Microbiome dynamics of disease suppressive soils

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Fall seven times and stand up eight

-Japanese proverb-

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Chapter 1

General introduction

(Part of this chapter was submitted for publication)

Soils constitute a huge reservoir of microbes and their population, densities and activities are greatly influenced by plants through the discharge of root exudates (Doornbos et al., 2012). Although some of the components in the released root exudates can stimulate pathogen growth and disease, root exudates constituents can also attract mutualistic microorganisms that promote plant growth or confer protection against pathogens. Beneficial microbes can operate directly through the production of metabolites that kill or inactivate the pathogen, or indirectly by competition for nutrients, niche colonization and inducing systemic resistance in plants (Alabouvette et al., 2006; Raaijmakers et al., 2009; Pieterse et al., 2014; Ciancio et al., 2016). Thus, beneficial plant-associated microorganisms are of importance for plant health and have been considered as a sustainable alternative to control plant diseases and to enhance crop productivity (Berendsen et al., 2012; Mendes et al., 2013; Mueller and Sachs, 2015).

The phenomenon of 'disease suppressive soils' is a clear example of microbe-mediated protection of plants against soil-borne pathogens. Suppressive soils are present worldwide and have been associated with suppression of a broad number of root diseases caused by different pathogens including fungi such as *Rhizoctonia solani* (Postma et al., 2010; Mendes et al., 2011), *Gaeumannomyces graminis* var *tritici* (Raaijmakers and Weller, 1998; de Souza et al., 2003), and *Fusarium oxysporum* (Scher and Baker, 1980; Klein et al., 2013), oomycetes such as *Pythium ultimum* (Martin and Hancock, 1986)and *Phytophthora cinnamomi* (Broadbent and Baker, 1974), bacteria such as *Streptomyces scabies* (Weinhold et al., 1964) and *Ralstonia solanacearum* (Shiomi et al., 1999), and nematodes such as *Meloidogyne incognita* (Pyrowolakis et al., 2002) and *Criconemella xenoplax* (Kluepfel et al., 1993).

Baker and Cook (1974) defined disease suppressive soils as "soils in which the pathogen does not establish or persist, establishes but causes little or no damage, or establishes and causes disease for a while but thereafter the disease is less important, although the pathogen may persist in the soil". In conducive (or nonsuppressive) soils, disease readily occurs under conditions favorable to the pathogen. Every natural soil has the ability to suppress a pathogen to a certain extent (Berendsen et al., 2012). This type of disease suppression is known as "general suppression" and it is due to the activity of the collective microbial community; no specific microbial groups are directly responsible for conferring suppressiveness as general suppression often derives from competition between pathogenic and non-pathogenic microbes for available resources (Mazzola, 2002; Weller et al., 2002). Thus, this type of suppression is often boosted by addition of organic matter (i.e. manure, seed meal, animal waste meal, compost and peat) (Henis et al., 1967;

Osunlaja,1990; Bonanomi et al., 2010; Postma et al., 2014; Postma and Schilder, 2015), or by certain management practices, including crop rotation, tillage or improvement of soil fertility (Weinhold et al., 1964; Scholte, 1992; Kremer and Li, 2003; Weller et al., 2011). Conversely, "specific suppression" is due to the activities of selected groups of microorganisms that are antagonistic against (a) specific pathogen(s). Specific suppression is commonly referred to as induced suppressiveness when disease suppressiveness is induced (and maintained) by crop monoculture (Mazzola, 2002; Weller et al., 2002). The presence of the pathogen during the successive mono-cropping has been proposed as a requirement to induce suppressiveness (Weller et al., 2002). The proposed underlying mechanism is that the pathogen causes a disease outbreak that in turn leads to the recruitment and activation of a specific microbial population able to combat that particular pathogen (Raaijmakers and Mazzola, 2016).

The intrinsic characteristics of general and specific suppression have remarkable similarities to the innate and adaptive immune responses in animals. That is, the innate immune response gives a primary and non-specific defensive response similar to what occurs in general suppression. The adaptive immune response in animals and specific disease suppression in soils both require highly specialized cells to eliminate the pathogen, require time and have a memory (Lapsansky et al., 2016; Raaijmakers and Mazzola, 2016). Hence, suppressive soils constitute a valuable source of microorganisms with antagonistic properties to limit plant infections by specific pathogens. Thus, identifying the microbial consortia, traits and genes involved in providing plant protection, may enable us to engineer the plant microbiome to promote their antagonistic activities and plant health.

History and origin of disease suppressive soils

The first suppressive soil was reported in 1892 by Atkitson and was suppressive to *Fusarium* wilt disease of cotton (Atkinson, 1892; Scher and Baker, 1980; Amir and Alabouvette, 1993; Lemanceau et al., 2006). In 1931, Henry noticed that addition of one unsterilized agricultural soil into another soil containing spores of the fungal pathogen *Helminthosporium sativum* made it suppressive (Henry, 1931a; 1931b). This was the first study that showed the transferability of disease suppressiveness from one soil to another, suggesting a role of soil microorganisms. Similarly, Walker and Snyder (1933) recognized differences in disease development between certain soil types, ones in which pea wilt occurred quickly, and others in which the disease developed slowly or not at all (Walker and Snyder, 1933; Hornby, 1983). For the soil-borne pathogen *Rhizoctonia solani*, which is

the subject of this thesis, Emden (1967) was the first to describe a suppressive soil for potato (Emden, 1967; Jager and Velvis, 1983a). Since these initial studies, specific suppression has been reported for a range of pathogens, including fungi (*G. graminis* var *tritici*, *F. oxysporum, F. solani*, *Verticillium dahliae, V. albo-atrum*, *Pyrenochaeta lycopersici, Pyrenochaeta terrestris*, *Phymatotrichum omnivorum*, *Pseudocercosporella herpotrichoides, R. solani*), oomycetes (*P. cinnamomi*), bacteria (*S. scabies*), protists (*Plasmodiophora fragariae*) and nematodes (*M. incognita*) (Shipton, 1977; Wijetunga and Baker, 1979; Scher and Baker, 1980; Chern and Ko, 1989; Mazzola, 2002; Pyrowolakis et al., 2002; Weller et al., 2002; Ciancio et al., 2016; Giné et al., 2016).

When it comes to the origin of disease suppressive soils, both biotic and abiotic components in the soil have been studied for their involvement in suppressiveness attainment. Among the abiotic factors most commonly studied are soil pH, carbon and nitrogen content, soil texture (proportion and type of clay), soil moisture, and levels of cations and trace elements (i.e. P, Mg, K, Al, Fe, Na, Zn, Cu, B or Ca) (Hornby, 1983). However, the relative importance of these abiotic factors in specific disease suppression is still unclear as they are more commonly associated with general disease suppression (Höper and Alabouvette, 1996; Janvier et al., 2007). For example, suppressiveness of soils to clubroot disease caused by *Plasmodiophora brassicae* has been associated with higher levels of gentisic acid (Young et al., 1991), calcium (Hsieh and Wang, 1986; Young et al., 1991; Niwa et al., 2007) and magnesium (Young et al., 1991), with soil pH (Hsieh and Wang, 1986; Niwa et al., 2007), with lower levels of humic acids (Murakami et al., 2000) or lower concentrations of CO_2 in soil (Osozawa et al., 1994). These factors in turn affect total microbial activity and thus general suppression. The microbiological basis of general and specific suppression has been widely demonstrated by soil sterilization *via* autoclaving, gamma irradiation, steam pasteurization or the application of biocides (Scher and Baker, 1980; Alabouvette, 1986; Mazzola, 2002; Weller et al., 2002; Garbeva et al., 2004). Soil transplantation experiments are used to further discriminate between general and specific suppression. In contrast to general suppression, specific suppression of soils can be transferred to a conducive or sterilized soil by adding small amounts (0.1 – 10% (w/w)) of suppressive soil (Scher and Baker, 1980; Alabouvette, 1986; Wiseman et al., 1996; Weller et al., 2002; Mendes et al., 2011). Soil transplantation causes specific changes in the plant microbiome composition which in turn leads to an enhanced level of protection against the causal pathogen (Mendes et al., 2011; van der Voort et al., 2016). Following these observations, numerous studies have been conducted to identify the microbes involved, to resolve the underlying mechanisms and to unravel how these disease suppressive

microbes interact with other soil microbes and with the plant host. Further insight in the microbial and chemical basis of disease suppressiveness can be exploited to design strategies for achieving suppressiveness by re-introducing microbes in conducive soils or by augmenting the indigenous disease suppressive microbes and their activities in different agro-ecosystems.

Mechanisms involved in disease suppressiveness

For the majority of disease suppressive soils studied to date, the mechanisms involved in suppressiveness remain unknown or unproven. For certain suppressive soils, however, key mechanisms have been elucidated. These mechanisms include competition, antibiosis, parasitism, predation and induced systemic resistance (Kloepper et al., 1980; Scher and Baker, 1980; Neeno-Eckwall et al., 2001; Mazzola, 2002; Alabouvette et al., 2009; Junaid et al., 2013; Jambhulkar et al., 2015).

Competition

Soil environments are typically oligotrophic, which leads to competition for nutrients (e.g. carbon sources) and trace elements (e.g. iron) that are metabolically essential for microbial growth (Lemanceau et al., 2006). Nutrient availability in soils may be so limited, that for survival most of the microorganisms inhabiting soils make structures, such as sclerotia or spores, that can resist harsh conditions. Suppressive soils have been proposed to harbour higher microbial biomass so that competition, and as a result reduction of growth and virulence of the pathogen, is stronger in suppressive soils than in conducive soils (Lemanceau et al., 2006).

Although competition for carbon is associated with general suppression, specific suppression has also been correlated with carbon competition. Alabouvette (1986) observed that specific suppression in the *Fusarium* wilt suppressive soil from Châteaurenard (France) was explained, at least in part, by competition for carbon between pathogenic and non-pathogenic *F. oxysporum* strains. More specifically, the non-pathogenic *F. oxysporum* were more competitive in obtaining carbohydrates than the pathogenic *F. oxysporum* (Couteaudier and Alabouvette, 1990). Sivan and Chet (1989) demonstrated that the reduction in germination of chlamydospores of *F. oxysporum* in the rhizosphere of cotton and melon by application of *Trichoderma* T35 was attributed to competition for nutrients. Competition by non-pathogenic *Streptomyces* strains was also proposed as one of the mechanisms involved in suppressiveness of soils to the bacterial

potato scab pathogen *S. scabies* (Neeno-Eckwall et al., 2001). The role of competitive interactions between the pathogen and the resident microbiota in disease suppression has been further demonstrated by providing a surplus of carbon sources into suppressive soils, thereby favouring spore germination of the pathogen and thus leading to an increase in disease severity. For example, addition of glucose to soil reduced *Fusarium* wilt suppressiveness by stimulating chlamydospore germination of pathogenic *F. oxysporum* and *F. solani* (Alabouvette et al., 1985). Similarly, addition of sucrose and asparagine reduced suppressiveness of soil to damping-off disease of cucumber by stimulating the spore germination of the pathogen *P. ultimum* (Chen et al., 1988). Mandelbaum and Hadar (1990) further showed that application of a mixture of glucose and asparagine to compost led to an increase in the germination of *Pythium* oospores and an increased disease severity.

