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MASTER THESIS

Influence of root-colonizing bacteria on root architecture of *Nicotiana attenuata.*

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Abstract:

Bacterial plant growth regulators such as auxins, cytokinins and ethylene can change root growth and morphology. To date, very little is known about this phenomenon in native plant-bacteria interactions. The main objective of this study was to investigate the impact of native root colonizing bacteria of *Nicotiana attenuata* on its root architecture. To this end, bacterial strains isolated from the roots of field grown jasmonate (JA)-deficient irAOC and jasmonate competent empty vector (EV) *N. attenuata* plants were studied. First, the impact of 16 different bacterial strains on root architecture of irAOC and EV plants was investigated *in vitro* and under glass house conditions. Second, the ability of the different strains to produce indole-3-acetic acid (IAA), ethylene, salicylic acid (SA), abscisic acid (ABA) and jasmonic acid (JA) was tested. Third, the diffusion of IAA from bacterial cultures into the environment was studied *in vitro*. Finally, principal component analysis (PCA) was used to compare the changes in root architecture due to bacterial and exogenous application of hormones.

From this study, it has been found that several of the tested bacterial strains influence the root architecture of *N. attenuata* seedlings *in vitro* and in the glass house condition. *In vitro*, the strongest effects were observed in terms of primary root length and growth direction as well as lateral root density. EV and irAOC plants generally responded in a similar manner. However, for a subset of bacteria, plant genotype specific responses were observed: The most striking effect was that irAOC primary root bent more strongly than EV when growing towards certain bacterial patches. Hormonal screening revealed that several bacterial strains produce significant amounts of ethylene, IAA and SA, but not JA and ABA. IAA was also found to be released into the growth medium, indicating active secretion. Multivariate analysis of all analyzed root traits revealed a clustering of root systems exposed to high ethylene and IAA producing bacteria, indicating a potential role of these two hormones in root architecture modification. However, the application of individual synthetic hormones to the growth medium was not sufficient to mimic the observed root phenotypes, suggesting that as yet unknown bacterial components may be necessary to modulate root architecture. Taken together, this study demonstrates the potential of native bacteria to influence root

architecture of *N. attenuata*, with potential consequences for water and nutrient uptake and ultimately plant reproductive output in nature.

Zusammenfassung:

Bakterielle Pflanzenwachstumsregulatoren wie Auxine, Cytokenine und Ethylen haben die Fähigkeit, das Wurzelwachstum und die Wurzelmorphologie zu ändern. Hauptziel dieser Arbeit war es, den Einfluss der heimischen Bakterien von *Nicotiana attenuata* auf deren Wurzelarchitektur zu untersuchen. Dazu wurden Bakterienstämme untersucht, welche aus den Wurzeln von irAOC (Jasmonat (JA)-defizient) und EV (transgene Kontrolle) *Nicotiana attenuata* Pflanzen isoliert wurden. Zuerst wurde der Einfluss von den 16 verschiedenen isolierten Bakterienstämmen auf die Wurzelarchitektur von irAOC- und EV-Pflanzenlinien von *N. attenuata* untersucht, sowohl unter *in vitro*- als auch unter Glashausbedingungen. Zweitens wurde getestet, ob diese Bakterienstämme die Fähigkeit zur Produktion von Indol-3-essigsäure (IAA), Ethylen, Salicylsäure (SA) und Jasmonsäure (JA) besitzen. Des Weiteren wurde das Diffusionsverhalten von bakteriell gebildetem IAA in die Umgebung untersucht. Schlussendlich wurde noch eine Hauptkomponentenanalyse (PCA) durchgeführt, um Veränderungen der Wurzelarchitektur – hervorgerufen durch die bakterielle Aktivität – mit jenen Veränderungen zu vergleichen, die durch extern hinzugefügte Phytohormone (*in vitro*) verursacht wurden.

Aus dieser Studie wurde herausgefunden, dass einige der untersuchten Bakterienstämme beeinflussen die Wurzelarchitektur von *N. attenuata* Sämlinge *in vitro* und in dem Glashausbedingung. *In vitro* wurden die stärksten Effekte bei der Primärwurzellänge und Wachstumsrichtung sowie Seitenwurzeldichte beobachtet. EV und irAOC Pflanzen reagierten im Allgemeinen in einer ähnlichen Weise. Doch für eine Teilmenge der Bakterien, Pflanzen- Genotyp spezifische Reaktionen beobachtet: Der auffälligste Effekt war, dass irAOC Primärwurzel gebogen stärker als EV beim Anbau auf bestimmte bakterielle Patches. Hormonelle Screening ergab, dass einige Bakterienstämme erzeugen erhebliche Mengen an Ethylen, IAA und SA, aber nicht JA und ABA. IAA wurde auch gefunden, in das Wachstumsmedium freigesetzt werden, was auf aktive Sekretion. Multivariate Analyse aller untersuchten Wurzelmerkmaleergab eine Häufung von Wurzelsystemen hohe Ethylen und IAA -produzierenden Bakterien ausgesetzt, was auf eine mögliche Rolle dieser beiden Hormone im Wurzelarchitektur

Modifikation. Die Anwendung der einzelnen synthetischen Hormonen in das Wachstumsmedium war jedoch nicht ausreichend, um die beobachteten Phänotypen Wurzel imitieren, was darauf hindeutet, dass noch unbekannten Bakterienkomponentenkann notwendig sein, um die Wurzelarchitektur zu modulieren. Zusammengenommen zeigt diese Studie das Potenzial von nativen Bakterien Wurzelarchitektur von *N. attenuata* zu beeinflussen, mit möglichen Folgen für die Wasser -und Nährstoffaufnahme und letztlich zu pflanzen Reproduktionsleistung in der Natur.

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1. INTRODUCTION:

1.1 Plant-bacteria interaction:

Both abiotic and biotic factors influence growth and development of terrestrial plants (Vandegehuchte et al., 2010). A remarkable example for biotic determinants of plant growth and development are soil bacteria (Bonfante and Anca, 2009). Conversely, plants influence underground microbial communities by means of selective rhizodeposition leading to the recruitment of specific bacteria (Hartmann et al., 2009). From a plant's perspective, interactions with bacterial communities can either be beneficial (Meldau et al., 2012), neutral or pathogenic (Block et al., 2005; Hückelhoven, 2007). Examples of beneficial plant-bacteria interactions are: 1) bacteria that can promote plant growth by fixing nitrogen without any organ formation, 2) nodule forming nitrogen fixing bacteria, 3) endophytic bacteria that improve plant development. Based on the benefits they elicit in the plants, some of these bacteria are termed plant growth promoting bacteria (PGPB). The mechanistic basis of PGP has been documented extensively (Bisseling et al., 2009; Hartmann et al., 2009).

However, apart from promoting growth, bacteria can also influence root architecture (López-Bucio et al., 2007). Since root architecture plays an important role in plant fitness and productivity (Lynch, 1995), these effects are of potential ecological relevance. Modification of root architecture can lead to an increase in absorption of water and nutrients or and help plants to endure stress conditions such as drought and disease (Lynch and Ho, 2005). However, so far most studies on this aspect have been conducted on artificial combinations of plants and bacteria, and little is known about the interplay in native systems (Meldau et al., 2012).

1.2 Interaction of endophytic bacteria with plants:

Bacteria that colonize the internal tissues of plants are termed endophytes. These endophytes can either be harmful or beneficial to plants (Hallmann et al., 1997). Although many bacterial communities can be found in the soil, studies report specific recruitment of endophytes by plants (Rudrappa et al., 2008). For instance recruitment of actinobacteria and proteobacteria to the rhizosphere of *Arabidopsis thaliana* was observed (Lundberg et al., 2012). Endophyte abundance varies not only from plant to plant but also within tissues of the same plant (Mano et al., 2007). Endophytes are believed to promote plant growth either indirectly by helping in nutrient like phosphate uptakes (Wakelin et al., 2004) and inducing plant resistance against pathogens (van Loon et al., 1998) or directly by shielding the plant from pathogens (Bent and Chanway, 1998). Endophytes can produce plant growth regulators and can thereby influence plant root architecture (Camehl et al., 2010; Spaepen and Vanderleyden, 2011).

1.3 Bacteria producing plant growth regulators:

Bacteria can produce plant growth regulators, including auxin (IAA- indole 3- acetic acid) which has been reported in many studies in recent years (Spaepen and Vanderleyden, 2011). Over 80% of the bacteria strains from the rhizosphere have been reported to be capable of synthesizing IAA production (Khalid et al., 2004). The ability of auxin to form adventitious roots in plants is well described (Geiss et al., 2009), and bacterial IAA may therefore change root architecture. In general, the communication between host plants and microorganisms occurs through secretion of signaling factors in the form of metabolites or volatile organic compounds. Apart from auxin (Spaepen and Vanderleyden, 2011), other plant growth regulators such as ethylene (Camehl et al., 2010; Glick et al., 2007) and jasmonic acid (Sun et al., 2006) represent an additional classes of signaling molecules that influences beneficial plant-bacteria interactions. The impact of bacterial plant growth regulators on root morphogenesis includes over production of root hair and lateral roots (Persello-Cartieaux et al., 2003), which may lead to increased plant performance in challenging environments (Lynch and Ho, 2005).

1.4 Role of auxin, ethylene and jasmonate in plant root growth:

An influence of plant hormones on plant- microbe interaction was reported 70 years ago already (MacDougal and Dufrenoy, 1944). Since then, the role of auxin and ethylene as growth regulators of plants has been discussed in numerous reviews, including aspects of auxin synthesis, transport and signaling (Overvoorde, Fukaki, and Beeckman 2010). In general, the role of auxin in root development can be defined as an inhibition of root elongation and promotion of lateral root development (De Smet, 2012). Ethylene on the other hand influences root hair formation and elongation (Alonso et al., 2003). Though jasmonate (JA) known for its defensive mechanism in plants (Paschold et al., 2007), it is also known for influence in plant root growth (Raya-González et al., 2012). As far now, jasmonate signaling in plants was known for its root inhibition behavior (Staswick et al., 1992) especially reduction of primary root and lateral root positioning in *Arabidopsis thaliana* (Raya-González et al., 2012).

1.5 *N. attenuata* and its interaction with bacteria:

Nicotiana attenuata is a wild tobacco species native to the Great Basin Desert located in eastern North America. Seeds germinate in post-fire environments that are characterized by nitrogen rich soil patches (Lynds and Baldwin, 1998). These soil conditions support plant growth and play a critical role in bacterial community enrichment. Previous studies on the interaction between *N. attenuata* and native bacteria have documented a potential role of IAA (Long et al., 2008) and ethylene (Long et al., 2010). In Long et al., 2010, it was proposed that ethylene homeostasis along with soil composition determines bacterial communities in the roots of *N. attenuata*. Another study investigated the native bacterium *bacillus sp*., B55 on *N. attenuata* growth and its role in recovering a healthy root system in 35S-etr1 (ethylene insensitive transgenic line) *N. attenuata* (Meldau et al., 2012). To date, little is known about the role of IAA in this system.

1.6 Aim of this study:

In this study, we investigated the interaction between native endophytic bacteria and *N. attenuata.* Particularly, the influence of native bacteria on root architecture of *N. attenuata* was investigated. We used irAOC line: a jasmonate deficient inverted repeat allene oxide cyclase line and EV line: transgenic control line transformed with an empty vector construct of *N. attenuata* under *in vitro* condition and glasshouse conditions, as the endophytic bacteria were isolated from the root tissue of irAOC and EV lines in the field. We first checked for the ability of these native endophyte bacteria to synthesize plant growth regulators. Later, we examined the effect of exogenous phytohormone application on root architecture of irAOC and EV lines of *N. attenuata* to understand which component plays root architecture modification. This study will help to understand the importance of native bacteria from root system of *N. attenuata* and its influence on the root architecture of the plant.

2. MATERIALS AND METHODS:

2.1 Plant material:

The following plant lines were used for this study: a jasmonate deficient inverted repeat allene oxide cyclase line (irAOC, line A-07-457-1) (Kallenbach et al., 2012) and a transgenic control line transformed with an empty vector construct (EV , line A-03-9-1).

