the natural cues and exclude the effect that the native microbial community might have on the colonization pattern of certain PGPB. Therefore, care should be taken when extrapolating results obtained using exudates to explain what occurs in the rhizosphere (Bertin et al., 2003; Jaeger et al., 1999; Rovira, 1969). Many factors affect the amount and composition of RE, including the plant species, age, nutrition and root damage (Rovira, 1969), the presence of microorganism, the culture solution (Groleau-Renard et al., 2000; Rovira, 1969) and the presence of microbial metabolites (Philips et al., 2004; Walker et al., 2003). Because microorganisms are known to increase the amount of exudates fourfold (Rovira, 1969) and to solubilize different compounds (Groleau-Renard et al., 2000) results from a non-sterile approach should be used to achieve a more precise picture of what really happens in the rhizosphere.

A number of plant associated-bacteria have been shown to have a chemotactic response to plant root and seed exudates, including the symbiotic bacteria, *Rhizobium* spp. (Currier and Strobel, 1976) and *Bradyrhizobium japonicum* (Barbour et al., 1991); and potential bacterial biocontrol agents like *Pseudomonas* spp. (De Weert et al., 2002). The motility test and the swarming plate assay performed in this study showed that *Bacillus sp.* B55 was non motile neither a chemotactic bacterium. According to Cleary et al. (2002) most *Bacillus* species are motile; therefore *Bacillus sp*. B55 may have undergone a spontaneous mutation which could affect the motility and chemotactic behavior of the strain (Dr. Liyan Ping, personal communication, July 9, 2012). Due to the lack of motility of B55, most likely the cells reach *Nicotiana attenuata* roots by passive carriage or other mechanism like adsorption, anchoring by pili or agglutination (Brimecombe et al., 2007).

5. Conclusions

- There was no consistent effect of the RE on *Bacillus sp*. B55 growth among the different experiments performed with WT plants.
- *Bacillus sp.* B55 grew better on “youngest” RE than on “oldest” RE.
- Root exudates from transgenic lines impaired in production and perception of ET (*ir-aco1* and *35S-etr1*) have a positive effect on *Bacillus sp*. B55 growth depending on the growth medium and growth state of the plants.
- By means of the motility test and swarming plate assay used in this study, it is possible to conclude that *Bacillus sp*. B55 is a non-motile bacterium which may have undergone a spontaneous mutation. Nevertheless, to confirm this hypothesis a molecular analysis is necessary.

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Selbstständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und nur unter Verwendung das angegebenen Hilfsmittel und Literatur angefertigt habe.

Jena, November. 2012

María del Pilar Bonilla Arbelaez