Besides carbon, competition for other trace elements may be involved in disease suppression. For example, Sagova-Mareckova et al. (2015) compared the chemical composition of two soils suppressive and two soils conducive to potato scab, and showed that the suppressive soil had significant lower concentrations of total C, N, Ca and Fe. Mizuno and Yoshida (1993) further observed that suppression of potato scab was related with lower pH and higher levels of exchangeable aluminium. Among all trace elements, iron in particular has received considerable attention. Availability of iron in soils is generally low (Colombo et al., 2014) and a reduction of available iron often leads to a decrease of the growth of fungal pathogens in soil (Elad and Baker, 1985; Naureen et al., 2015). Several rhizobacteria can produce siderophores that have a higher affinity for iron than siderophores produced by plant pathogenic fungi (Loper and Buyer, 1991). Fluorescent *Pseudomonas* spp. are very efficient in iron acquisition through the release of different types of siderophores (e.g., pseudobactins). By production of those siderophores, these rhizobacteria can deprive pathogens from iron, adversely affecting growth and reducing disease development (Van Loon and Bakker, 2003). For example, Kloepper et al. (1980) observed that the addition of either *Pseudomonas* strain B10, isolated from a take-all suppressive soil, or its siderophore (pseudobactin) into a take-all or a *Fusarium* wilt conducive soil, rendered these two soils suppressive to each of the respective fungal pathogens. Also Scher and Baker (1982) demonstrated that the suppressiveness of the Salinas Valley *Fusarium* wilt suppressive soil (California) was attributed to competition for iron. Amending conducive soil with the iron-chelating chemicals EDDHA, FeEDDHA or a siderphore-producing *Pseudomonas putida* isolated from the *Fusarium* suppressive soil, led to increased suppressiveness to *Fusarium* wilt of radish, flax and cucumber. The role of

competition for iron was also demonstrated in the Châteaurenard *Fusarium* wilt suppressive soil. Although the major mechanism involved in this French suppressive soil was competition for carbon sources (Couteaudier and Alabouvette, 1990), Lemanceau et al. (1988) also observed that addition of EDDHA into a conducive soil resulted in an increased level of disease suppression. Conversely, addition of $FeCl_3$ to a *Fusarium* wilt suppressive soil reduced its suppressiveness by favouring germ tube elongation of *F. oxysporum* f. sp. *lini* (Scher and Baker, 1982). Furthermore, addition of EDTA-chelated iron led to an increase in disease incidence, indicating that competition for iron was a secondary mechanism involved in the Châteaurenard suppressive soil (Lemanceau et al., 1988).

In addition to competition for carbon or trace elements, competition for root infection sites has been proposed as a mechanism involved in disease suppression (Alabouvette et al., 2009). A successful infection demands the establishment of a parasitic relationship between the invading pathogen and the plant host. Therefore, out-competing the invading pathogen for space will reduce disease development. Davis et al. (2010) showed that a decrease of *V. dahliae* wilt on potato correlated with an increase in root colonization by non-pathogenic *Fusarium equiseti*. Sneh et al. (1989) reported that the hypovirulent *Rhizoctonia* sp. isolate 521 provided significant protection on radish against virulent *Rhizoctonia* strains by densely colonizing the root surface. The hypovirulent *R. solani* AG4 (R3) could control disease caused by *R. solani* AG4 (RhFag) on radish (Cardinale et al., 2006). Further studies revealed that its control apparently relies in spatial and nutrient competition and on systemic induced resistance, and not on its ability to degrade cell walls or its hypovirulence due to the presence of double stranded RNAs (ds RNAs)(Cardinale et al., 2006). Non-pathogenic *F. oxysporum* strains were proposed to out-compete pathogenic strains by better colonization ability on celery (Schneider, 1984), flax (Eparvier and Alabouvette, 1994) and carnation (Postma and Luttikholt, 1996). Competition for infection sites has also been proposed as a mechanisms involved in potato scab suppressive soils (Neeno-Eckwall et al., 2001). In addition, mycorrhizal fungi can protect plant roots from diseases by providing not only a physical but also chemical barrier against the invading pathogen. For example, Takakura (2015) demonstrated that the ectomycorrhizal fungus *Tricholoma matsutake* controls plant pathogenic *R. solani* by producing hydrogen peroxide through its fruiting bodies.

Parasitism

Fungi can be parasitized by viruses (mycoviruses) causing reduced virulence of their fungal host (Kulshrestha et al., 2014; Kumar and Chandel, 2016). For example, Ikeda et al. (2005)

obtained isolates of *Rosellinia necatrix*, the causal agent of white root rot disease in many crops, from soils with different levels of disease incidence and from infected pear tree roots. Their results showed that *R. necatrix* isolates that contained viral double stranded RNA segments were hypovirulent and found in lower frequency in infected roots than in soil samples.

In soil environments, propagules of pathogenic fungi, including spores, conidia or sporangia are in close association with other soil organisms (Jambhulkar et al., 2015). The colonization of these propagules can prevent germination and decrease the inoculum density of the pathogen (Lockwood, 1990; Alabouvette et al., 2009). Several studies have demonstrated the role of mycoparasites in suppressing diseases caused by *Sclerotinia* spp. and other sclerotia-forming fungi (Whipps et al., 2008; Alabouvette et al., 2009). For example, application of the mycoparasitic fungus *Coniothyrium minitans* reduced disease and the number of sclerotia of *Sclerotinia sclerotiorum* in field plots undergoing rotation of potato, bean, carrot and chicory (Gerlagh et al., 1999). This mycoparasite also reduced stem rot on rapeseed caused by *S. sclerotiorum* (Li et al., 2006), and *Sclerotinia* blight of peanut caused by *S. minor* (Partridge et al., 2006). *Verticillium bigutattum* is a common mycoparasite of *R. solani* that acts by killing its sclerotia (Velvis and Jager, 1983) or by penetrating *Rhizoctonia* hyphae as well as sclerotial tissue with penetration pegs, resulting in death of *Rhizoctonia* (Boogert et al., 1989). Application of *V. bigutattum* also reduced sclerotia formation on potato tubers (Jager and Velvis, 1985; 1986) and controlled black scurf in potato caused by *R. solani* (Scholte, 1992). Further research suggested that antibiosis through production of the antifungal compounds bigutol and methylbigutol (Morris et al., 1995) as well as extracellular enzymes such as chitinases, proteases and β-1,3-glucanases (McQuilken and Gemmell, 2004) by *V. bigutattum* could play a role in suppressing *Rhizoctonia* disease.

Parasitism by fungi is typically associated with the production of extracellular enzymes, including hydrolytic cell wall degrading enzymes (CWDEs) that can lyse cell walls of fungi and oomycetes (Elad et al., 1982; Van Loon and Bakker, 2003; Naureen et al., 2015). For example, Chet and Baker (1981) demonstrated that *Trichoderma hamatum* produces β-(1-3) glucanase and chitinase that damage the mycelium of *R. solani*. Similarly, Elad et al. (1982) demonstrated that *Trichoderma harzianum* produces β-1,3-glucanase, chitinases, proteases and lipases, responsible for cell wall degradation of *Sclerotium rolfsi*. The role of CWDEs in disease suppression is supported by the findings that transformants of *Trichoderma longibrachiatum* overexpressing β-1,4-endoglucanases were more effective in controlling *P. ultimum* on cucumber (Migheli et al. 1998), whereas a transformant of *T.*

harzianum with a disruption in the endochitinase gene *ech42* was less effective than wildtype *T. hamatum* in biocontrol of *Botrytis cinerea* on bean leaves (Woo et al., 1999). Picard et al. (2000) found that the mycoparasite *Pythium oligandrum* antagonized *Phytophthora parasitica* by penetrating its hyphal cell wall and by the production of cellulolytic enzymes degrading the host cell wall. Rey et al. (2005) further showed that *P. oligandrum* could colonize sclerotia of *B. cinerea*, *S. sclerotiorum* and *S. minor* causing chitin degradation of the host cells walls.

In addition to viruses and fungi, also bacteria can exhibit parasitic activities. For example, Fradkin and Patrick (1985) demonstrated that bacterial colonization reduced germination and virulence of spores of several *Cochliobolus* spp., causal agents of root rot of grasses. Toyota and Kimura (1993) further showed that colonization of chlamydospores of *F. oxysporum* f. sp. *raphani* by *Pseudomonas stutzeri* and *Pimelobacter* spp. reduced spore germination. Like parasitic fungi, several bacterial species secrete hydrolytic enzymes (i.e. chitinases and proteases) to feed on living fungi, a process known as mycophagy. *Stenotrophomonas maltophilia* W81 was found to reduce damping-off disease on sugar beet caused by *P. ultimum* due to the secretion of an extracellular protease (Dunne et al., 1997). Also the nematicidal effects of *S. maltophilila* G2 towards *Bursaphelenchus xylophilus* were due to the production of a protease, in this case a serine protease (Huang et al., 2009). Likewise, the ability of *Pseudomonas fluorescens* CHA0 to reduce root-knot caused by *M. incognita*, was found to be due, in part, to the production of an extracellular protease (Siddiqui et al., 2005). Bacteria within the genus *Collimonas* produce chitinases and have been reported to feed on *Chaetomium globosum*, *Fusarium culmorum*, and *Mucor hiemalis* (De Boer et al., 2001), *Laccaria bicolor* (Deveau et al., 2007) and *Glomus mosseae* (Pivato et al., 2009). Introduction of mycophagous *Collimonas* in soil caused a shift in the fungal community composition indicating that *Collimonas* has feeding preferences for certain fungal species (Höppener-Ogawa et al., 2009; Rudnick, 2015). Previous studies reported not only the lysis but also the engulfment of fungal hyphae of *R. solani* and conidia of *Cochliobolus miyabeanus* by myxobacteria (Homma, 1984).

Besides enzymes, there are other compounds known to be involved in bacterial parasitism. For example, *Collimonas fungivorans* can also inhibit the growth of *Aspergillus niger* by producing volatile organic compounds (VOCs). Transcriptomic analysis showed that exposure of *A. niger* to *C. fungivorans* volatiles caused up-regulation of *A. niger* genes involved in membrane permeability and fluidity and down-regulation of genes involved in the synthesis of the cell membrane and cell wall pointing to changes in the integrity of the cell wall of *A. niger* (Mela et al., 2011). In addition, oxalic acid, a virulence factor produced by

many (pathogenic) fungi (Cessna et al., 2000; Nagarajkumar et al., 2005; Schoonbeek et al., 2007; Li et al., 2014; Nakajima and Akutsu, 2014) was suggested to play an important role in the interaction between *Collimonas* and fungi by attracting *Collimonas* towards the hyphal tip (Rudnick, 2015). Exposure of *C. fungivorans* to volatiles of *A niger* caused upregulation of *C. fungivorans* genes involved in degradation of oxalate, reinforcing the idea that pathogenic volatiles are recognised and trigger defense mechanisms such as oxalic acid degradation (Mela et al., 2011). Besides the oxalate degrading genes, also fungal growth inhibiting volatile organic compounds (VOCs) were stimulated by the presence of the fungi, indicating that production of volatiles by *Collimonas* spp. could play an important role in its mycophagous lifestyle (Garbeva et al., 2014). However, although parasitism is a powerful mechanism to control plant pathogens, its role in disease suppressive soils is yet not well understood and requires further investigation.