2.2 Seed germination:

Before germination, the seeds were surface sterilized in a solution containing 100 mg of dicholoroisocyanuricacid, 50µl of 0.5g/100ml Tween 20 in 5ml of distilled water. Five minutes later, the seeds were rinsed three times with autoclaved water before smoke-treating them during one hour. 5ml of a 50-times diluted smoke (House of Herbs) solution supplemented with 50 µl of 0.1M gibberellic acid (Duchefa Biochemi). Before plating, the seeds were rinsed three times with autoclaved water. Seeds were used either for petri dish or green house experiments (See description below).

2.3 Preparation of petri dishes:

For the *in vitro* petri dish experiment, seeds were surfaced sterilized and smoke treated as described above before transferring them in culturing media (0.5g/l of Gamborg B5, 6 g of plant agar, pH 6.80). After autoclaving (121ºC for 20 minutes), the autoclaved media was poured into square petri dish plates (12cmX12cm) (Greiner, Germany). After removing the top 2 cm layer, either one or two seeds were plated onto the media surface resulting in either one or two seeds were placed onto the media surface resulting in either one (Fig 1a) or two seedlings per plate (Fig 1b). Petri dish plates were then sealed with parafilm (Parafilm M) and incubated vertically in a Percival chamber (27ºC, 16/8 hours day/night cycle). 7 days later, germinated seeds were used for treatment with bacteria or phytohormones as described below.

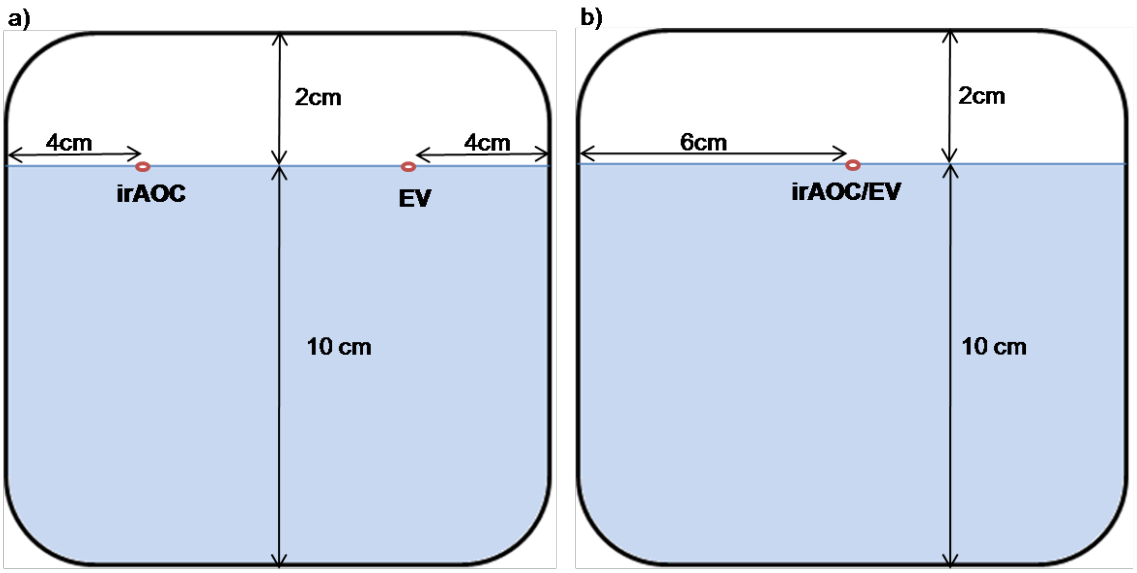

Figure 1: Seed germination media plate preparation with Gamborg B5 agar media.

2.4 Bacterial strains.

Bacterial strains used in this study were isolated from Utah field station grown irAOC and EV *N. attenuata* plants (Santhanam et al. unpublished). In brief, plants at different stages were excavated, the excess of soil was removed by smoothly shaking them and root systems were wrapped with moist paper tissue. Shoots and roots from different plants (n=14) were packed individually and shipped to Max Planck Institute for chemical ecology, Jena, Germany. Endophytic bacterial isolation was carried out as described (Long et al., 2010). In short, plant tissue was surface sterilized with ethanol: water (70:30) for 60 sec and sodium hypochlorite solution(3%) for 300 sec. Later it was washed thrice with sterile water and aseptically sectioned into smaller fragments and distributed in three different isolation media: tap water-yeast extract agar (Coombs and Franco, 2003), *Streptomycetes* isolation media (D'Costa et al., 2006) and glucose-yeast extract agar (Okoro et al., 2009) (Sigma, Steinheim, Germany).Plates were incubated at 28˚C for 4 d. After incubation, single colonies were sub-cultured until plates with apparently only single species were obtained. Stock cultures were stored in 50%

glycerol solution at -80˚C. The isolated bacterial strains were identified using 16S rRNA gene sequencing. Genomic DNA was extracted from bacterial isolates and 16S rRNA PCR amplifications were performed according to Kim & Goodfellow (2002) with minor modifications. Amplification of 16S rRNA gene was performed in a 20 µL final volume of ReadymixTaq PCR reaction mix (SigmaAldrich) containing 2 µL of template DNA, 50 µM of primer 27F (5´-AGAGTTTGATCCTGGCTCAG- 3´) and 1492R (5´-GGTTACCTTGTTACGACTT- 3´, Lane DJ, 1991). A negative control PCR mixture with sterile water was included in all PCR experiments. PCR products were purified using the QIAquick™ Gel Extraction Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. Direct sequencing using the primer 783R (5´-CTACCAGGGTATCTAATCCTG -3´) was conducted with Big Dye Mix (Applied Biosystems, Foster City, CA, USA), and purification of the sequencing reactions was performed using the Nucleo-SEQ Kit (Macherey-Nagel, Düren, Germany). Analysis of all sequences was carried out with the EzTaxon server (Kim et al, 2012). More than 200 bacterial strains were identified. From them, 14 were selected for this study (Table 1). Apart from these 14 bacterial strains, *Bacillus sp*. B55 (GenBank accession number: JX101913) (Long et al., 2010; Meldau et al., 2012)and *Bacillus megaterium* (Obtained accidently from a contaminated petri dish*)* were included in this study. The bacterial strains were sub-cultured from single colonies in nutrient agar (Roth) and stored in 50% glycerol stock.

Table 1: List of bacterial strains used in this study.

S.no.	Name of the Bacterial strains	Bacterial strain-labels	Isolated Plant line of *N. attenuata*	Tissue of isolation
1,	*Kocuria palustris*	B56	irAOC	Root
2,	*Pseudomonas koreensis*	A151	EV	Root
3,	*Pseudomonas koreensis*	A21	irAOC	Root
4,	*Pseudomonas frederiksbensis*	A176	EV	Root
5,	*Micrococcus luteus*	D4	EV	Root
6,	*Micrococcus cohnii*	D13	irAOC	Root
7,	*Kocuria marina*	D102	irAOC	Root
8,	*Bacillus Cereus*	C88	irAOC	Root
9,	*Kocuria marina*	B14	irAOC	Root
10,	*Achromobacter spanius*	B30	EV	Root
11,	*Pseudomonas frederiksbensis*	A127	EV	Root
12,	*Rhizobium phenanthrenilyticum*	AN1	irAOC	Root
13,	*Rhizobium phenanthrenilyticum*	BN3	EV	Root
14,	*Kocuria palustris*	B6	ir AOC	Root
15,	*Bacillus sp.*	B55	-	-
16,	*Bacillus megaterium*	B.M.	-	-

2.5 Bacterial treatment on root system of *N. attenuata*.

To test the hypothesis that native bacterial strains influence root architecture of *N. attenuata* in a distance specific manner, we treated petri dish cultured *N. attenuata* seedlings with native bacteria (section 2.4) at two distances from the root system (Figure 2) and measured different root architecture traits. To treat the seedlings with native bacteria, 25µl of a bacterial solutions were applied to 7 days old seedlings at two different distances from main root tip: next to growing main root and in the path of growing main root (Figure 2) (n = 6). To prepare bacterial solutions, 72 hours grown bacteria were harvested and suspended in nutrient broth (75g/l) to obtain a bacterial suspension with $O.D_{600nm}$ 2.0. 9 days later, the plates were scanned and the images were analyzed using Fiji (Image J, Win64, USA). The root parameters considered in this study includes: primary root length, lateral root density and primary root deviation (Fig 3).

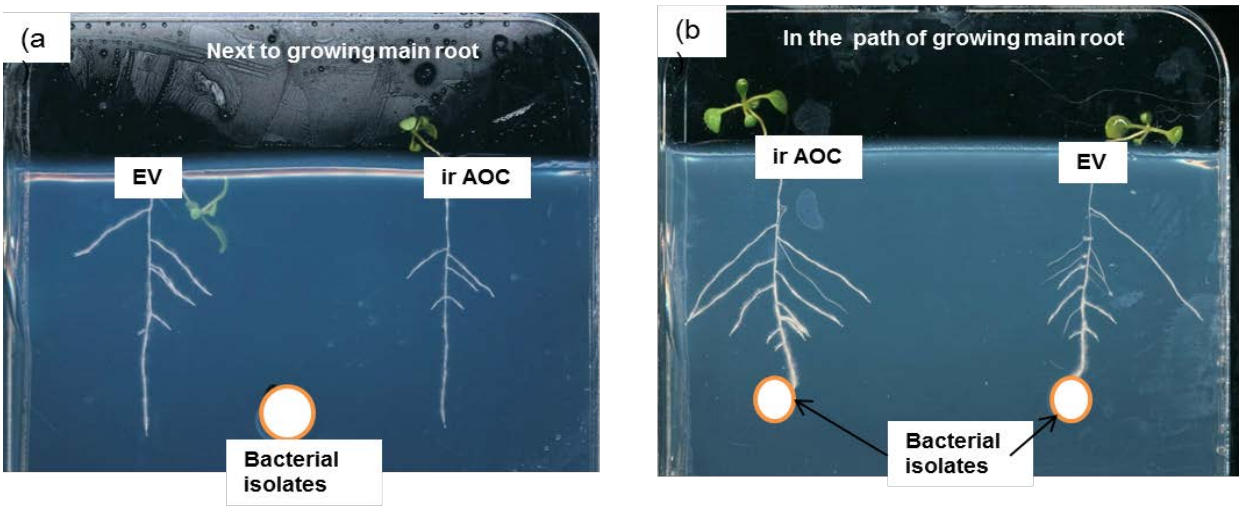

Figure 2: *In vitro* experimental set up for the bacterial treatment on root system of *N. attenuata.* **a)** Bacterial colony away from root tip and **b)** bacterial colony on the way of root tip.

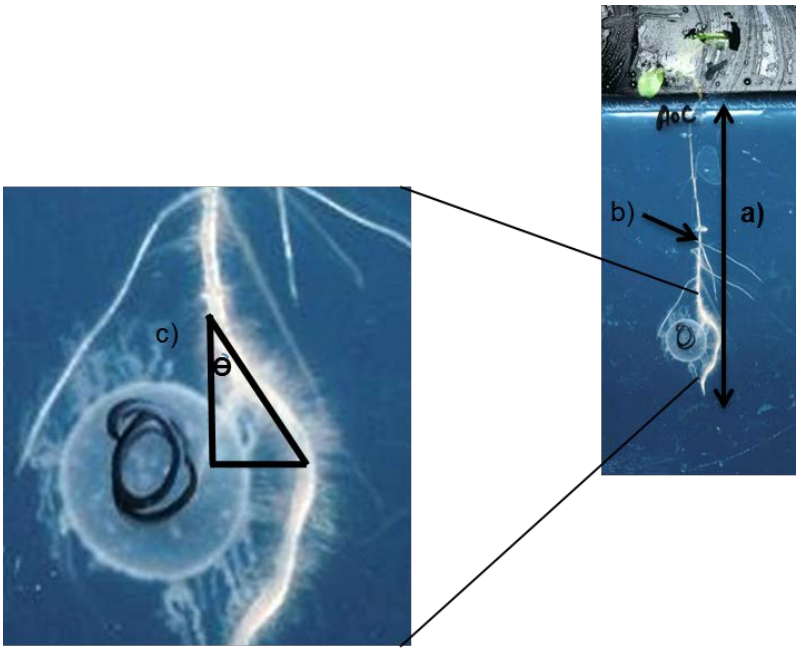

Figure 3: Root parameters considered in avoidance and attraction in vivo experimental set up; **a)** Primary root length , **b)** Lateral root density (Number of secondary root per unit primary root length), **c)** primary root deviation (ϴ).

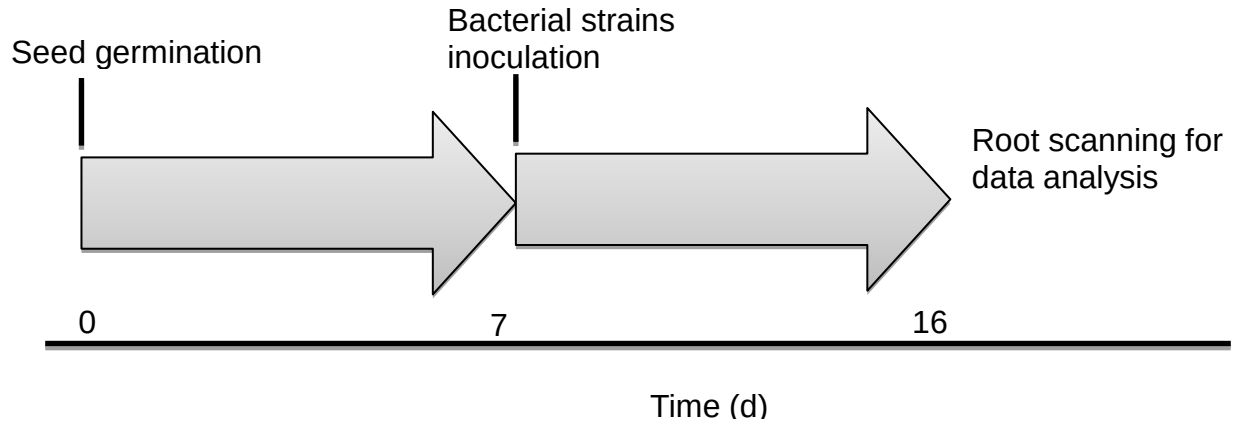

Figure 4: Time period for bacterial strains treatment on irAOC and EV lines of *N. attenuata in-vitro* condition.

2.6 Phytohormone measurement from bacteria.

2.6.1 Indole-3-acetic acid (IAA), salicylic acid (SA), abscisic acid (ABA) and jasmonic acid (JA) meaurement:

To assess whether the bacteria used in this study produce phytohormones, we cultured bacterial strains and extracted and quantified the phytohormones produced (Machado et al., 2013). For this, the bacterial mass was produced by culturing single colonies on nutrient agar (NA) media for 72 hours at 28ºC. 100 mg of bacteria from each strain (*n*=6) were extracted with 1 mL ethylacetate: formic acid (99.5:0.5) containing the following phytohormones standards: 40ng of 9,10-D_2-9,10-dihydrojasmonicacid (JA), 40 ng of D_4-salicylic acid (SA), 40 ng of D_6-Abscissic acid (ABA) (Santa Cruz Biotechnology, USA), 8 ng of jasmonic acid-[$^{13}C_6$] isoleucine (JA-Ile) and 20 ng of D_5-indole-3-acetic-acid (IAA).JA-[$^{13}C_6$]-Ile conjugate was synthesized as described by using [$^{13}C_6$]-Ile (Sigma). Samples were vortex for 10 min and centrifuged at 14,000 rpm for 10 minutes at 4ºC. Extraction buffer was evaporated completely using the SpeedVac at room temperature (Eppendorf 5301, Germany). Remaining pellets were re-suspended in 50µl methanol: water (70:30) and dissolved using ultra sonic bath (Branson1210, USA) for 5 mins. IAA, JA, SA and ABA were analyzed using high performance liquid chromatography (Agilent 1260 infinity, HPLC system (Agilent Technologies) coupled to a mass spectrometer (API 5000, Applied Biosystems) using a

procedure adapted from Vadassery et al 2012. Briefly, separation was performed on a Zorbax Eclipse XDB-C18 column (50 x 4.6 mm, 1.8 mm; Agilent). Mobile phases A and B were 0.05% acetic acid in water and acetonitrile, respectively. Compared to Vadassery et al. (2012), acetic acid was chosen as mobile phase additive instead of formic acid since it was shown to provide higher sensitivity for all hormones measured. The following gradient was employed: 0 to 0.50 min, 5% B; 0.50 to 9.50 min, 5% to 58% B; 9.50 to 9.52 min, 58% to 100% B; 9.52 to 11.00 min, 100% B; and 11.10 to 14.00 min, 5% B the mobile phase flow rate was of 1.1 $mL.min^{-1}$ and the column temperature was maintained at 25°C. The API 5000 tandem mass spectrometer (Applied Bio systems, Carlsbad, CA, US) equipped with Turbospray ion source was operated in negative ionization mode. The ion spray voltage was set at -4500 eV and the turbo gas temperature at 700°C. Nebulizing gas, curtain gas and collision gas were set at 60, 25 and 7 psi respectively. Multiple reaction monitoring was used to monitor analyte parent ion → product ion: mass-to charge ratio [m/z] 136.93 → 93.0 (collision energy [CE], 22 V; declustering potential [DP], 35 V) for salicylic acid; m/z 140.93 → 97.0 (CE, 22 V; DP, 35 V) for D4-SA; m/z 209.07 → 59.0 (CE, 24 V; DP, 35 V) for JA; m/z 213.07 → 56.0 (CE, 24 V; DP, 35 V) for 9,10-D2-9,10-JA; m/z 263.0 → 153.2 (CE, 22 V; DP, 35 V) for ABA; m/z 269.0 → 159.2 (CE, 22 V; DP, 35 V) for D6-ABA; m/z 322.2 → 130.1 (CE, 30 V; DP, 50 V)for JA-Ile; and m/z 328.2 → 136.1 (CE, 30 V; DP, 50 V) for JA-[13C6]Ile. Both Q1 and Q3 quadrupoles were maintained at unit resolution. IAA was analyzed using the same system and solvents except that the elution profile was as follows: 0 to 0.50 min, 5% B; 0.50 to 6.00 min,5% to 37.4% B; 6.00 to 6.02 min, 37.4% to 100% B; 6.02 to 7.50 min, 100% B; 7.50 to 7.60 min, 100 to 5% B; and 7.60 min to 10.50 min, 5 % B. The mass spectrometer was operated in the positive ionization mode, with an ion spray voltage of + 5500 eV and turbo gas temperature was at 700°C. Nebulizing gas, curtain gas and collision gas were set at 60, 30 and 4 psi respectively. IAA was measured by monitoring the transition m/z 176,00 → 130,00 (CE,19 V; DP, 31 V) for IAA; m/z 181,00 → 135,00 (CE, 19 V; DP, 31 V) for D5-IAA. Both Q1 and Q3 quadrupoles were maintained at unit resolution. Data acquisition and processing was performed using Analyst 1.5 software (Applied Biosystems, Carlsbad, CA, US). Phytohormones were quantified using the signal of their corresponding internal standard. Quantified unit was ng/gFW (nano gram per gram fresh mass).

2.6.2 Ethylene measurement:

To test whether the bacteria used in this study produce ethylene (gaseous phytohormone), we cultured bacterial strains and measured ethylene production using an ethylene sensor (sensor-sense ethylene sensor ETD 300, Netherlands). For this, we produced 5ml screw cap GB5 agar (0.5g/l) slants tubes in air tight condition (Fig. 5b) and inoculated with bacterial solution (see Section 2.4) (n=6). 24 hours later, accumulated ethylene was measured using sensor-sense ethylene sensor (ETD-300). The sensor-sense ethylene sensor is a photoacoustic trace gas sensor optimized to measure ethylene from biogenic sources. This sensor uses a CO_2 laser and in the infrared spectral region molecules have characteristic absorption spectra that can be used for a qualitative and quantitative detection. The absorbed photon energy is transformed into translational energy by collisions, resulting in an *i*ncrease in gas-temperature (200 ºC). Such temperature rise causes an increase of pressure*.* Modulation of the radiation source at an acoustic frequency results in a periodical pressure *variation* that is directly related to the concentration of absorbing molecules (ethylene) in the photoacoustic cell. Using a sensitive microphone to measure the signal, very low concentrations can be detected. Interfering gases that may influence the results because of an overlap between their spectral absorption and the CO_2 laser wavelengths have been eliminated with help of filters and scrubbers in the air-way of the measuring system. In this study, ethylene sensor has CAT catalyzer (removes small hydrocarbon in air flow using catalyst Pt/SiO_2), VC valve controller (controls air flow at 2.5l/h) connected to the sample and ethylene detector (ETD-300) which detects ethylene (photoacousticdetection) in scale of ppbV (parts per billion Volume). Data acquisition and processing was performed using Valve controller software (Sensor sense, Netherlands).

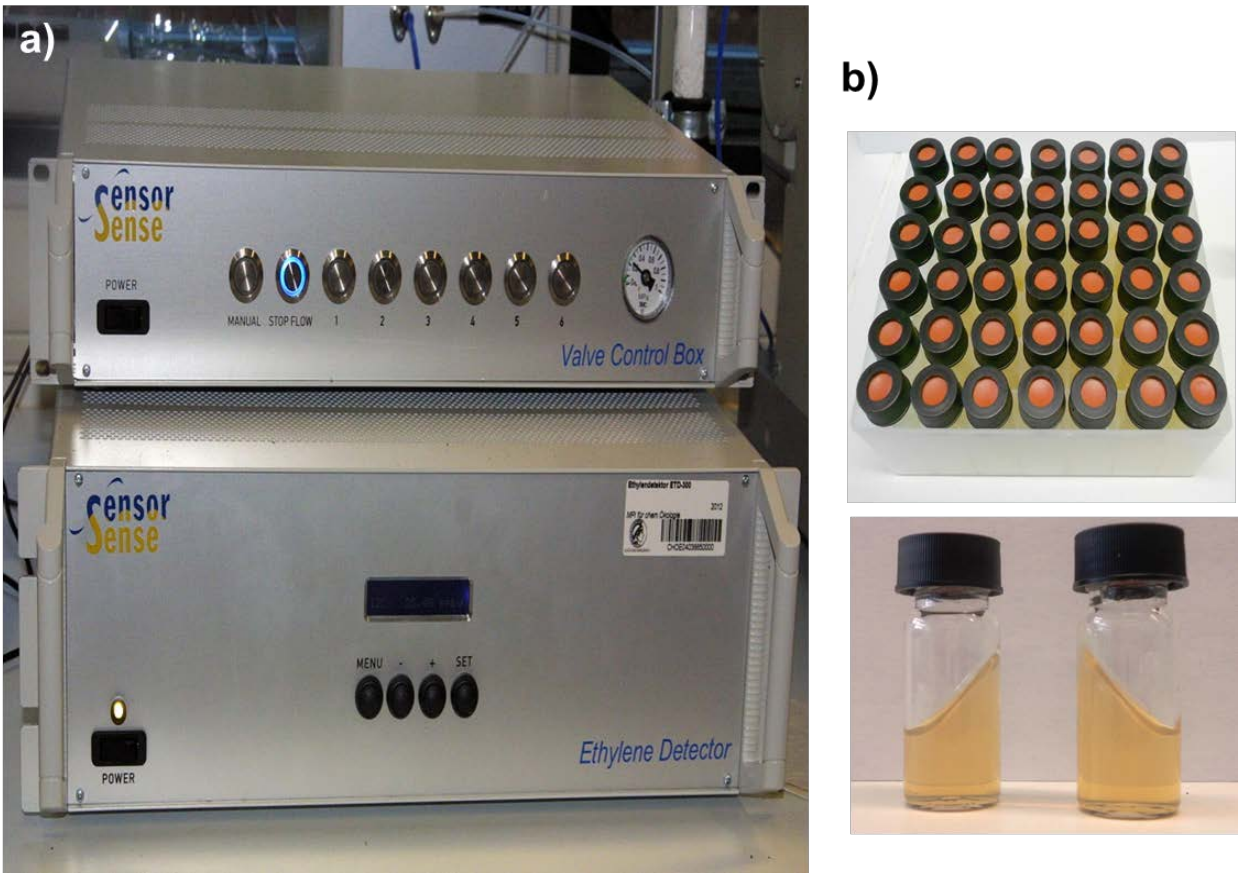

Figure 5: **a)** Sensor-Sense Ethylene sensor; **b)** GB5 agar slants with bacterial inoculants in air tight condition for ethylene measurement.