Predation

Predation of plant pathogenic fungi has been described for several higher organisms, such as amoebae, nematodes and microarthropods (Curl and Old, 1988; Geisen et al., 2016). For example, Homma and Ishii (1984) observed that hyphae and sclerotia of *R. solani* and conidia of *Cochliobolus miyabeanus* were perforated and the interior consumed by the vampyrellid amoebae *Arachnula impatiens*. Furthermore, higher abundances of mycophagous amoebae *Saccamoebae* spp. and *Thecamoeba granifera* ssp. *minor* were associated with an increased number of perforated *G. graminis* var. *tritici* hyphae from a take-all suppressive soil (Chakraborty and Warcup, 1983; 1984). Geisen et al. (2016) demonstrated that soil amoebae within the genera *Cercomonas*, *Leptomyxa*, *Mayorella* and *Thecamoeba* could thrive on spores of *F. culmorum*. The role of mycophagous nematodes such as *Aphelenchus avenae* in suppressing plant diseases has been studied in more detail. For example, high amounts (10^4 to 10^6 per pot) of *A. avenae* could suppress diseases of alfalfa caused by *R. solani* and *F. solani* (Barnes et al., 1981; Lee and Pankhurst, 1992). *R. solani* disease could also be suppressed on potato by *A. avenae* and also by the collembola *Folsomia finmetaria* (Lootsma and Scholte, 1997). The collembolans *Proisotoma minuta* and *Onychiurus encarpatus* were more attracted to *R. solani* than to beneficial fungi such as *Trichoderma* (Curl and Old, 1988; Lartey et al., 1989). Furthermore, they readily consumed the mycelium of *R. solani* and reduced *R. solani* disease when they were in soil at densities of 1000-2000 kg^{-1} (Curl and Old, 1988).

Antibiosis

Antibiosis is the most widely studied mechanism of microbial interactions in the context of disease suppressive soils. Antibiosis is defined here as inhibition of the growth and/or activity of one organism by another organism *via* the production of specific or nonspecific metabolites, including volatiles and other diffusible compounds (Fravel, 1988; Thomashow and Weller, 1996; Lemanceau et al., 2006).

Of the antibiotics with a role in disease suppressive soils, 2,4-diacetylphloroglucinol (2,4-DAPG) and phenazine-1-carboxylic acid (PCA) have been studied in most depth (Haas and Defago, 2005; Raaijmakers and Mazzola, 2012). Both 2,4-DAPG and PCA are produced by several strains of (fluorescent) *Pseudomonas* associated with suppressiveness of soils to take-all disease of wheat or *Fusarium* wilt of flax (Raaijmakers and Weller, 1998; Weller et al., 2002; Lemanceau et al., 2006). The densities and diversity of the *Pseudomonas* spp. producing these antibiotics were determined for soils suppressive and conducive to different fungal pathogens. In take-all suppressive soils, phenazine-producing strains were not detectable whereas 2,4-DAPG-producing strains were found in multiple take-all suppressive soils in the USA and the Netherlands (Raaijmakers et al., 1997; de Souza et al., 2003; Bergsma-Vlami et al., 2005). Furthermore, Raaijmakers et al. (1999) also detected 2,4-DAPG in the take-all suppressive soil at concentrations of 0.62 ng per 10^5 CFU indicating that 2,4-DAPG is produced *in situ* on roots of wheat in the take-all suppressive soil. For the *Fusarium* wilt suppressive soil, Mazurier et al. (2009) found that the frequencies of bacterial populations positive for 2,4-DAPG (2,4-DAPG+) were similar between conducive and suppressive soils, whereas populations positive for PCA (PCA+) were detected only in the *Fusarium* wilt suppressive soil. Subsequent bioassays with isolated PCA+ strains showed that although they could not suppress *Fusarium* wilt by their own, they acted synergistically in disease suppression when combined with the non-pathogenic *F. oxysporum* Fo47. Similarly, Latz et al. (2012) quantified the *Pseudomonas* populations harbouring the *phlD* or *prnD* genes (encoding 2,4-DAPG and pyrrolnitrin, respectively) by qPCR in soils with different levels of suppressiveness towards *R. solani* and found a positive correlation between the frequency of these genes and the level of disease suppression. 2,4-DAPG and also the antibiotic pyoluteorin have been implicated in the suppressiveness of the Morens soil in Switzerland against black root rot of tobacco caused by *Thielaviopsis basicola* (Laville et al., 1992; Haas and Defago, 2005).

Antifungal VOCs also play an important role in antagonistic interactions between microbes and in disease suppressiveness of soils (Garbeva et al., 2011; Mendes et al., 2013;

Weisskopf et al., 2016). Approximately 30-60% of the soil isolates are able to produce antifungal VOCs (Garbeva et al., 2011). VOCs can diffuse through aqueous solutions and soil air spaces, so their effective range of action can be up to meters, allowing the VOC producers to suppress pathogens that are physically separated (Garbeva et al., 2011; Schmidt et al., 2015; Weisskopf et al., 2016). The first serious indication for a role of volatile fungistatic substances in suppressing the development of soil-borne pathogens was based on the study by Kouyeas and Balis (1968), in which they observed that the passage of an air stream over soil and removing microbial volatiles nullified its fungistatic effect (Lockwood, 1977). Hora and Baker (1970) demonstrated for the first time the existence of a volatile factor existing in soil that significantly contributed to reduced spore germination of twenty fungal species. Later studies demonstrated that the diversity of VOCs produced by microorganisms is extensive and comprises alcohols, aldehydes, acids, esters, terpenes, ketones, alkenes, benzenoids, pyrazines, and sulfur-containing compounds (Effmert et al., 2012). Some of these compounds suppress the growth of several plant pathogens. Early studies reported ammonia, an inorganic volatile substance, for its involvement in disease suppression. For example, Ko et al (1974) observed that ammonium chloride was contributing to the fungistatic activity of an alkaline soil by inhibiting spore germination of *Neurospora tetrasperma*, *Mucor rammanianus*, *Aspergillus fumigatus* and *Penicillium frequentans*, but not of *Fusarium solani* f. sp. *phaseoli* or *Calonectria crotalariae*. Also Howell et al. (1988) described that ammonia produced by *Enterobacter cloacae* was involved in suppression of *P. ultimum* damping-off in cotton. In addition, Duffy et al. (1997) analyzed the biocontrol activity of *Trichoderma koningii*, isolated from a take-all suppressive soil, on eight acidic soils and observed that its biological control activity was positively correlated with the amount of nitrate-nitrogen in these soils. Their results confirmed that alkaline soils tend to release more ammonia than acidic soils, reducing the germination of conidia, as it was observed for the biocontrol agent *T. hamatum* (Papavizas, 1985).

Hydrogen cyanide (HCN), a highly toxic compound that acts by blocking the cytochrome oxidase pathway, is one of the first volatiles studied for its role in disease suppression. HCN production by *P. fluorescens* CHA0, a strain originally isolated from a tobacco black root rot suppressive soil, is one of the metabolites involved in inhibition of root infections caused by *T. basicola* (Voisard et al., 1989). Another study pointing to a role of VOCs in disease suppressiveness of soils is from Chuankun et al. (2004), who analyzed 146 soils with different levels of fungistasis towards the fungal pathogens *Paecilomyces lilacinus* IPC, *Pochonia chlamydospora* ZK7, and *Clonostachys rosea* GR87. They found that

the VOCs trimethylamine, 3-methyl-2-pentanone, dimethyl disulfide, methyl pyrazine, 2,5-dimethyl-pyrazine, N,N-dimethyloctylamine and nonadecane were typically found in fungistatic soils. Benzaldehyde was found in most of the samples, but its relative concentration was higher in fungistatic soils. Minerdi et al. (2009) showed that the volatiles produced by the non-pathogenic *F. oxysporum* MSA35, a strain isolated from a *Fusarium* wilt suppressive soil, suppressed pathogenic *F. oxysporum*. The VOCs produced by the non-pathogenic *F. oxysporum* included sesquiterpenes, mainly carophyllene. More recently, Cordovez et al. (2015) analyzed the VOC profiles of *Streptomyces* species obtained from a *R. solani* suppressive soil and showed significant inhibition of hyphal growth *in vitro*. Profiling of the VOCs produced by two *Streptomyces* strains revealed the production of the antifungal VOCs methyl 2-methylpentanoate and 1,3,5-trichloro-2-methoxy benzene. Whether these or other VOCs are also produced *in situ* at concentrations that are sufficient to inhibit fungal growth remains to be tested. Hol et al. (2015) realized dilution-to-extinction in seven different soils leading to a decrease of low-abundance microbial species and observed that only communities with a high microbial richness were able to produce volatiles able to inhibit the growth of *F. oxysporum*. Among the volatile compounds suspected of being involved in fungal inhibition they found 2-methylfuran, 2-furaldehyde, 2-(methylthio)benzo thiazole, and muurolol.

Biosurfactants are amphiphilic compounds involved in cell adhesion, dispersion, flocculation, cell aggregation and defence against predators (Raaijmakers et al., 2010). Besides influencing basic traits such as motility, biofilm formation or colonization, biosurfactants can also affect the availability of nutrients, enhancing their uptake, or enhancing the biological activity of other metabolites such as antibiotics (D'Aes et al., 2011). Moreover, biosurfactants can also have a direct antimicrobial activity. For example, the biosurfactant cyclic lipopeptide viscosinamide produced by *P. fluorescens* DR54 was shown to reduce mycelial density and intracellular activity, and induced encystment of *Pythium* zoospores, thereby contributing to suppression of the pathogen and survival of sugar beet (Thrane et al., 2000). Viscosinamine and thanamycin, a nine amino acid chlorinated lipopeptide produced by *Pseudomonas* SH-C52, were also suggested to be involved in the suppression of *R. solani* on sugar beet (Thrane et al., 2001; Mendes et al., 2011; Watrous et al., 2012). Michelsen et al. (2015) isolated *P. fluorescens* In5 from a soil suppressive to *R. solani* AG3 and demonstrated that its activity *in vivo* was due to the production of the cyclic lipopeptide nunapeptin. The application of purified rhamnolipid biosurfactants in the re-circulating nutrient solution could reduce disease due to *Pythium aphanidermatum*, *Plasmopara lactucae-radicis* and *Phytophthora capsici* in cucumber by

breaking the plasma membrane of their zoospores (Stanghellini et al., 1996; Stanghellini and Miller, 1997), and application of either rhamnolipid or saponin biosurfactant in hydroponic systems could fully suppress disease due to *P. capsici* on pepper by killing its zoospores (Nielsen et al., 2006).