2.7 Indole-3-acetic acid diffusion assay.

To determine whether indole-3-acetic acid (IAA) produced by bacterial strain diffuses in the petri plate to reach *N. attenuata* root system, we cultured bacterial strains and measured IAA levels at three different locations: at the inoculation point and at two distances from bacterial colony (Fig. 6).Bacterial strains were sub-cultured freshly from glycerol stock this experiment and re-suspended in nutrient broth (75g/l) (LB carl ROTH GMBH) to obtain bacterial solution with an $O.D._{600nm}$ of 2.0. 25µl of the bacterial solutions were inoculated in GB5 agar media (0.5g/l) and incubated at 28ºC (*n*=3). 3 days after incubation, IAA from the culturing media surface was extracted and quantified as described (See phytohormone measurements, section 2.6.1).

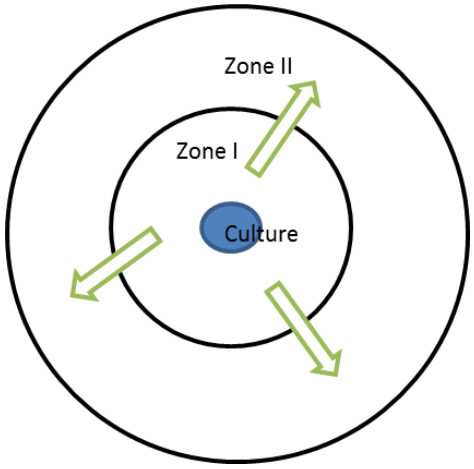

Figure 6: IAA diffusion assay: IAA synthesized and diffused from bacterial strain colonies on 15% GB5 media during the time period of 3 days.

2.8 Phytohormonal and bacterial treatment on *N. attenuata* root system.

To test whether phytohormonal treatments alter *N. attenuata* root system in a similar manner as native bacterial treatments do, we produced either EV or irAOC seedlings (Fig. 1b) and treated them with either bacteria (See bacterial treatments on *N. attenuata* root system) (n=9) (Fig.4a) or different phytohormones (n=15) (Fig.4b) and root architecture traits were measured (Fig. 5). For the phytohormonal treatments, 50ng/plate of following phytohormones were used: indole-3-acetic acid (Duchefa), 6-Benzylamino purine (synthetic cytokinin) (Duchefa), methyl jasmonate (Doukian); 2-chloroethyl-phosphonic acid (synthetic ethylene) (Sigma). Plants treated with 25µl of autoclaved water (phytohormonal treatment) or nutrient broth (75g/l) (bacterial treatment) was used as control. At the end of the experiment, plates were scanned and analyzed in Fiji (Image J, Win64, USA). 8 root parameters were considered in this study (Fig.8). The parameters “primary root deviation” was measured as sum of root deviation from axis on grid lines in respective region (Grid lines with 1.33 cm spacing was used) (Fig.8). A principle component analysis (PCA) (R commander- Factor MinoER) was used to visualize the overall effect on the different root traits due to treatments.

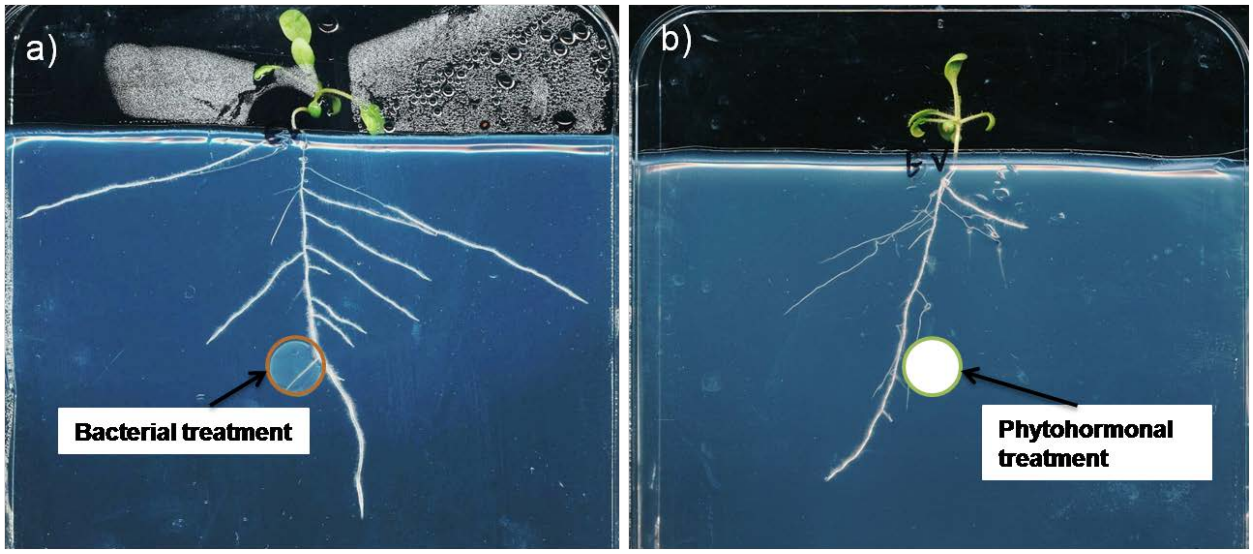

Figure 7: Treatment on root system of irAOC and EV lines of *N. attenuata:* **a)** Bacterial treatment, **b)** Phytohormonal treatment.

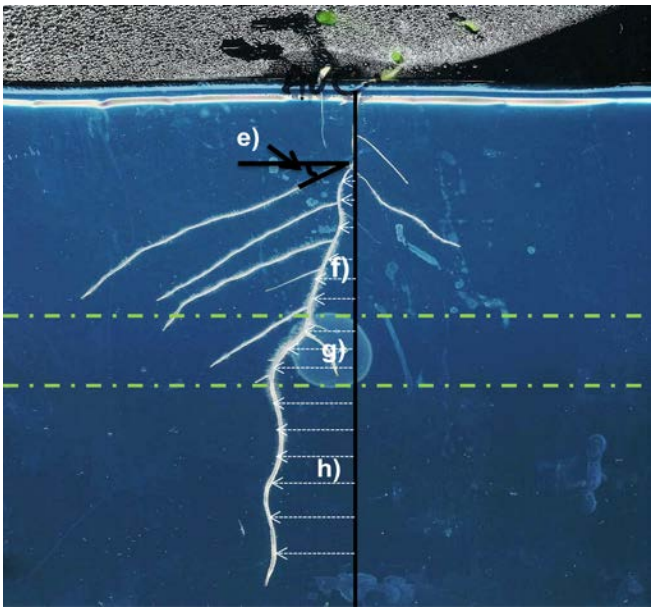

Figure 8: Root parameters considered in: **a)** Primary root length, **b)** Number of secondary roots, **c)** Average length of secondary root, **d)** Root hair density, **e)** Average secondary root angle from primary root, **f)** Primary root deviation before treatment, **g)** Primary root deviation on treatment, **h)** Primary root deviation after treatment.

2.9 Glass house experiment:

To evaluate whether native bacteria alter *N. attenuata* root system under glass house condition, we treated greenhouse grown rosette stage plants with bacterial solution and measure root architecture traits. For this, irAOC and EV seeds were germinated as described (see seed germination, section 2.2) and plated in GB5 media (3g of GB5/l, 6 g of plant agar, pH 6.8). The seedlings were transferred to Teku pots (Poeppelmann GmbH & Co. KG, Lohne, Germany). 10 day after germination and seedlings were planted into 1 liter pot filled with autoclaved sand (1.2mm grain size, Raiffeisen, Germany). Plants were grown at 45-55% relative humidity and temperature of 23-25ºC during day and 19-23ºC during night under 16 h of light as described by (Krügel et al., 2002). Plants were watered once every day by a flood irrigation system. Fertilizer contained 0.6g of $Ca(NO_3)_2.4H_2O$ and 0.3g Flory basisdünger 1 (Euflor, Munich, Germany). Once the plants reached rosette stage, bacterial solution were applied as shown (Fig. 9). To prepare bacterial solution, the bacterial strains A21 and A151 (table 1) from glycerol stock were cultured on nutrient agar media at 28ºC for 24 hours. Bacterial mass produced was re-suspended in nutrient broth (75g/l) (LB ROTH) to obtain a solution with $O.D._{600nm}$ of 2.0. 1ml/pot of bacterial strains were inoculated. Liquid nutrient broth was used as control (*n* = 12). 10 days after inoculation, root system of the treated plants were harvested and scanned in an A4 scanner for further analysis. Data extraction was done using Fiji.

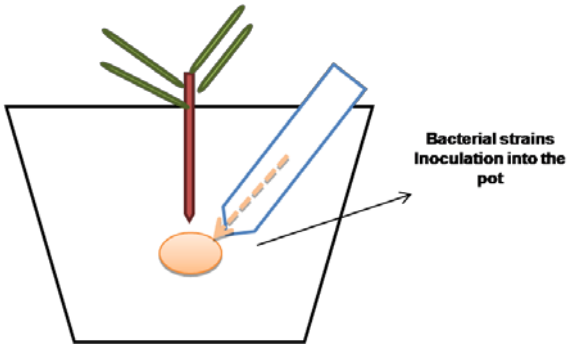

Figure 9: Diagrammatic representation of bacterial strains treatment on root system of *N. attenuata* in glass house condition.

2.10 Statistical analysis:

Statistical analysis was performed using Sigma Plot 12.0. Data are shown as mean ± standard error. Two-way ANOVAs (multiple comparisons versus control, Holm-Sidak method) were performed to find the difference in bacterial and phytohormonal treatments on root system of *N. attenuata*. One way ANOVAs were performed for estimating phytohormone measurements versus control. During this study, all statistical analysis was tested for normality by means of Shapiro-Wilk test.

3. RESULTS:

3.1 Bacterial treatment on root system of *N. attenuata*:

Bacillus sp. B55, a native plant growth promoting bacteria has been shown to alter root architecture by reducing primary root length and increased lateral root density in *N. attenuata* (Meldau et al., 2012). To test whether other native endophytic bacteria also modify *N. attenuata* root architecture, we inoculated different native bacteria (see table 1) at two different distances from the main root tip and measured root architecture traits. When we inoculated the bacterial strains in the path of growing main root, we observed that D4, A21, A127, A151, A176, AN1, BN3, B55, B14, D13, D102 and B.M. treated seedlings had shorter primary roots compared to non-treated plants in a JA independent manner (Fig. 10 a). We also observed that the lateral root density was also significantly increased in A21, A127, A151, A176, AN1, B55, D13, D102 and B.M. treated seedlings compared to control. Interestingly, JA deficiency led to an increased lateral root density in B55 and D102 treated seedlings (Fig. 10 b).

On the other hand, bacterial treatment significantly alters the primary root deviation. Apart from B55, B.M., and C88 treatments, all the other strains significantly altered the primary root by deviating it away from the treatment compared to control (Fig. 10 C). A151, AN1 and D13 treated irAOC seedlings showed high root deviation compared to A151, AN1 and D13 treated EV seedlings (Fig. 10c).

When the bacterial colonies were inoculated next to growing main root we did not find any other modification of the root architecture traits evaluated in this study (Fig. 11 a, b, c).