Induced systemic resistance

Plants naturally possess defence mechanisms to combat pathogen intrusion. However, in some cases pathogens are able to circumvent the defence barriers raised by the plant so disease occurs. Upon recognition of a pathogen plants activate a defense response known as systemic acquired resistance (SAR), that enables the plant to react rapidly to a second confrontation with the pathogen (Pieterse et al., 2014). However, defence in plants can also be activated prior to pathogen attack by exposure to non-pathogenic rhizobacteria. This type of defence is activated upon recognition of bacterial determinants that trigger an induced systemic resistance (ISR) response (Pieterse et al., 2014). Hence, ISR constitutes an important mechanism involved in disease suppression. To date, the role of ISR in disease suppressive soils is still not well understood. Work by Larkin et al. (1996) showed that non-pathogenic *F. oxysporum*, when physically separated from the pathogenic *F. oxysporum* in a split-root experimental design, suppressed *Fusarium* wilt on watermelon. Whether this mechanism also operates in suppressive soil has, to my knowledge, not been conclusively demonstrated yet.

Approaches to study disease suppressive soils

The interest in unraveling the underlying mechanisms of disease suppressive soils already goes back to the first study in 1892 by Atkinson. Knowledge of the mechanisms that lead to suppressiveness can be instrumental in managing disease suppressive soils. Here, different methods and approaches to study the microbiological basis of disease suppressive soils are summarized.

The role of microbes in disease suppressive soils was first demonstrated by heat treatments (pasteurization, steam, autoclaving or gamma irradiation), and by application of biocides that eliminate specific microbial groups. This was clearly exemplified in the work of Mendes et al. (2011) where heating of a *R. solani* suppressive soil for 1 hour at 50°C or 80°C led to a partial or complete loss of disease suppressiveness, respectively. A second approach used to demonstrate the role of microbes in disease suppressive soils comprised

the transfer of suppressiveness to a conducive soil by mixing 10% (w/w) or less of the suppressive soil into the conducive soil or into a pasteurized suppressive soil. The first example of disease-suppressiveness transplantation was published by Henry (1931a), who observed that the addition of small amounts of non-sterilized soil into a sterilized soil inhibited the capacity of the latter to suppress the sporulation of *Helminthosporium sativum*. To further study the mechanism of suppressiveness, Henry (1931b) isolated bacteria, fungi and actinomycetes from the soil suppressive to *H. sativum* and confirmed that each of them, when tested individually, reduced the severity of root-rot infection on wheat. Furthermore, combining all these organisms resulted in an even stronger suppression of *H. sativum*. For *R. solani*, Mendes et al. (2011) showed that suppressiveness could also be transplanted to a conducive soil and that *Pseudomonas* isolates were involved, at least in part, in this suppression. Lemanceau and Alabouvette (1991) tested the ability of different fluorescent *Pseudomonas* isolated from the Châteaurenard soil in suppressing *Fusarium* wilt, alone and in combination with the non-pathogenic *F. oxysporum* Fo47. Results indicated that although the majority of the *Pseudomonas* isolates could not suppress *Fusarium* wilt, several of the *Pseudomonas* isolates could suppress *Fusarium* wilt when introduced alone, and some others suppressed *Fusarium* wilt only when re-inoculated with the non-pathogenic *F. oxysporum* Fo47 (Lemanceau and Alabouvette, 1991). Liu et al. (1996) isolated *Streptomyces* strains from soils suppressive and conducive to potato scab and showed that some of these strains, when re-introduced in conducive soil, were able to control the pathogenic *S. scabies* both under greenhouse and field conditions. Non-pathogenic *F. oxysporum* f. sp. *melonis* strains were isolated from a soil suppressive to *Fusarium* wilt obtained upon mono-cropping of melon, and their re-inoculation in conducive soil could enhance protection against pathogenic *F. oxysporum* f. sp. *melonis* on melon (Sneh et al., 1987). Yang et al. (2012) isolated *Pochonia chlamydosporia* var. *chlamydosporia* from a *M. incognita*-suppressive soil, and its re-inoculation in conducive soil was shown to significantly reduce disease caused by *M. incognita*. Mghalu et al. (2007) isolated *Trichoderma* species from an induced suppressive soil against *R. solani* and *S. rolfsii* and observed that a *T. viride* strain isolated from that suppressive soil could suppress disease in radish caused by *S. rolfsii* and *R. solani* whereas a *T. koningii* strain isolated from the same suppressive soil could only suppress *S. rolfsii.*

These studies showed that first the microbial basis needs to be determined of disease suppressiveness of soils, and subsequently isolations, and phenotypic and genotypic characterization are required to elucidate the mechanisms of pathogen suppression. Numerous studies have followed this line of research resulting in the

identification of several microbial genera and species with a role in disease suppressiveness of soils. The most prominent are the (fluorescent) *Pseudomonas* spp., *Streptomyces* spp., *Bacillus* spp., non-pathogenic *F. oxysporum* and *Trichoderma* spp. (Alabouvette et al., 2009).

However, disease suppression is not attributed to just one microbe but rather to a microbial community. In the early 30's, Henry already suggested the role of multiple saprophytic microorganisms in suppressing soil-borne diseases (Henry, 1931a; 1931b). Alabouvette (1986) stated that suppressiveness in the Châteaurenard soil was attributed to the complementary association of competition for nutrients between the total microflora and the entire *Fusarium* population as well to the intrageneric competition between pathogenic and non-pathogenic *Fusarium*. Thus, different microbial genera appeared to be responsible for the disease suppression observed in the Châteurenard suppressive soil. Comparative metataxonomic analyses of the microbial communities between suppressive and conducive soils have also suggested that suppressiveness is caused by microbial consortia rather than by the activity of individual microbial genera. For example, Mendes et al. (2011) showed that Actinobacteria, Gammaproteobacteria, Acidobacteria, Alphaproteobacteria, Bacteroidetes, Betaproteobacteria and Planctomycetes were among the most dynamic bacterial phyla associated with a *R. solani* disease suppressive soil (The Netherlands). Other meta-analyses on the microbial communities of *Rhizoctonia* suppressive soils have also detected other microbial groups associated with disease suppression (Donn et al., 2014; Penton et al., 2014; Chapelle et al., 2015; van der Voort et al., 2016).

Although several microorganisms that have been isolated from suppressive soils efficiently control the pathogen under greenhouse conditions, the majority of them fail under field environments. This inconsistency in *in vivo* activity has been attributed to a reduced efficacy to colonize the rhizosphere or to express their protective characteristics under field conditions. Also deficiencies in the formulation, timing, mode of application and dosage may contribute to this inconsistency (Alabouvette et al., 2009). Moreover, this approach does not take into account the non-culturable fraction of the soil microbial community. Whether the non-culturable microbial fraction plays a role in disease suppressiveness of soils has, to my knowledge, not been investigated yet.

To go beyond the one-microbe-at-a-time approaches and to analyse disease suppressive soils from a community perspective, other technologies have to be used. These technologies include community profiling by terminal restriction fragment length polymorphism (T-RFLP) or denaturing gradient gel electrophoresis (DGGE), quantitative

PCR (Q-PCR), PhyloChip analysis, 16S and ITS amplicon sequencing, metagenomics, metatranscriptomics, metaproteomics and metabolomics. Each technique has its advantages and disadvantages. For example, Donn et al. (2014) used microarray, T-RFLP and qPCR analyses to study soils with different levels of disease suppression towards *R. solani* and found that abundance of *Pseudomonas* spp. showed conflicting results between microarray data and the qPCR, most probably due to the specificity of the target (Donn et al., 2014).

In the past years, several metagenomic studies have been conducted to compare the microbial (mainly bacterial) community composition of soils suppressive and conducive against different plant pathogens, including *F. oxysporum*, *G. graminis* var. *tritici*, *T. basicola* or *R. solani*. Among the microbial taxa most frequently found in higher abundance in suppressive soils than in conducive soils are Alphaproteobacteria (*Azospirillum*, *Gluconacetobacter*, *Sphingomonadaceae*), Betaproteobacteria (*Burkholderia*, *Oxalobacteraceae*, *Comamonadaceae*), Gammaproteobacteria ((fluorescent) *Pseudomonadaceae*, *Xanthomonadaceae*), Actinobacteria (*Streptomyces*), Verrucomicrobia (*Opitutaceae*), Acidobacteria (Gp4, Gp5), Firmicutes (*Bacillus*, *Tumebacillus*, *Thermoanaerobacter*), Planctomycetes, Nitrospira, Chloroflexi, Armatimonadetes (*Chthonomonas*) and Gemmatimonadetes (Kyselkova et al., 2009; Sanguin et al., 2009; Mendes et al., 2011; Li et al., 2015; Shen et al., 2015).

However, the community analyses conducted to date (including, Q-PCR, DGGE, T-RFLP, amplicon sequencing and metagenome sequencing) have not provided insight into the metabolically active microbial groups or into specific activities involved in disease suppressiveness. To target the active microbial communities, DNA-Stable Isotope Probing (DNA-SIP) (Radajewski et al., 2000) or metatranscriptomics (Ofek-Lalzar et al., 2014; Ofek et al., 2014; Tkacz et al., 2015) can be applied. Several studies (Ofek-Lalzar et al., 2014; Ofek et al., 2014; Tkacz et al., 2015) highlight that polyphasic strategies, combining different approaches and technologies, should be adopted to investigate the microbial ecology of complex ecosystems including disease suppressive soils.

Rhizoctonia suppressive soils

The genus *Rhizoctonia* constitutes a complex mixture of filamentous fungi characterized by brown, wide and septate hyphae with 90° lateral branching, the lack of production of asexual spores and the formation of sclerotia as survival structures. Its teleomorph stage is known as *Thanatephorus cucumeris*. Infection is often initiated by mycelium or sclerotia

(Fig. 1). Hyphae can sense the presence of the host plant *via* exudates released by the roots. Infection typically starts belowground (seeds, hypocotyls and roots), although above ground parts (stems, leaves and fruits) can also be infected. *R. solani* often enters its host through intact tissue, although it can also enter through wounded tissue, lenticels and stomata (Ogoshi, 1987).

R. solani was first described in 1858 by Julius Kühn on potato and is a devastating soil-borne plant pathogenic fungus in agriculture, horticulture and forestry. It is distributed worldwide and survives as saprotroph and facultative parasite (Ogoshi, 1987). It has an extensive host range, including both ornamental (hortensias, tulips, gladiolos or chrysanthemum) and agronomic species (rice, potato, sugar beet, bean, lettuce, soybean, tobacco, tomato, cotton, corn, wheat or strawberry, among others). *R. solani* causes several types of diseases including root rot, bare patch, crown rot, and damping-off of seedlings (Cao et al., 2004). The extent of the host range and the type of disease symptoms vary between anastomosis group (AG) (Pannecoucque and Höfte, 2009). To date, 14 *R. solani* anastomosis groups (AGs) have been reported, which are further classified in several intraspecific groups (Bolton et al., 2010; Tuncer and Eken, 2013). *R. solani* can be controlled, in part, by the use of resistant cultivars and by fungicides. However, both strategies are not very effective or, in the case of fungicides, not preferred due to adverse environmental effects. Hence, there is a need to develop more sustainable approaches to control diseases caused by this devastating pathogen. Understanding the microbes and mechanisms operating in *Rhizoctonia* disease suppressive soils may lead to the development of new measures that, in conjunction with other management practices, provide a more consistent and durable control of this pathogen. Previous studies done on *Rhizoctonia* suppressive soils aimed at unraveling which microbes contribute to disease suppression and through which mechanisms they contribute. Although several microorganisms have been found to be more abundant in *Rhizoctonia* suppressive soils, the mechanisms through which they may confer disease suppression are still poorly understood. The findings from previous research are listed in Table 1 and summarized below.

Penton et al. (2014) studied the fungal community composition of soils suppressive and conducive to *R. solani* AG8 using 454-sequencing targeting the 28 LSU rRNA gene and T-RFLP. Their results showed significant differences in the fungal community composition between the suppressive and the conducive soils, and between the soil type/location. The differences associated with disease suppression were attributed to less than 40 genera, including a number of endophytic species and mycoparasites (Penton et al., 2014). Similarly, Yin et al. (2013) used metataxonomic analysis to analyze the

bacterial community of soils with different levels of disease suppression towards *R. solani* AG8 on wheat. Their results showed that *Acidobacteria* Gp7 and *Flavobacterium*, were found in higher abundance in recovered patches and outside patches, and this was validated by qPCR (Penton et al., 2014).

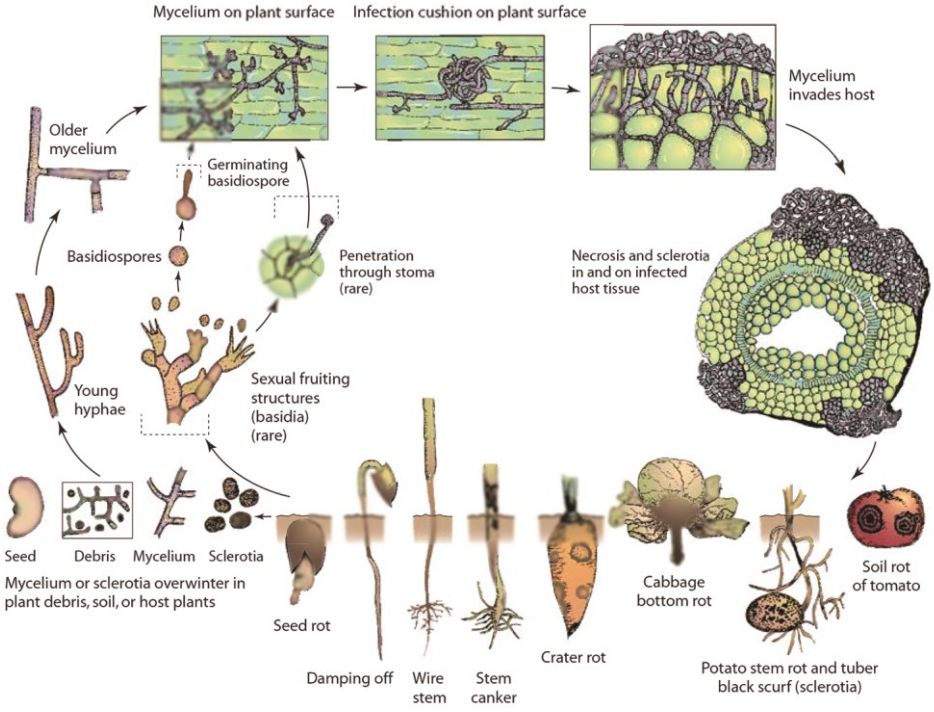

Figure 1. Disease cycle of *Rhizoctonia solani* (adapted from Agrios, 2005).

qPCR targeting genes encoding antibiotics showed that soils with highest suppressiveness were those where both 2,4-DAPG and pyrrolnitrin gene abundances were highest (Latz et al., 2012). Scherm et al. (2009) used qRT-PCR to target genes upregulated in *T. harzianum* when confronted with *R. solani* and showed that increased expression of genes encoding acetyl xylan esterase and endoglucanases were correlated with higher antagonistic activities of different *Trichoderma* strains. Donn et al. (2014) determined the bacterial composition of soil samples from different sites with different levels of disease suppression to *R. solani* by microarray and T-RFLP analysis. When comparing the bacterial community in the suppressive soil from Avon, Australia (sampled after 25 years of mono-cropping) and the conducive soil (obtained from the margins of the field), they observed that *Paenibacillus borealis*, the genus *Asaia* and the family *Cystobacterineae* were found in higher abundance in suppressive soil. Conversely, *Acetobacteraceae*, *Rhizobiaceae*, *Bradyrhizobiaceae* and Actinobacteria were found in higher abundance in conducive soil (Donn et al., 2014). Additionally, Donn et al. (2014) compared the bacterial communities from inside *Rhizoctonia* diseased patches and from outside these patches in the region of Galong. Although disease severity was equivalent in pot assays when using soil from either inside the patches, outside the patches or in a 1:1 mixture of both, the soils harbored different bacterial communities. For example, samples from outside the patches contained higher amounts of Proteobacteria, Cyanobacteria, Firmicutes, Bacteroidetes and Actinobacteria, whereas samples from inside *Rhizoctonia* diseased patches contained a higher amount of Betaproteobacteria, some Gammaproteobacteria and Deltaproteobacteria. Samples with a 1:1 mixture of both soils produced an intermediary community (Donn et al., 2014). Contrarily to the important role of *Pseudomonas* spp. in suppressing *Rhizoctonia* damping-off shown by Mendes et al. (2011), Donn et al. (2014) found that several probes targeting *Pseudomonas* were found in higher abundance in the soil inside the diseased patches than in soils supporting healthy plants outside the patches.

To date, metataxonomic studies on *Rhizoctonia* suppressive soils are limited. For example, Yin et al. (2013) analyzed the bacterial communities of soils suppressive and soils with different levels of disease due to *R. solani* AG8, causing bare patch and root rot disease on wheat. Based on metataxonomic analyses, *Acidobacteria* and *Gemmatimonas* were found in higher abundance in the rhizosphere of healthy plants whereas *Dyella* and *Acidobacteria* subgroup Gp7 were found in higher abundance in recovered patches. Conversely, the rhizosphere of diseased plants showed higher abundances of *Chitinophaga*, *Pedobacter*, *Oxalobacteriaceae* and *Chyseobacterium*. Penton et al. (2014) analyzed the fungal community composition of conducive and suppressive soils to *R.*

solani AG8 on wheat at two different sites and found that suppressive soils had higher abundances of *Xylaria*, *Peethambara*, *Anthostomella*, *Ascolobus*, *Zygopleurage*, *Parasola* or *Bionectria*, among others.

For *R. solani* suppressive soils, (Chapelle et al., 2015) performed both metagenomic and metatranscriptomic analyses to determine the transcriptional changes in the bacterial community in the rhizosphere of sugar beet seedlings exposed to the fungal pathogen. They found that upon addition of *R. solani*, stress-related genes were upregulated, particularly in the bacteria belonging to the *Oxalobacteraceae*, *Sphingobacteriaceae*, *Burkholderiaceae*, *Alcaligenaceae*, *Cystobacteraceae*, *Sphingomonadaceae*, *Cytophagaceae*, *Comamonadaceae* and *Verrucomicrobia* subdivision 3. Based on these results they proposed a model in which *R. solani* grows towards the root system secreting oxalic and phenylacetic acid, activating specific rhizobacterial families and, directly or indirectly, exerting oxidative stress in both the rhizobacterial community as in the plant. This stress in turn leads to the activation of survival strategies of the rhizobacterial community including enhanced motility, biofilm formation and production of secondary metabolites.

Table 1. Overview of the microbial groups found to be associated with *Rhizoctonia* suppressive soils in various studies and proposed mechanisms involved in conferring specific suppression.

Rhizoctonia AG	Crop	Location	Type of study	Microbes	Putative mechanism	Reference
AG1-1B, AG1-1C, AG-2, AG4	Radish	Japan	Isolation	*Trichoderma viride, T. harzianum, T. hamatum*	Unknown	(Mghalu et al., 2007)
AG2-2IIIB	Sugar beet	The Netherlands	PhyloChip	Actinobacteria, Gammaproteobacteria, Acidobacteria, Alphaproteobacteria, Bacteroidetes, Planctomycetes, Chloroflexi	Unknown	(Mendes et al., 2011)
AG2-2IIIB	Sugar beet	The Netherlands	Isolation	*Pseudomonas* SH-C52	Cyclic lipopeptide (thanamycin)	(Mendes et al., 2011)
AG2-2IIIB	Sugar beet	The Netherlands	Metagenome, metatranscriptome	*Oxalobacteraceae, Burkholderiaceae, Sphingobacteriaceae, Sphingomonadaceae*	Acid stress response and oxidative stress response	(Chapelle et al., 2015)
AG2-2IIIB	Sugar beet	The Netherlands	PhyloChip	*Streptomycetaceae, Micrococcaceae, Mycobacteriaceae, Solibacteriaceae*	Unknown	(van der Voort et al., 2016)
AG2-2IIIB	Sugar beet	The Netherlands	Isolation	*Streptomyces* spp.	Antibiosis through volatiles (methyl 2-methylpentanoate, 1,3,5-trichloro-2-methoxy benzene)	(Cordovez et al., 2015)
AG2-2IIIB	Sugar beet	The Netherlands	Isolation	*Trichoderma* spp.	Hyperparasitism	(Bakker et al., 2006)
AG2-2IIIB	Bentgrass	Japan	Isolation	*Pseudomonas fluorescens* HP72	Antibiosis (2,4-DAPG)	(Murakami et al., 2007; He et al., 2004)