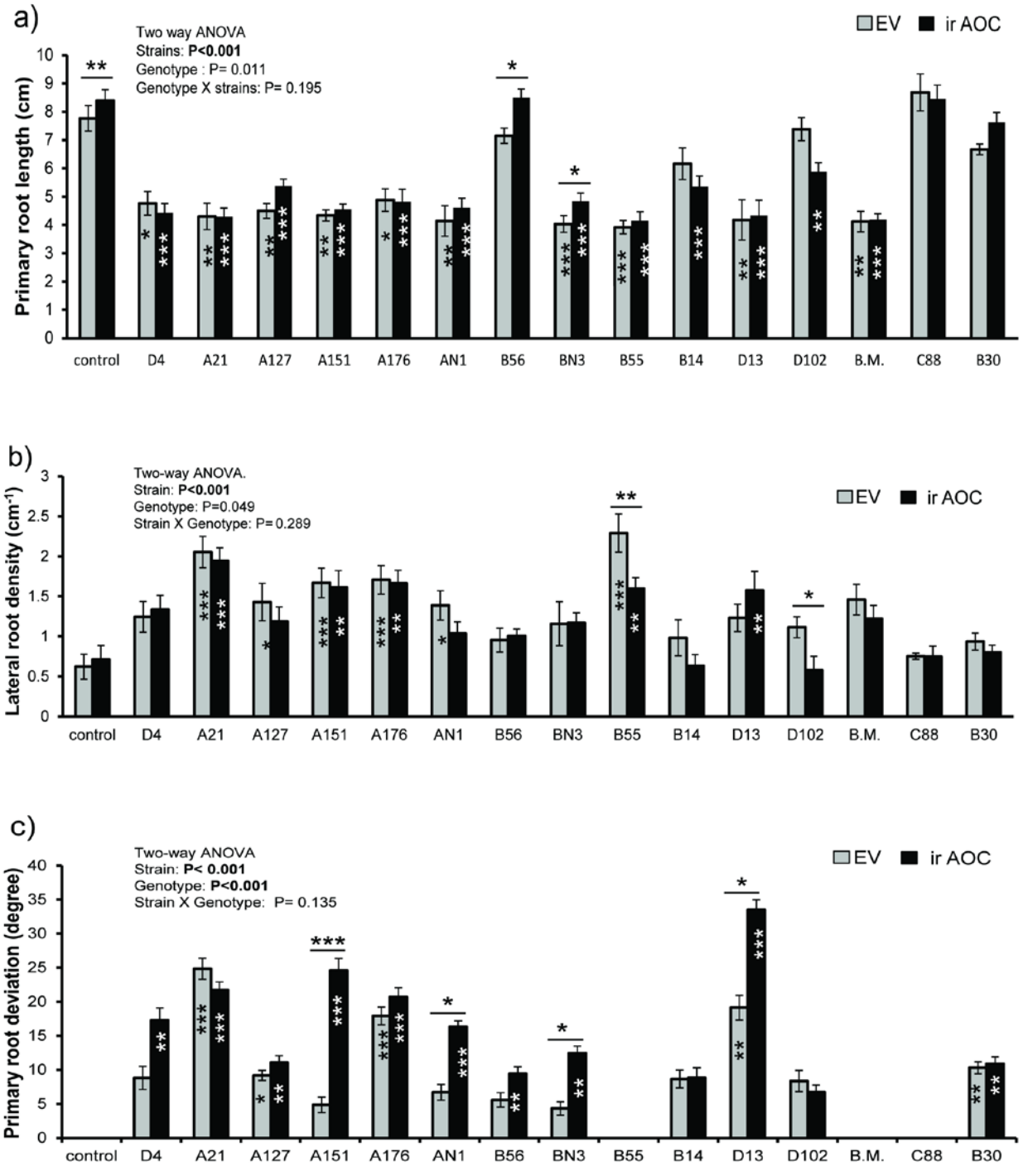

Figure 10: Bacterial isolate treated plants in the path of growing main root: **a)** mean (± SE) primary root length, **b)** mean (± SE) lateral root density **c)** mean (± SE) primary root deviation (mean ± SE).Two way ANOVA- Holm-Sidak test. ***, P≤.001: **, P≤ 0.01: *, P≤0.05;

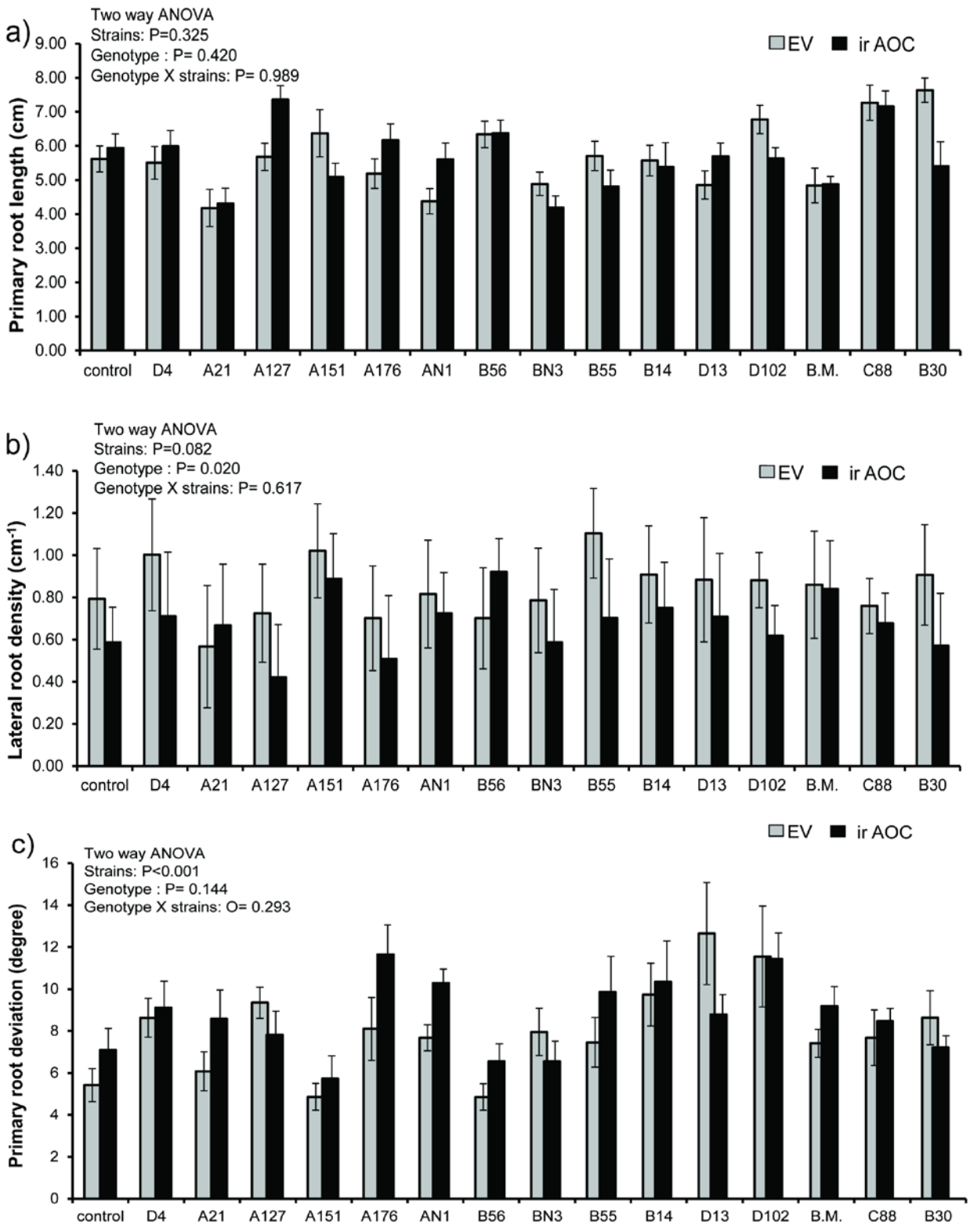

Figure 11: Bacterial isolate treated plants next to growing main root: **a)** mean (± SE) primary root length, **b)** mean (± SE) lateral root density, **c)** mean (± SE) primary root deviation; Two way ANOVA- Holm-Sidak test.

3.2 Phytohormones produced by native bacteria.

From phytohormone analysis, we found that the tested native bacterial strains synthesis IAA (Fig 12 a). The strains A21, A127, A151, A176, AN1 and B56 produce IAA (0.1-0.2 µg/gFW). Interestingly, the strain D4 produces high amount of IAA (1.4µg/gFW). On the other hand, we found that the strains A151, AN1, B30 produced higher level of SA compared to other strains. The bacterial strains A21, A127, A176, AN1, BN3, B55 and B.M. produced ethylene in the range of 0.2-1.2 ppbV (Fig. 12 b). There was not any evidence for the production of JA, ABA (abscisic acid) or OPDA (12-oxo-phytodienoic acid) (JA precursor) (Fig. 13).

3.3 IAA diffusion assay:

To determine whether indole-3-acetic acid (IAA) produced by bacterial strain diffuses in the petri plate to reach *N. attenuata* root system; we cultured bacterial strains and measured IAA levels at three different locations. We found a positive correlation between IAA levels and distance from the inoculation point: 100-times and 20-times higher IAA levels were found in the outer zone (Zone II) and the middle zone (Zone I) respectively, compared to the zone where bacterial colony was inoculated (Fig. 14).

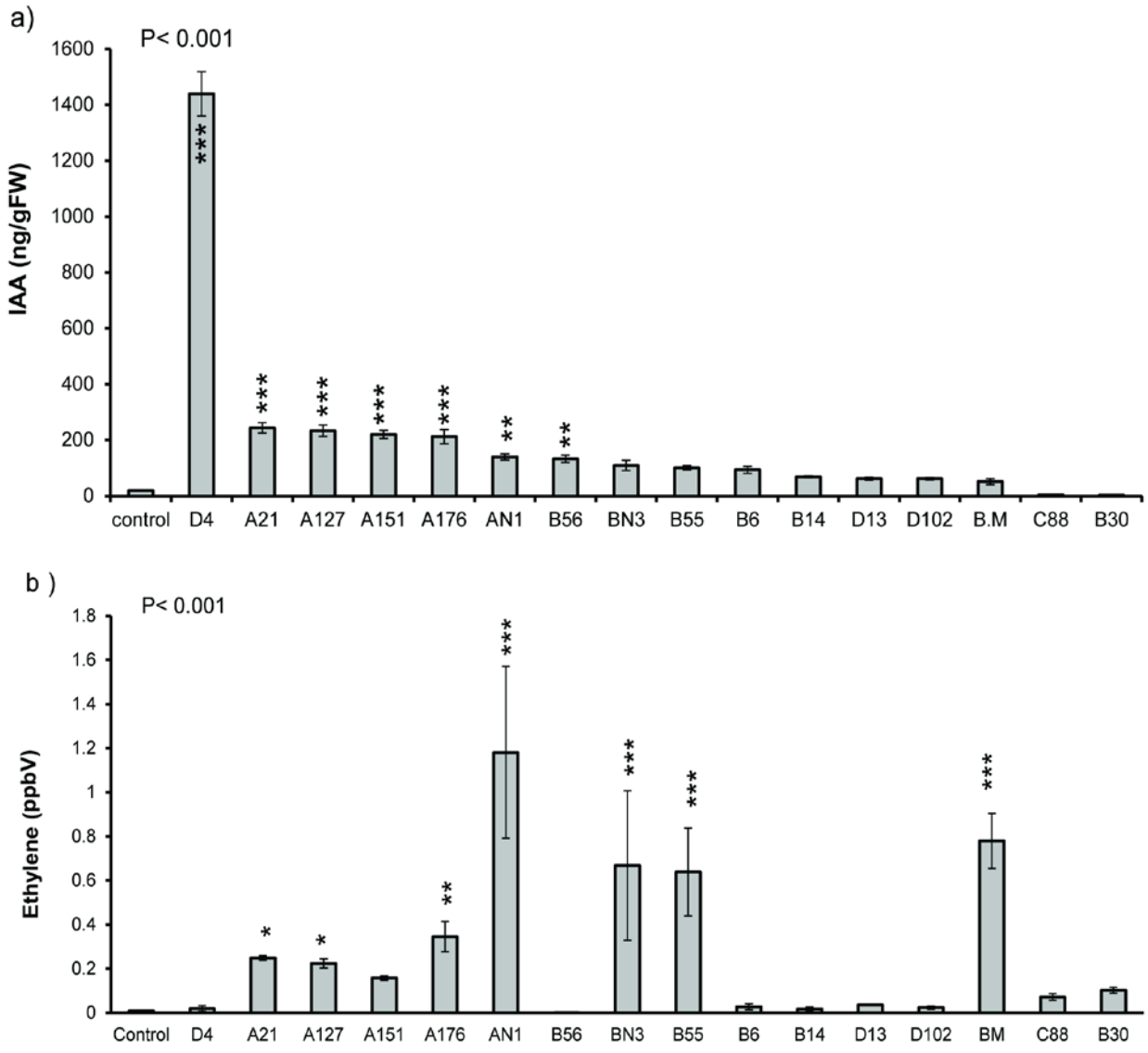

Figure 12: Mean (± SE); **a)** IAA and **b)** ethylene produced from Bacterial strains in Gamborg's B5 (GB5) media; Control- GB5; One way ANOVA - Holm-Sidak test; ***,P≤.001 : **, P≤0.01 : *, P≤0.05 .

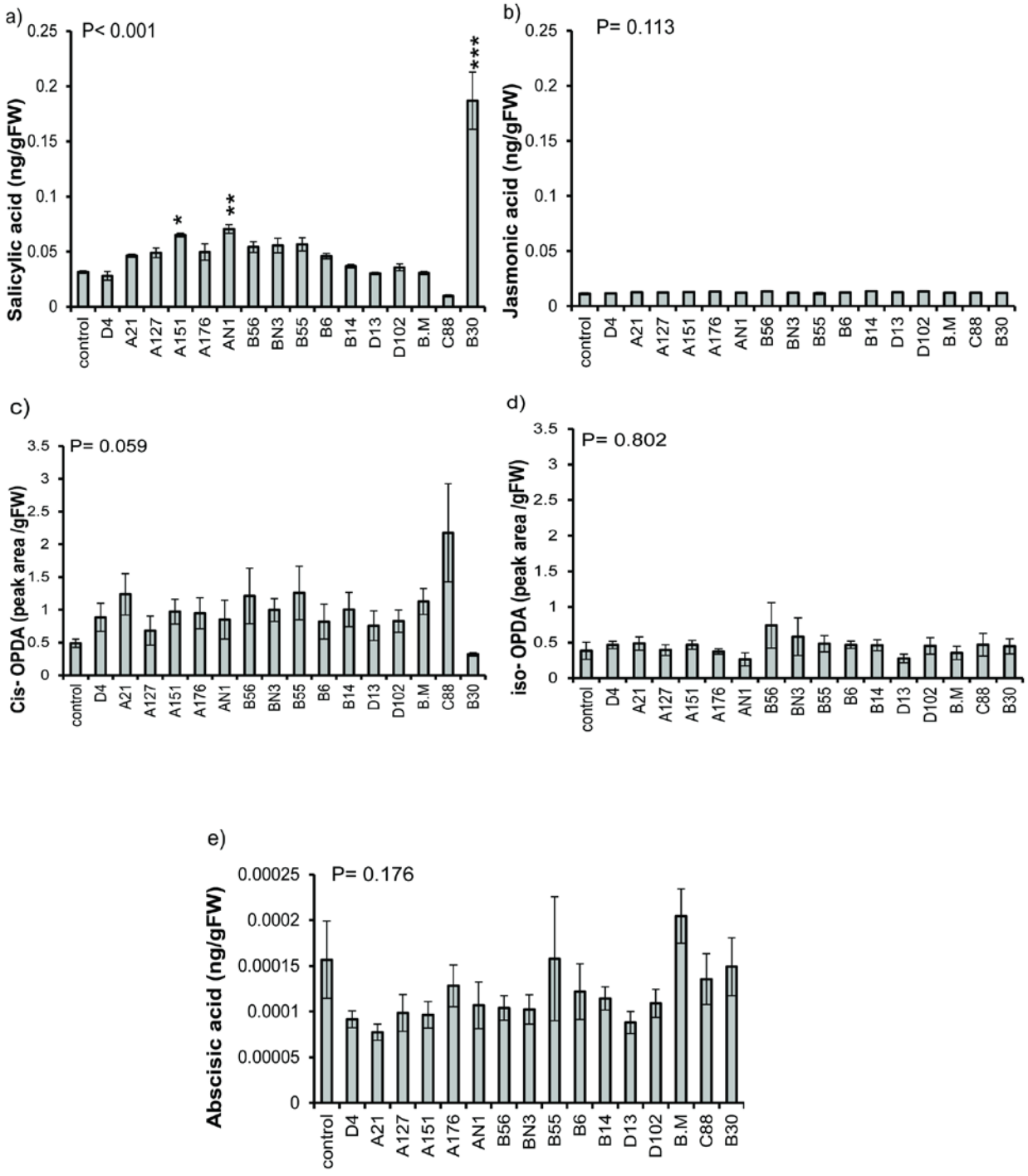

Figure 13: Plant growth regulators produced from bacterial strains in Gamborg's B5 (GB5) media; **a)** mean (±SE) salicylic acid; **b)** mean (± SE) jasmonic acid; **c)** mean (± SE) cis-OPDA; **d)** mean (± SE) iso-OPDA; **e)** mean (± SE) abscisic acid. One way ANOVA - Holm-Sidak test. ***, P≤.001: **, P≤0.01: *, P≤0.05.

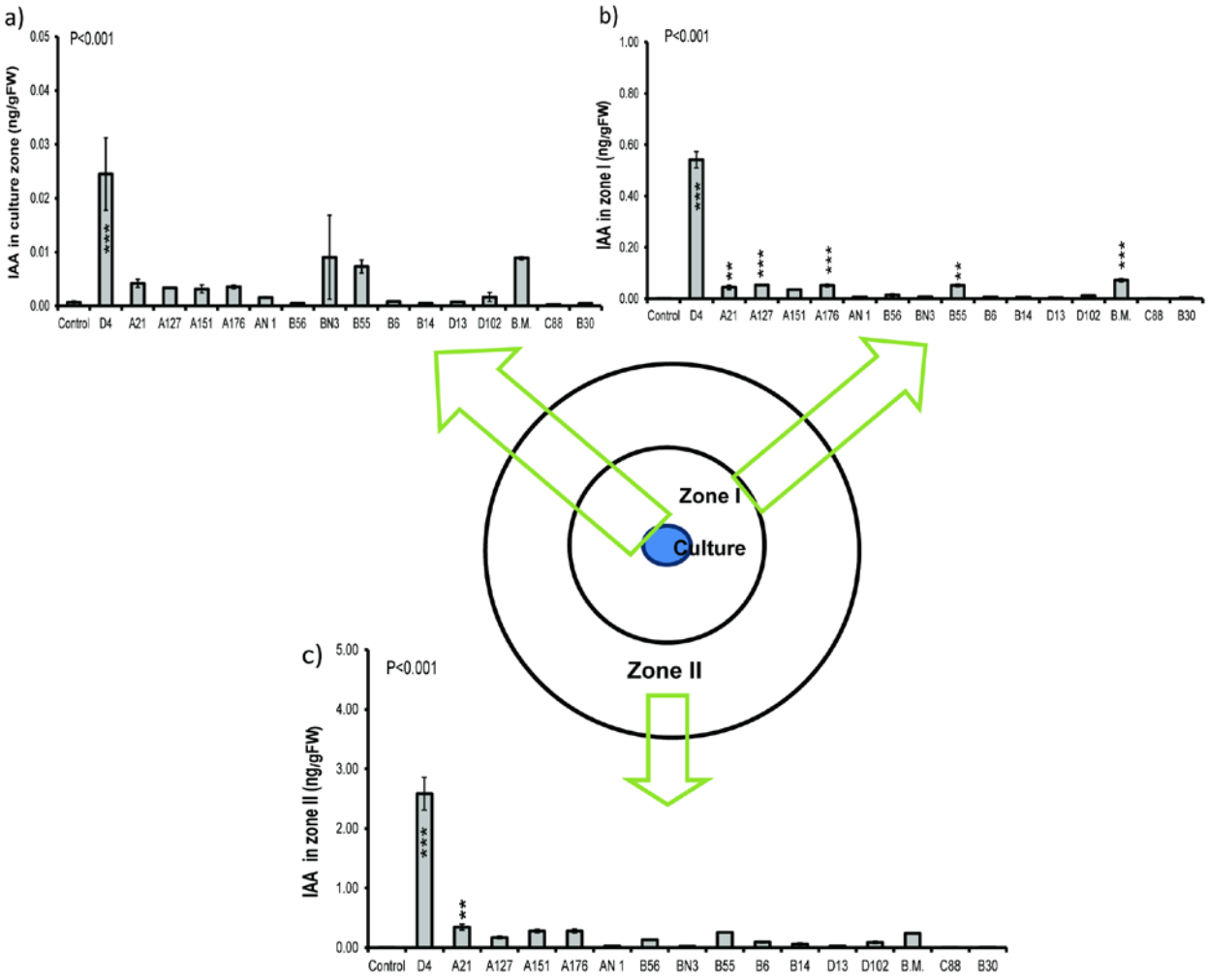

Figure 14: Mean (±SE): IAA diffusion assay. **a)** IAA in culture zone **b)** IAA in Zone I **c)** IAA in Zone II. One way ANOVA- Holm-Sidak test; ***, P≤001: **, P≤0.01: *, P≤0.05.

3.4 Phytohormonal and bacterial treatment on *N. attenuata* root system:

We found that the bacteria tested in this study produce IAA, ethylene and SA. To test whether exogenous application of phytohormones alters root architecture similarly as bacterial treatment do, we treated *N. attenuata* root system with bacterial strains and for exogenous phytohormones and different root architecture traits measured.

3.4.1 Bacterial treatment:

We found that, *N. attenuata* seedlings treated with A21, A127, A176, AN1, BN3, B55, B14 and D13 showed reduced primary root length in JA independent manner. On the other hand D4, A151 and B.M. treatment seedling had reduced primary root length in JA dependent manner (Fig 15 a). There was not significant change in number of secondary roots and average secondary root length *N. attenuata* seedlings due to individual treatment compared to control. However when overall treatment effect on these two traits were considered, there was significant modification due to treatment in number of secondary root ($P<0.001$) and average secondary root length ($P=0.04$) (Fig 15 b and c). Also, in general average secondary root length of irAOC seedling were higher than the EV plants ($P<0.001$) (Fig 15 c).Next trait, root hair density was increased significantly ($P<0.001$) due to bacterial treatment when compared with control (Fig 15 d). Secondary root angle from the primary root which was not modified due to individual treatment compared with control, but there was an overall influence due to bacterial treatment ($P=0.003$). Especially in few treatments like A151, A176, B.M., and C88 irAOC plants was having high secondary root angle compared to EV plants ($P<0.001$) (Fig 15 e). Primary root deviation was not found before bacterial treatment (Fig 15 f), but there was high deviation on bacterial treatment ($P<0.001$) (Fig. 15 g) and after treatment root deviation was not found.

3.4.2 Phytohormonal treatment:

During phytohormonal treatment on root system of *N. attanuata*, there was no significant modification in most of the root traits considered in this study compared to control. Only, primary root deviation was observed after phytohormone treatment ($P=0.003$) (Fig. 16 h). On the other hand, irAOC plants were having longer primary root ($P=0.034$) and more number of secondary roots ($P=0.001$) compared to EV plants irrespective of any phytohomonal treatment (Fig. 16 a & b).