Rhizoctonia AG	Crop	Location	Type of study	Microbes	Putative mechanism	Reference
AG2-2, AG4	Cocoyam	Cameroon	Isolation	*Pseudomonas* spp. CMR12a	Cyclic lipopeptides & phenazines	(Perneel et al., 2006)
AG3	Potato	Greenland	Isolation	*Pseudomonas fluorescens* In5	Cyclic lipopeptide (nunapeptin)	(Michelsen et al., 2015)
AG3	Grass	The Netherlands & Sweden	Isolation	*Pseudomonas, Streptomyces, Bacillus*	Cell wall degrading enzymes and competition for iron	(Adesina et al., 2007)
AG3	Grass	The Netherlands & Sweden	Isolation	*Pseudomonas jessenii*	Unknown	(Adesina et al., 2009)
AG3	Potato	The Netherlands	Isolation	*Verticillium bigutattum*	Parasitism	(Velvis and Jager, 1983a; Velvis and Jager, 1983b)
AG3	Potato	The Netherlands	*In vivo* assays	*Verticillium bigutattum*	Parasitism	(Velvis and Jager 1983b; Jager and Velvis, 1986)
AG4	Carnation	Colombia	Isolation	*Trichoderma hamatum*	Unknown	(Chet and Baker, 1981)
AG4	Radish, Cucumber	USA	Isolation	*Trichoderma* spp.	Unknown	(Liu and Baker, 1980)
AG5	Wheat	USA	Isolation	(fluorescent) *Pseudomonas* spp.	Unknown	(Mazzola and Gu, 2002)
AG8	Wheat	Australia	Isolation	*Pantoea agglomerans, Exiguobacterium acetylicum, Microbacteriaceae*	Unknown	(Barnet et al., 2006)
AG8	Wheat	United States	16S amplicon	Acidobacteria (Gp7), *Gemmatimonas, Dyella*	Unknown	(Yin et al., 2013)
AG8	Wheat	Australia	Taxonomic microarray	*Asaia* spp., *Cystobacterineae, Paenibacillus borealis*	Unknown	(Donn et al., 2014)
AG8	Wheat	Australia	28S amplicon	*Xylariaceae, Bionectriaceae, Hypocreaceae*	Unknown	(Penton et al., 2014)

Thesis outline

Disease suppressive soils occur worldwide, but the underlying mechanisms and the microorganisms involved are not well understood for most of these soils. The overall aim of my thesis was to identify the role of specific bacterial genera and their modes of action in the suppressiveness of soils to damping-off disease of sugar beet caused by the fungal root pathogen *Rhizoctonia solani.* Understanding the microbial consortia and microbial activities involved in disease suppressive soils may provide practical means to engineer soil and plant microbiomes to control soil-borne plant diseases. This introductory chapter (Chapter 1) gives a historical perspective of disease suppressive soils, with a focus on *Rhizoctonia* suppressive soils, and an overview of the underlying mechanisms known to date. In this Chapter, also relevant approaches used to study the nature of disease suppressive soils are discussed.

To date, several microbial groups have been proposed to play a role in suppressiveness of soils to *R. solani.* These include the bacterial genera *Lysobacter*, *Pseudomonas* and *Streptomyces*. Chapter 2 focuses on the role of different *Lysobacter* spp. in disease suppressiveness to *R. solani*. *Lysobacter* spp. were previously found in higher abundance in soils suppressive to *R. solani* and several isolates have shown activity *in vitro*. In Chapter 2, the ability of different *Lysobacter* spp. to produce lytic enzymes and antimicrobial metabolites is investigated, as well as their ability to suppress damping-off disease caused by *R. solani.* Furthermore, this chapter explores the capacity of *Lysobacter* spp. to promote growth of different plant species. Although most of the strains exhibit antimicrobial activities *in vitro*, no disease suppression by each of the individual isolates was observed *in vivo*.

Chapter 3 evaluates whether combinations of *Lysobacter* strains or combinations of *Lysobacter*, *Pseudomonas* and/or *Streptomyces* strains can lead to an increased or more consistent level of disease control. To this end, the ability to reduce hyphal growth of *R. solani* was tested for the single strains and for several strain combinations, referred to as synthetic communities or SynComs. The effects of these SynComs on suppression of damping-off disease caused by *R. solani* was also investigated in this chapter. The results presented show that none of the SynComs tested provided significant disease control *in vivo,* suggesting that other, yet unknown bacterial genera play a more prominent role in *Rhizoctonia* suppressive soils.

To identify these unknown bacterial genera and functions, in Chapter 4 a reproducible, small-scale and short-term bioassay was established in which *Rhizoctonia* suppressiveness was induced in a timeframe of six successive plant growth cycles of three

weeks each. Subsequent experiments were performed to elucidate the temporal dynamics in the rhizobacterial community composition and functions during the transition of the soil from the conducive to the disease-suppressive state. Only minor shifts in rhizobacterial community composition were observed, whereas major shifts in functions were found. Among the functions found up-regulated during the induction of *Rhizoctonia* soil suppressiveness were enzymes involved in the degradation of oxalic acid. Oxalic acid is an organic compound secreted by *R. solani* and other plant pathogenic fungi that acts as a virulence factor.

Chapter 5 investigates the role of oxalotrophic bacteria, i.e. bacteria able to use oxalic acid as a sole carbon and energy source, in suppressing damping-off disease caused by *R. solani*. Firstly, we determined the phylogenetic diversity of oxalotrophic bacteria in an agricultural field soil and evaluated their potential to suppress *Rhizoctonia* damping-off disease of sugar beet. To this end, bacterial communities that can use oxalate as a sole carbon source were enriched and extracted from soil. Subsequently, the oxalate-enriched bacterial community was isolated and their efficacy to control *R. solani* damping-off disease was assessed.

Chapter 6 summarizes the major findings of this thesis and addresses the specificity of *Rhizoctonia* suppressive soils. The importance of integrated 'omics analyses to better understand the mechanisms underlying disease suppressive soils is highlighted and directions for further research are given.

Chapter 2

Diversity and activity of *Lysobacter* species from disease suppressive soils

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Abstract

The genus *Lysobacter* includes several species that produce a range of extracellular enzymes and other metabolites with activity against bacteria, fungi, oomycetes and nematodes. *Lysobacter* species were found to be more abundant in soil suppressive against the fungal root pathogen *Rhizoctonia solani*, but their actual role in disease suppression is still unclear. Here, the antifungal and plant growth-promoting activities of 18 *Lysobacter* strains, including 11 strains from *Rhizoctonia* suppressive soils, were studied both *in vitro* and *in vivo*. Based on 16S rRNA sequencing, the *Lysobacter* strains from the *Rhizoctonia* suppressive soil belonged to the four species *L. antibioticus*, *L. capsici, L. enzymogenes* and *L. gummosus*. Most strains showed strong *in vitro* activity against *R. solani* and several other pathogens, including *Pythium ultimum*, *Aspergillus niger, Fusarium oxysporum* and *Xanthomonas campestris*. When the *Lysobacter* strains were introduced into soil, however, no significant and consistent suppression of *R. solani* damping-off disease of sugar beet and cauliflower was observed. Subsequent bioassays further revealed that none of the *Lysobacter* strains was able to promote growth of sugar beet, cauliflower, onion and *Arabidopsis thaliana*, either directly or *via* volatile compounds. The lack of *in vivo* activity is most likely attributed to poor colonization of the rhizosphere by the introduced *Lysobacter* strains. In conclusion, our results demonstrated that *Lysobacter* species have strong antagonistic activities against a range of pathogens, making them an important source for putative new enzymes and antimicrobial compounds. However, their potential role in *R. solani* disease suppressive soil could not be confirmed. In-depth omics'- based analyses will be needed to shed more light on the potential contribution of *Lysobacter* species to the collective activities of microbial consortia in disease suppressive soils.

Keywords: *Lysobacter*, *Rhizoctonia solani*, *Beta vulgaris*, disease suppression, plant growth promotion.

Introduction

Lysobacter are Gram-negative bacteria widely distributed in diverse ecosystems, including soil, rhizosphere and freshwater habitats (Reichenbach, 2006). The genus *Lysobacter* was first described in 1978 by Christensen and Cook and included four species. *Lysobacter* spp. are closely related to members of the genus *Xanthomonas* and were initially misclassified as *Cytophaga*, *Sorangium* or *Myxobacter* (Christensen and Cook, 1978). Currently, 30 *Lysobacter* species have been taxonomically accepted (for updates see http://www.bacterio.net/lysobacter.html) and new *Lysobacter* species have been recently identified (Du et al., 2015; Lin et al., 2015; Singh et al., 2015) but are not yet included in the database. Various members of this bacterial genus have activity against a range of other (micro)organisms, including Gram-negative and Gram-positive bacteria, fungi, oomycetes and nematodes (Reichenbach, 2006). They are well-known for the production of a variety of extracellular enzymes and antimicrobial compounds. Enzymes identified for *Lysobacter* include chitinases (Zhang and Yuen., 2000; Zhang et al., 2001), glucanases (Palumbo et al., 2005), proteases (Stepnaya et al., 2008; Gökçen et al., 2014; Vasilyeva et al., 2014), lipases (Folman et al., 2003; Ko et al., 2009) as well as elastases, keratinases, phosphatases, endonucleases, endoamylases and esterases (Reichenbach, 2006). Antimicrobial compounds described for *Lysobacter* include lysobactin, tripopeptin, xanthobaccin, maltophilin, dihydromaltophilin, phenazine, lactivicin (Xie et al., 2012), HSAF (Li et al., 2008), and WAP8294-A (Zhang et al., 2011). Currently, WAP8294-A is in phase I/II clinical trials for controlling methicillin-resistant *Staphylococcus aureus* (MRSA) (Zhang et al., 2011; Wang et al., 2013).

In terms of ecosystem services, Postma et al. (2010a) showed a correlation between the abundance of three *Lysobacter* species (*L. antibioticus*, *L. capsici* and *L. gummosus*) in soil and the level of suppressiveness against *Rhizoctonia solani*, a devastating fungal pathogen of numerous economically important crops such as sugar beet, potato and rice. Also in the study by Mendes et al. (2011), the Xanthomonadaceae family, to which *Lysobacter* belongs, was found more abundant in a soil suppressive against *R. solani* on sugar beet. Several studies have shown that application of *Lysobacter* spp. reduced diseases caused by different plant pathogens in several crops such as cucumber (Folman et al., 2004; Postma et al., 2009), bean (Yuen et al., 2001), rice (Ji et al., 2008), pepper (Ko et al., 2009), grapevine (Puopolo et al., 2014), tomato (Puopolo et al., 2010), and sugar beet and spinach (Islam et al., 2005). To date, however, few data are available on the frequency and diversity of *Lysobacter* species in natural habitats and little

is known about the ecology and the determinative role of *Lysobacter* species in plant growth promotion and disease suppressive soils. The work described here focused on elucidating the role of *Lysobacter* spp. in protecting plants against soil-borne diseases and in stimulating plant growth. To that end, we determined (i) the genetic and phenotypic diversity of 18 different *Lysobacter* strains obtained from soil and plant-associated environments, (ii) their activity against a range of pathogens, (iii) if these *Lysobacter* strains alone can suppress damping-off disease of sugar beet and cauliflower caused by *R. solani*, and (iv) if *Lysobacter* can promote plant growth *via* direct contact and/or *via* production of volatile compounds.