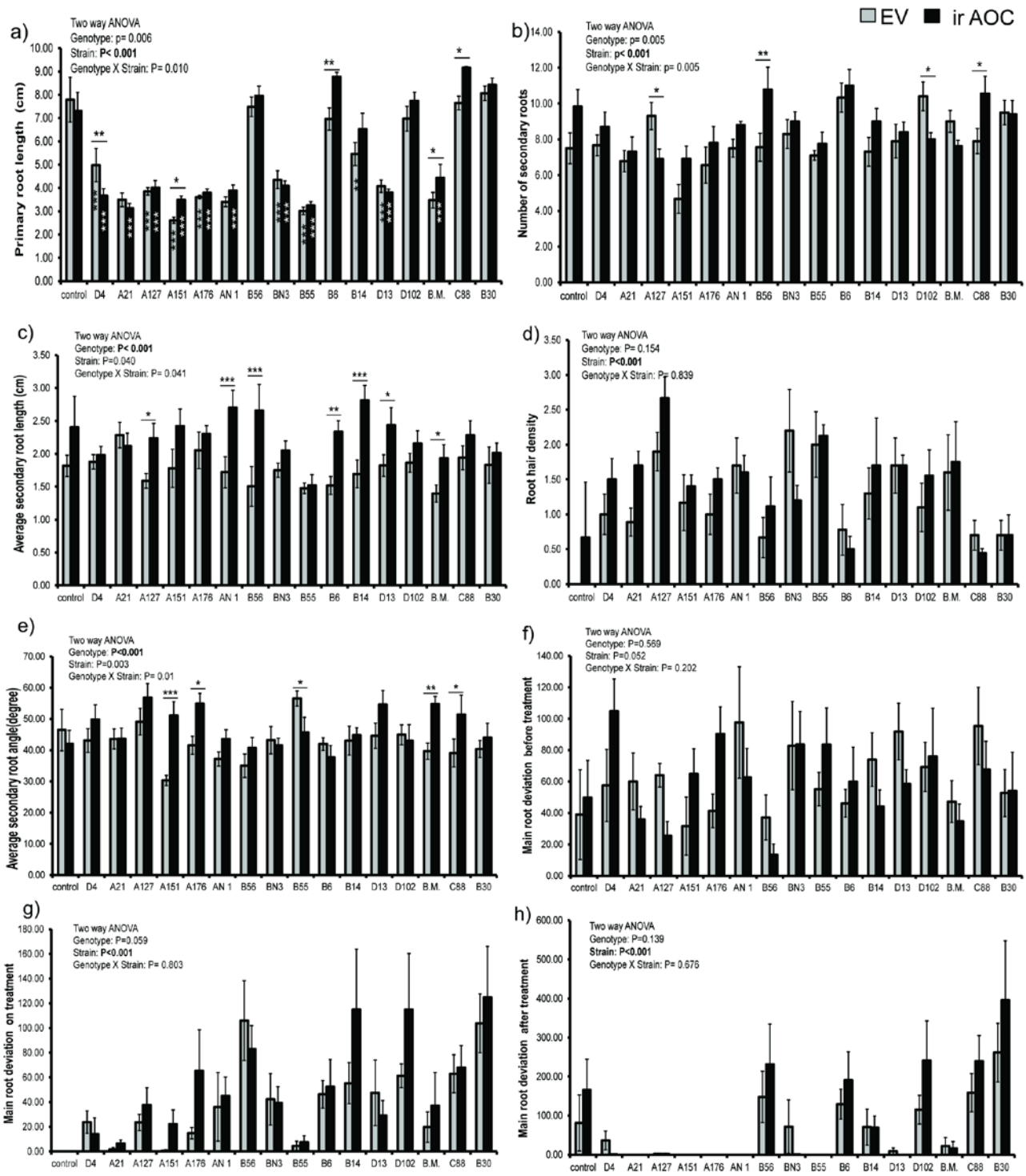

Figure 15: Bacterial treatment on *N. attanuata* root system: mean (± SE): **a)** primary root length, **b)** number of secondary root number, **c)** secondary root length, **d)** root hair density, **e)** secondary root angle from primary root, **f)** primary root deviation before bacterial treatment, **g)** primary root deviation on bacterial treatment, **h)** primary root deviation after bacterial treatment. Two-way ANOVA (Holm-Sidak test). ***, P≤.001: **, P≤0.01: *, P≤0.05.

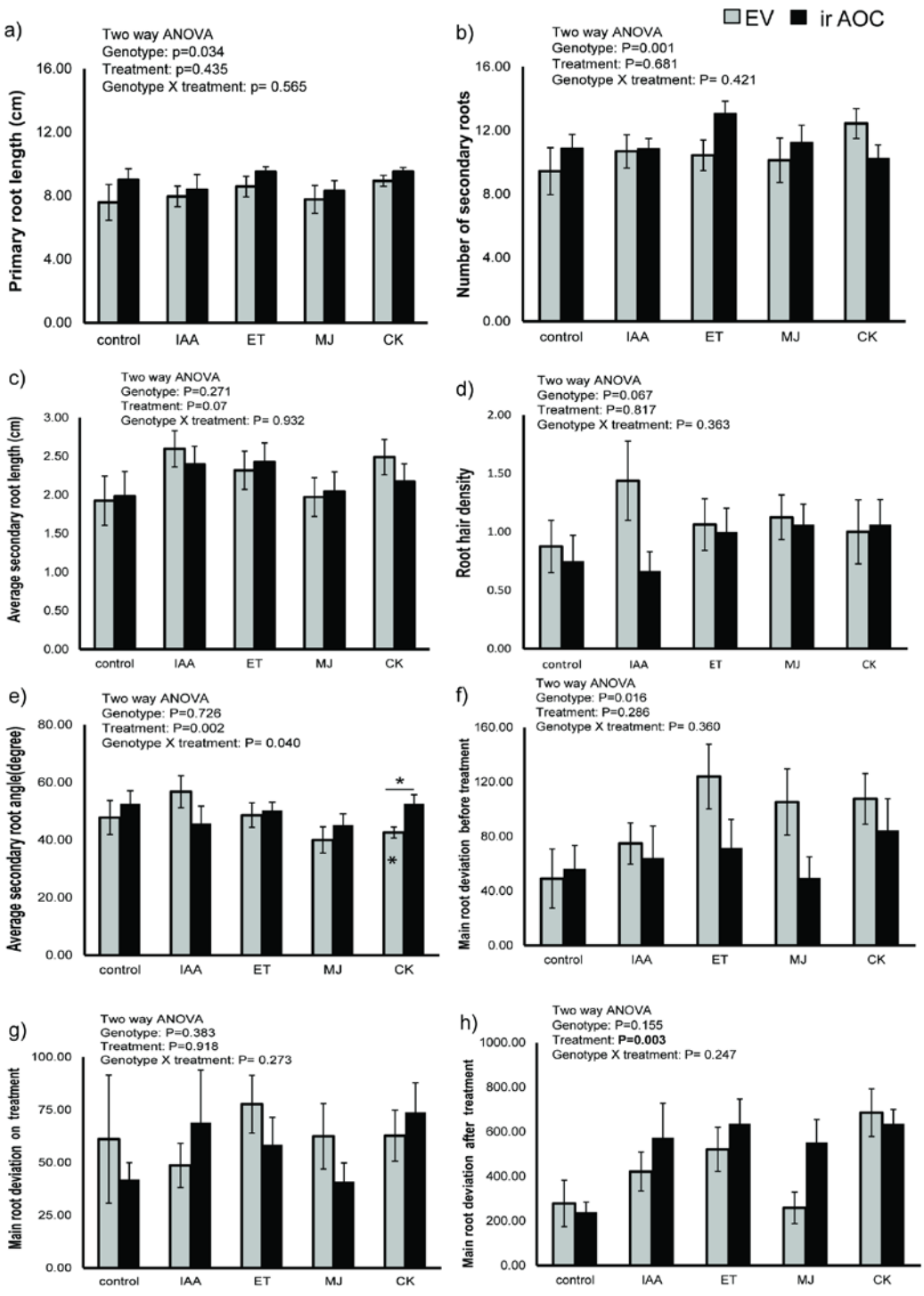

Figure 16: **Phytohormone treated plants** IAA: (Indole-3-acetic acid); ET (2-chloroethyl phosphate, ethylene emitting component); MJ (methyl jasmonate); CK: (6 benzyl amino purine, synthetic cytokinin); mean (± SE); **a)** primary root length, **b)** number of secondary root number, **c)** average secondary root length, **d)** root hair density, **e)** average secondary root angle from primary root, **f)** primary root deviation before treatment, **g)** primary root deviation on treatment , **h)** primary root deviation treatment ; Two way ANOVA (Holm-Sidak test). *, $P \leq 0.05$.

3.5 Principle component analysis (PCA).

To characterize overall changes in root architecture due to bacterial and phytohormonal treatment, we carried out a principle component analysis. The results shows that the bacterial strains which were producing auxin and ethylene fall into one distinct cluster (Fig. 17a) characterized by a reduction in primary root length, number of secondary roots and average length of secondary roots and an increase in root hair density (Fig 17 b). On the other hand, IAA and methyl jasmonate treatment increases root hair density and reduces primary root length, number of secondary roots and average secondary root length (Fig. 18 b). Ethylene and cytokinin treatment on the root system of *N. attenuata* increases the primary root deviation before the treatment, on the treatment and after the treatment (Fig 18. b).

3.6 Glass house experiment.

While testing the bacterial influence on the root system of *N. attenuata* under glass house condition, we found that there was primary root length reduction due to treatment (P=0.022) (Fig. 19 a). On the contrary to *in vitro* condition, the lateral root density was reduced due to overall bacterial treatment compared to control under glass house condition (P=0.01) (Fig. 19 d). Main root deviation was not modified as *in vitro* condition and root dry mass was not altered (Fig 19 b & c).

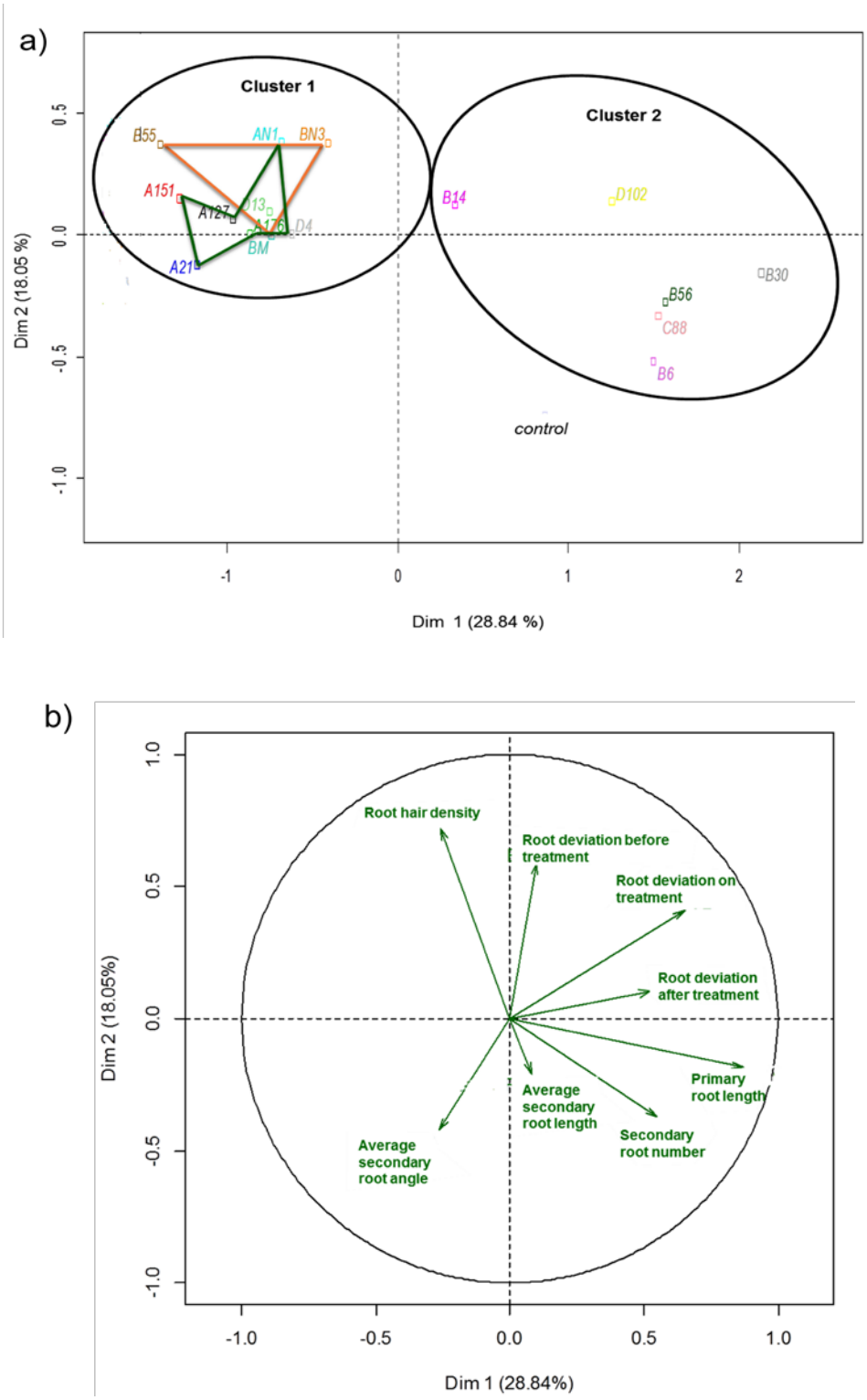

Figure 17: Principle component analysis (PCA) for bacteria treated plants: **a)** Bacterial strains distribution based on effect, **b)** Vector diagram of modified root parameters due to treatment. **Orange line** connected strains are high ethylene producers. **Green line** connected strains are high IAA producers.

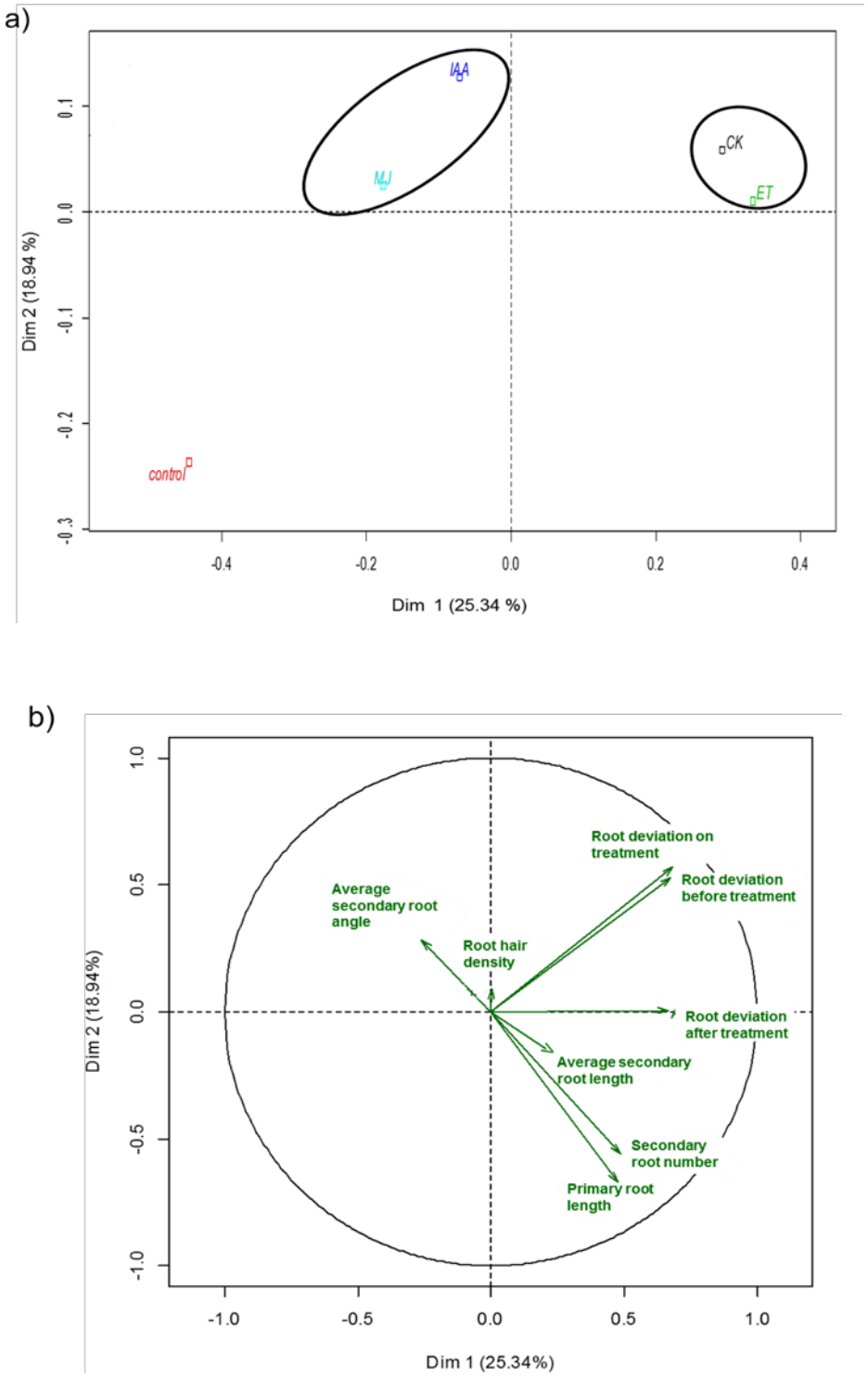

Figure 18: Principle component analysis (PCA) for exogenous phytohormone treated plants, **a)** phytohormone distribution based on effect, **b)** Vector diagram of modified root parameters due to treatment.

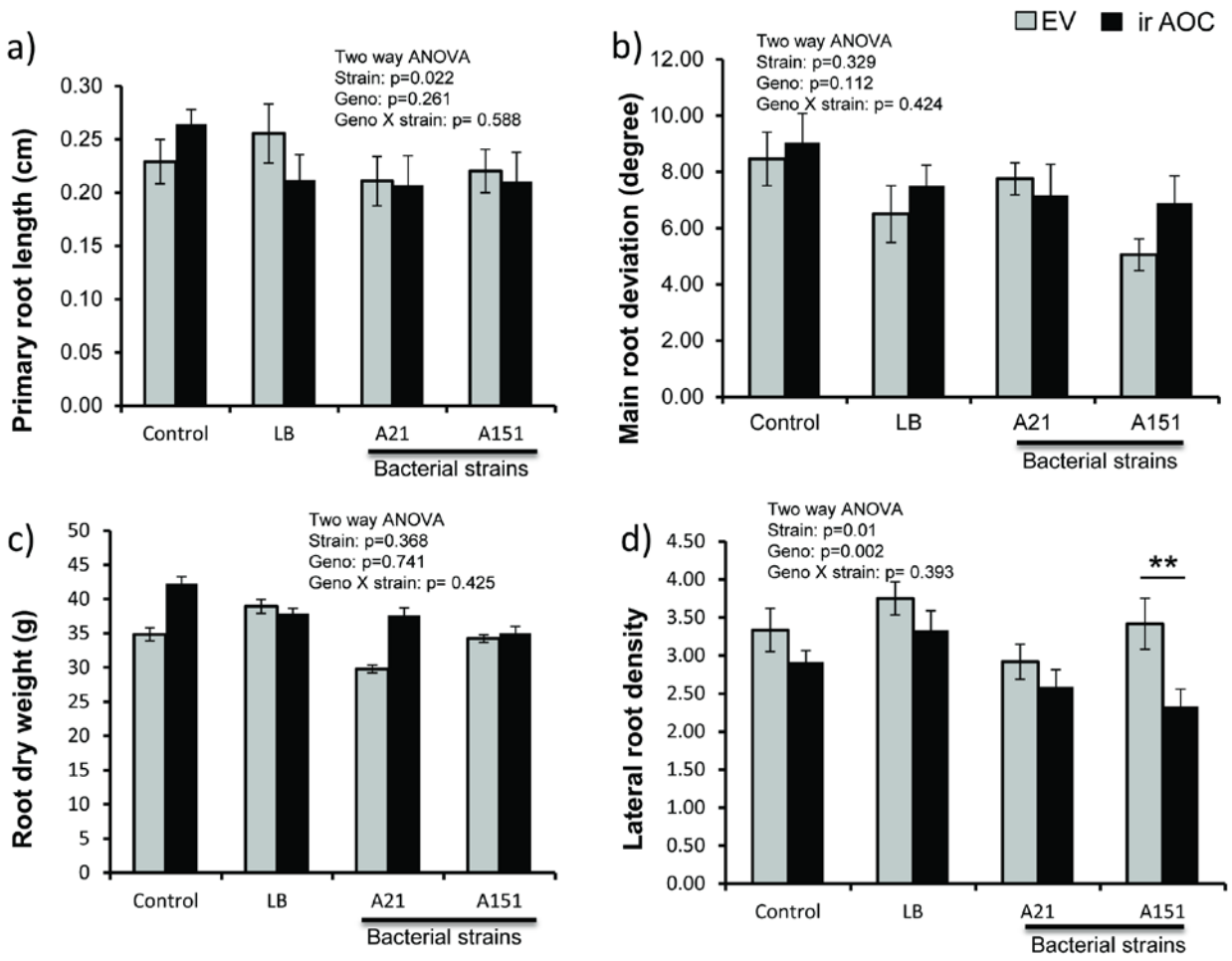

Figure 19: Glass house experiment: mean ± SE: **a)** primary root length **b)** main root deviation **c)** root dry weight **d)** lateral root density: Control- water; LB – Liquid broth; Genotype – Geno: **, P≤0.01

4. DISCUSSION:

Rhizosphere microorganisms can have remarkable effects on plant performance and its fitness by influencing root growth and development (López-Bucio et al., 2007). Root development and alteration based on ethylene and auxin role has been well described (Stepanova et al. 2005, López-Bucio et al. 2002, Sukumar, Legue et al. 2013).

Form this study, we found that native bacteria used in this study influence on root architecture of *N. attenuata* (Figure 10 a, b, c) in distance specif manner. We also found that a high production of auxin (IAA) (upto 1.4µg/gFW) , ethylene (upto 1.2 ppbV) and SA (0.06-0.2 ng/gFM) from these native bacteria isolated of *N. attenuata* (Fig. 12).Apart from that, no evidence was found for the production of ABA, JA and its precusors Cis-OPDA and iso-OPDA (Fig. 13) from the bacteial strains. To evaluate whether IAA similar to ethylene is released by the bacteria into the environment, we performed a diffusion assay and found that the syntheszied IAA was 100 fold higher (from 0.25 ng/gFM to 25 ng/gFM) in the outer zone than the bacterial zone leads to formation of an IAA gradient (Figure 14). This IAA diffusion gradient might be one of the meachanism behind IAA in reaching plant root system.

To test whether phytohormones themselves are sufficient to trigger changes in root architecture similar to the tested bacteria, we applied synthetic hormones to the media and investigated how they influenced root growth. Our results show no statistically significant changes in all root parameters considered in this study. This effect may be explained by the low concentration (50ng) of phytohormones used in this experiment. In order to characerize overall changes in root architecture, we performed amultivariate statistical analysis (PCA-Principle component analysis) (Fig. 17 and 18). The results show that the bacterial strains which were producing auxin and ethylene fall into one distinct cluster1 (Figure 17 a) charcerized by a reduction of primary root length, number of secondary roots and average length of secondary roots and an increase in root hair density.

In the glasshouse experiment, the strains A21 and A151 were chosen, as these strains were producing auxin in the moderate range of 0.2µg/gFW and we observerd highly significant genotype effect (JA dependent manner) on the primary root deviation (Figure 10c).The results from the glass house experiment was not showing sigficant primary root deviatio, but there was statistical support for changes in other parameters, including primary root length (P=0.022) and lateral root density (P= 0.01). This result indicates that native bacterial may influence root architecture of *N. attenuata* under natural conditions.

With known role of jamonate (JA) for root growth inhibition in *A. thaliana* (Raya-Gonzalez et al., 2012) in this study, it was observed that the jasmonate deficient irAOC plants of *N. attanuata* line was having longer primary root length compared to control (EV lines) (Fig. 16a). This shows role of jasmonate in influencing *N. attenuata* root architecture. Also, it was found that the native bacteria used for the study were unable to synthesize JA or JA precursors (cis & iso-OPDA). Considering the 16 bacterial strains in this study, strains including D4, A151, A176, AN1, B55, D102 , D13 and B.M. modified root traits in JA depended manner (Fig. 10b, 10c, 15 a, c, e, g). Interestingly, among these strains D4, A151, A176, AN1 were high IAA producers and AN1, B55, B.M were high ethylene producers. Taken together from this study, there might be possibilities for phytohormonal cross talk between auxin, ethylene and jasmonate for the observed root traits modification.

Although auxin and ethylene have a strong impact on the root morphogenesis, there are other plant hormones which also play key roles in influencing root architecture like cytokinins (Persello Cartieaux et al. 2001) and gibberllins (Christian Löfke et al. 2013). These componentsremain to be tested for the production from the bacterial strains used in this study for further understanding. Adding to this, the role of jasmonates was not clear in many cases of bacterial treatment during the entire study and it can be concluded that jasmonate signaling from plants does not play amajor role in interacting with the native microbes studied here.

5. CONCLUSION:

- The root architecture of *N. attenuata* was significantly influenced by the native bacterial strains used in this studyin distance specific manner compared to control.
- Bacterial strains used in this study were producing indole-3-acetic acid (IAA), ethylene and salicylic acid.
- IAA produced by native bacteria forms an IAA gradient in GB5 media with respect to different concentration synthesized from bacteria under *in vitro* condition.
- The comparison of modified root architectute between bacterial and exogenous phytohormonal treatment using PCA does not show which of the above mentioned phytohormones are indeed responsible for such impact on root system of *N. attenuata*.
- Under glass house condition, bacterial isolate A21 and A151 treated *N. attenuata* root system showed statistically significant primary root length reduction. But contrary to *in vitro* condition, lateral root density was reduced in over all treatment and primary root deviation was not significant compared to control.

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