Materials and methods

Strains, culture and storage conditions

The *Lysobacter* strains used in this study (Table 1) were isolated from different Dutch soils suppressive to *R. solani*. Reference strains (Table 1) were obtained from the DSMZ strain collection (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). For the activity and plant growth promotion assays, *Lysobacter* strains were pre-cultured in tryptone soya broth (TSB, Oxoid) for 2-3 days at 25°C on a rotary shaker at 200 rpm and cells were washed 3 times with 0.9% NaCl unless mentioned otherwise. The fungal pathogens used in this study were mostly provided by the Institute of Sugar Beet Research (IRS). *Fusarium oxysporum* Forl1 was provided by the University of Turin, Italy (Clematis et al., 2009), *Verticillium dahliae* JR2 by B. Thomma [Wageningen University (WUR)], *Phytophthora infestans* by F. Govers (WUR) and *Aspergillus niger* was provided by L. de Graaf (WUR) (Table S1). The bacterial strains were kept in 40% (v/v) glycerol at -80°C; the fungi and oomycetes were kept in mineral oil at 10°C.

Table 1. Isolation details of the *Lysobacter* strains used in this study.

Code	Species	Strain	Soil type	Source	Crop	Origin	Location	Year	Reference
L02	*Lysobacter antibioticus*	3.2.10	clay	soil	grass/clover	Suppressive soil	Pietersbierum, NL	2003	Postma *et al.,* 2008
L08	*Lysobacter antibioticus*	76	clay	soil	cauliflower	Suppressive soil	Zwaagdijk, NL	2003	Postma *et al.,* 2010b
L23	*Lysobacter antibioticus*	4.1.2	clay	soil	potato	Suppressive soil	Marknesse, NL	2006	Postma *et al.,* 2008
L32	*Lysobacter antibioticus*	DSM2044	N.A.	soil	N.A.	Type strain	Ottawa, CA	N.A.	Christensen and Cook, 1978
173	*Lysobacter antibioticus*	173	clay	soil	no crop	Suppressive soil	Zwaagdijk, NL	2011	(this study)
174	*Lysobacter antibioticus*	174	clay	soil	no crop	Suppressive soil	Zwaagdijk, NL	2011	(this study)
L12	*Lysobacter capsici*	6.2.3	clay	soil	grass/clover	Suppressive soil	Hoensbroek, NL	2003	Postma *et al.,* 2010a
L13	*Lysobacter capsici*	1.3.3	clay	soil	grass/clover	Suppressive soil	Strijen, NL	2003	Postma *et al.,* 2010a
L14	*Lysobacter capsici*	55	clay	soil	cauliflower	Suppressive soil	Zwaagdijk, NL	2003	Postma *et al.,* 2010a
L31	*Lysobacter capsici*	DSM19286	N.A.	rhizosphere	pepper	Type strain	South Korea	2003	Park *et al.*, 2008
L19	*Lysobacter enzymogenes*	1.1.4	sand	soil	grass	Suppressive soil	Bakel, NL	2004	Nijhuis *et al.,* 2010
L28	*Lysobacter enzymogenes*	3.1T8	rockwool	root tip	cucumber	Suppressive soil	Wageningen, NL	1997	Folman *et al.,* 2003
L29	*Lysobacter enzymogenes*	C3	N.A.	leaf	turfgrass	Suppressive soil	Nebraska, USA	N.A.	Sullivan *et al.,* 2003
L30	*Lysobacter enzymogenes*	DSM2043	N.A.	soil	N.A.	Type strain	Ottawa, CA	N.A.	Christensen and Cook, 1978
L05	*Lysobacter gummosus*	2.4.7	clay	soil	grass/clover	Suppressive soil	Ijzendijke, NL	2003	Postma *et al.,* 2008
L15	*Lysobacter gummosus*	3.2.11	clay	soil	grass/clover	Suppressive soil	Pietersbierum, NL	2003	Postma *et al.,* 2008
L26	*Lysobacter gummosus*	10.1.1	clay	soil	pea	Suppressive soil	Ijzendijke, NL	2006	Postma *et al.,* 2008
L33	*Lysobacter gummosus*	DSM6980	N.A.	soil	N.A.	Type strain	Ottawa, CA	N.A.	Christensen and Cook, 1978

N.A. Not applicable/not available; NL = The Netherlands; USA = United States of America; CA = Canada

Soil collection and storage

The non-suppressive (conducive) soil to *R. solani* was collected from a pear orchard located in Zwaagdijk, The Netherlands (52°41'53.549" N, 5°6'58.643" E) in June 2012 at a depth of 10-40 cm. The soil, classified as clay soil with loam texture (29.9% of the particles are >50 µm, 26.4% of the particles are <2 µm), was air-dried, sieved (0.5 cm mesh) to remove plant/root material and stored at 8°C until use for the *in vivo* activity test of *Lysobacter* spp. against *R. solani* on cauliflower.

Genetic and phenotypic characterization of the *Lysobacter* strains

BOX-PCR

To determine the genetic variation among *Lysobacter* strains, the repetitive elements in their genome were analysed by BOX-PCR according to Rademaker et al. (2004). Amplification reactions were conducted in 25 µl volume composed of 1 µl BOX-A1R primer (10 µM), 1.25 µl dNTPs (25 mM each), 0.4 µl BSA (10 mg/ml), 2.5 µl 100% DMSO, 5 µl 5x Gitschier buffer, 0.4 µl Taq polymerase (5U/µl SuperTaq) and 14.45 µl miliQ water. DNA was added by a toothpick inoculation of bacterial cells in the reaction mix. The reaction volume was heated to 95°C for 2 min, followed by 30 cycles of 3 seconds at 94°C, 92°C for 30 seconds, 50°C for 1 min and 65°C for 8 min. The PCR reaction was finished with an 8 min incubation at 65°C for and then kept at 8°C. Five µl of the PCR product was loaded on an 1.5% (w/v) agarose gel and ran overnight at 40V.

Phylogenetic analyses

For each *Lysobacter* strain, the sequences of the 16S ribosomal RNA gene, the gene encoding a recombination/repair protein (*recN*) and the gene encoding the subunit C of the excinuclease ABC (*uvrC*) were amplified using primers described in Table 2. The markers *recN* and *uvrC* were chosen based on Zeigler (2003) who showed that these candidate genes will provide high fidelity for species prediction, and the 16S rRNA gene was included because of its broad use in taxonomic studies. Amplification reactions were conducted in 25 µl volume composed of 1 µl each of forward and reverse primer (10 µM), 1 µl dNTPs (5 mM each), 1.5 µl $MgCl_2$ (25 mM), 5 µl 5x GoTaq Flexibuffer, 0.125 µl GoTaq polymerase (5U/µl) and 15.375 µl miliQ water. DNA was added by a toothpick inoculation of bacterial cells in the reaction mix. The reaction volume was heated to 95°C for 3 min, followed by 35 cycles of: 1 min at 95°C, 58°C for 1 min, 72°C for 1.4 min (for 16S rRNA), 1 min at 95°C, 57.2°C

for 1 min, 72°C for 1.2 min (for *recN*), and 1 min at 95°C, 58°C for 1 min, 72°C for 2 min (for *uvrC*); The PCR reaction were finished with an 5 min incubation at 72°C for and then kept at 12°C. Five µl of the PCR product were visualized on an 1.5% (w/v) agarose and PCR products were sequenced by Macrogen Inc. (Amsterdam, The Netherlands). Phylogenetic trees were constructed with the three markers independently or concatenated using ClustalW alignments (Thompson et al., 1994) and neighbor joining tree constructions using the Tamura 3 parameter model and discrete Gamma distribution in MEGA6 (Tamura et al., 2013).

The sequences obtained during this study are deposited in NCBI GenBank under accession numbers KT851449 to KT851466 for *uvrC*, KT851467 to KT851484 for 16S rRNA and KT851485 to KT851502 for *recN*.

Table 2. Primer sets used for phylogenetic analysis

Gene target	Primer	Oligonucleotides sequence (5'→ 3')
16S rRNA	Forward	AGAGTTTGATCCTGGCTCAG
16S rRNA	Reverse	ACGGGCGGTGTGTACA
recN	Forward	CTCAAGCAATTCGCCGTC
recN	Reverse	CACCTGCACCGCGCTCTG
uvrC	Forward	CGGCAAGGCCTTCGTCAAGC
uvrC	Reverse	CGTGCAAGGCGGCGTAGAT

Swarming ability

Motility of the *Lysobacter* strains was assessed on soft standard succinate medium (SSM) as described in de Bruijn and Raaijmakers (2009). In brief, 5 µl of *Lysobacter* suspensions was spot-inoculated in the center of soft standard succinate medium (SSM) agar Petri dishes [(32.8 mM K_2HPO_4, 22 mM KH_2PO_4, 7.6 mM $(NH_4)_2SO_4$, 0.8 mM $MgSO_4$, 34 mM succinic acid (w/v)), adjusted pH to 7 and 0.6% agar (w/v)]. Petri dishes were incubated for 2 to 12 days at 25°C.

Enzymatic activity

Chitinase, glucanase and protease activity of the *Lysobacter* strains were tested as described in de Bruijn et al. (2015). In brief, 2-5 µl of *Lysobacter* suspensions (of stationary phase of growth) was spot-inoculated in the center of different media containing 1.5-2% agar. For chitinase activity, R2A (Oxoid) and 1/10th strength TSB agar Petri dishes were used containing 0.2% colloidal chitin prepared from crab shell chitin (Sigma) and Petri dishes were incubated for 3-7 days at 25°C. For glucanase activity, R2A medium containing 0.5% laminarin was used and Petri dishes were incubated for 3 days at 25°C. The colonies were removed by washing with water and the medium was stained with 1% congo red. After destaining, coloration of the medium was determined. For protease activity, bacteria were inoculated on 15 g/l skimmed milk powder, 4 g/l blood agar base and 0.5 g/l yeast extract and Petri dishes were incubated for 3-7 days at 25°C.

In vitro antagonistic activity

Lysobacter strains (Table 1) were grown in 5 ml TSB for 2 days at 25°C on a rotary shaker at 200 rpm. Suspensions were washed once by centrifugation at 3800 x *g* for 5 min and 10x concentrated in 0.9% NaCl.

To test activity against bacterial pathogens, R2A, 1/5th potato dextrose agar (PDA, Oxoid) and Luria-Bertani (LB, Difco) agar Petri dishes were prepared with an overlay of 1% water agar cooled down to 50°C to which washed cells of a culture of the bacterial pathogens (Table S1) were added. Subsequently, 2-5 µl of the *Lysobacter* cell suspensions (of stationary phase of growth) was spot-inoculated on the medium. Petri dishes were incubated for 3-7 days at 25°C and clearing zones surrounding the colonies were monitored.

To test inhibition of mycelial growth, oomycetes and fungal strains (Table S1) were grown on PDA at 25°C. Four 5 µl of the *Lysobacter* suspensions were spot-inoculated at the edges of Petri dishes containing 20 ml of R2A, 1/5th PDA or PDA and a fresh 5 mm agar plug with actively grown mycelium was placed in the middle of the Petri dish.

To test antagonism against fungal spores, fungi (Table S1) were grown on PDA until sporulation. To enhance spore production, *Cercospora* and *Stemphylium* were grown on vegetable juice agar Petri dishes [(vegetable juice (V8) solified with 1.5% agar)] (Beckman and Payne., 1983; Rossi et al., 2005) under 16 h photoperiod, and to enhance spore collection from *Verticillium* and *Aspergillus,* the spores of those two fungi were scratched from the mycelium and streaked on fresh PDA Petri dishes. Fungal spores were collected as described in Trifonova et al. (2008) with slight modifications. In brief, spores

were released from the mycelium by adding 10 ml of 0.9% NaCl and scratching the surface with a sterile spatula, collected, 10-fold diluted and added to the culture media (PDA, 1/5th PDA and R2A) of 48-55°C to a final concentration of 5% (v/v). Four 5 µl of the *Lysobacter* suspensions were spot-inoculated at the edges of Petri dishes containing 20 ml of medium with spores. For each assay, 3 replicates per media were used. Petri dishes without *Lysobacter* were used as controls. All Petri dishes were incubated at 25°C for one week and subsequent inhibitory halo formation was monitored.

In vivo activity of *Lysobacter* spp. against *Rhizoctonia solani*

Spontaneous rifampicin-resistant mutants of the *Lysobacter* strains were verified by BOX-PCR. These mutants exhibited chitinase activity to the same extent as their parental strains. The rifampicin-resistant mutants were grown in 10 ml of TSB supplemented with 50 µg/ml rifampicin for 2 days at 25°C on a rotary shaker at 200 rpm. Cultures were centrifuged, washed 3 times and resuspended in 0.9% NaCl. Cell suspensions were mixed in a potting soil:river sand (1:9, w/w) mixture at an initial density of 10^7 cells/g soil and approximately 20% hydration. Rectangle shape trays (19.5 x 6 x 3.5 cm) were filled with 250 g of the potting soil:sand mixture (8 replicates per treatment) and 16 sugar beet seeds coated with thiram, hymexazol and poncho-beta were sown in a row, 1 cm apart. Non-inoculated soil was used as a control. Trays were placed in boxes with transparent lids in a growth chamber at 24°C with a 16 h photoperiod. After 5 days, seeds germinated and a single fresh 1/5th PDA agar plug (5 mm) grown with *R. solani* AG2-2IIIB was placed touching the first seedling, with the mycelial side towards the plant. Spread of *R. solani* was scored at regular intervals during 2 weeks by scoring the number of diseased plants as well as the distance between the inoculum and the most distal plant suffering from damping-off. In addition, the area under the disease progress curve (AUDPC) was calculated to determine the disease dispersal over time as:

$$\left(Ak = \sum_{i=1}^{Ni-1} \frac{(y_i + y_{i+1})}{2} (t_{i+1} - t_i) \right)$$

where t_i are the time points in a sequence (days) and y_i are measures of the disease dispersal (cm). Therefore, y(0) is defined as the initial infection at t = 0 and A(tx) is the AUDPC (total accumulated diseased dispersal until t = t_x).

From each tray, the rhizospheres of two healthy sugar beet plants that were the closest to the last infected one were collected. Two replicates were pooled together in 4 ml 0.9% NaCl, vortexed for 1 min, sonicated for 1 min and vortexed for 15 sec. Fifty µl of a 10x, 100x and 1000x fold dilution were plated on selective medium, R2A supplemented with 50 µg/ml rifampicin, 200 µg/ml ampicillin, 25 µg/ml kanamycin and 100 µg/ml delvocid. Petri dishes were incubated at 25°C for one week. Colony forming units (CFU) were counted and CFU/g rhizosphere was calculated. The *in vivo* assay and the rhizosphere colonization test were done twice.

A similar experiment was performed in cauliflower using the same set up as described above with slight differences. Bacterial strains were grown in 10 ml of LB broth supplemented with 50 µg/ml rifampicin at 25°C for 3 days. The selected *Lysobacter* strains for this assay were L08, L14, L15, L19, L29. Bacterial strains were inoculated in Zwaagdijk conducive soil at an initial density of 10^5 and 10^7 cells/g soil. Sowing, *R. solani* AG2-1 inoculation, growth of the plants, disease scoring and AUDPC calculation was done as described above. The experiment was repeated twice, once with rifampicin resistant *Lysobacter* and once with non-rifampicin resistant *Lysobacter*. Statistically significant differences were determined by one-way ANOVA and post hoc Dunnet's analysis ($P<0.05$) performed in SPSS 22.0.

In vitro plant growth promotion assay

Seed preparation

Prior to surface sterilization, naked sugar beet (*Beta vulgaris*) seeds were soaked in 0.03 N HCl for 6 h under rotation, washed with sterile milliQ water and air-dried to enhance seed germination (Habib., 2010). Surface sterilization of sugar beet, cabbage (*Brassica oleracea*) and onion (*Allium cepa*) seeds was performed by washing the seeds in 2% sodium hypochlorite for 5 min and rinsing them with sterile milliQ water. Seeds were placed on Whatman filter paper moistened with 3 ml sterile milliQ water and pre-germinated at 25°C for 2-3 days. *Arabidopsis thaliana* (Columbia 0) seeds were sterilized in an exicator with 50 ml of commercial bleach (10% v/v) + 3% of concentrated HCl for 4 h, placed in wet Whatman filter paper and incubated at 4°C in darkness for 3 days.

Seed inoculation

Two day-old pre-germinated sugar beet seeds were soaked in 3 ml of *Lysobacter* suspensions of 10^9 cells/ml for 30 min. Subsequently, sugar beet seeds were placed in

cylinder shaped plastic containers (9 cm diameter, 8 cm height) with transparent lids containing 150 ml of 0.5 x Murashige and Skoog (MS) medium (including vitamins) (6 seeds per container), and incubated in a growth chamber at 24°C with a 16 h photoperiod. Fresh and dry weight of shoots and roots were determined after two weeks. The experiment was done twice, with 3 replicates per treatment.

Root tip inoculation

Two days-old pre-germinated sugar beet seeds were placed in square Petri dishes (10 x 10 x 2 cm) containing 50 ml of 0.5 x MS medium (4 seeds/ Petri dish). Petri dishes were incubated in vertical position in a growth chamber at 24°C with a 16 h photoperiod until the roots were approximately 1 cm long and 2 µl of the *Lysobacter* suspensions of 10^9 cells/ml were spotted onto each root tip and incubated for one week. Fresh and dry weight of shoots and roots was determined. The experiment was done once, with 3 replicates per treatment.

Volatile assay

Two days-old pre-germinated seeds of sugar beet, cauliflower and onion were placed in containers as described above containing either 150 ml of 0.5 x MS medium or 150 g of a sterile mixture of potting soil:sand (1:9) with 20% humidity. A small Petri dish (35 mm diameter), containing 4 ml of R2A medium was placed in the middle of the container, and the *Lysobacter* strains were inoculated into the small Petri dishes at a density of 10^7 cells/Petri dish. Containers were incubated in a growth chamber at 24°C with a 16 h photoperiod for 2 weeks and fresh and dry weight of shoots and roots as well as leaf area were determined. The experiment was performed 3 times for sugar beet, once for cauliflower and once for onion, with 5 replicates per treatment. For the volatile assay in *A. thaliana*, *L. antibioticus* L08, *L. capsici* L14, *L. gummosus* L15 and *Pseudomonas fluorescens* SBW25 (known by its ability in promoting plant growth in *A. thaliana* when growing on King´s B (KB) agar medium and used as a positive control (J.M. Raaijmakers, personal communication)) were used. Each bacterial strain was pre-cultured in LB broth for 2 days at 25°C, and then washed three times with 10 mM $MgSO_4$. A 10 µl drop of a bacterial suspension of 10^9 cells/ml was spotted in the small Petri dish (35 mm diameter) containing 4 ml of R2A, LB or KB agar medium and Petri dishes were incubated for one day at 25°C. Small Petri dishes were placed into big Petri dishes (150 mm diameter) containing 50 ml of 0.5 x MS medium and five 3-days-old pre-germinated seeds were sown per Petri dish. Petri dishes with medium but without bacteria were included as controls. Petri dishes were incubated in vertical position in a growth chamber at 21°C with a 16 h photoperiod for 21

days. After that period, fresh and dry weight of shoots and roots were determined. The experiment was repeated once with 5 replicates/treatment.

Seed colonization ability

Naked sugar beet seeds were surface sterilized as described above and soaked in 3 ml of bacterial suspensions containing 10^9 cells/ml for 30 min as described above for the seed inoculation assay (22 seeds/bacterial treatment). Six seeds from each bacterial suspension were placed in 4 ml 0.9% NaCl, vortexed 1 min, sonicated 1 min and vortexed 15 sec. Fifty µl of both undiluted suspensions and 10x, 100x, 1000x and 10000x time dilutions were plated on R2A agar dishes and incubated at 25°C for one week. The remaining seeds were sown in squared Petri dishes containing 50 ml of 0.5 MS (4 seeds/Petri dish, 4 replicates per treatment) and incubated as described above for the root tip inoculation assay. After one week, the roots of the seedlings from each Petri dish were excised and placed in 4 ml of 0.9% NaCl, vortexed 1 min, sonicated 1 min and vortexed 15 sec. Fifty µl of both undiluted suspensions and 10x, 100x, 1000x and 10000x fold dilution were plated on R2A agar dishes, incubated at 25°C for one week and the amount of colony forming units (CFU) per seed and per root were determined by colony counting.

Results

Genetic and phenotypic characterization of the *Lysobacter* strains

BOX-PCR profiling of the 18 *Lysobacter* strains revealed a high genetic diversity among the different *Lysobacter* species and between strains of a given species (Fig. 1A). *L. gummosus* strains showed the lowest intraspecific diversity whereas *L. enzymogenes* strains showed the highest diversity. Based on 16S rRNA sequences, the most phylogenetically distant species was *L. enzymogenes* (Fig. S1A). When using either *recN* or *uvrC* or the three molecular markers together, however, *L. antibioticus* was the most distant of the four species (Fig. 1B and Fig. S1B and C).

The *Lysobacter* strains did not show any motility after 4 days of incubation on soft SSM agar medium. After 12 days of incubation, however, *L. capsici* (L12, L13, L14 and L31) and *L. enzymogenes* (L19, L28, L29, L30) did spread from the point of inoculation, most likely due to gliding motility (Fig. 2). All *Lysobacter* strains used in this study showed extracellular chitinase and glucanase activities (Fig. 2). Most strains presented proteolytic activity except for two *L. gummosus* and four *L. antibioticus* strains (Fig. 2). Variation in

these three enzymatic activities among strains belonging to the same species was observed, especially for the *L. antibioticus* strains.

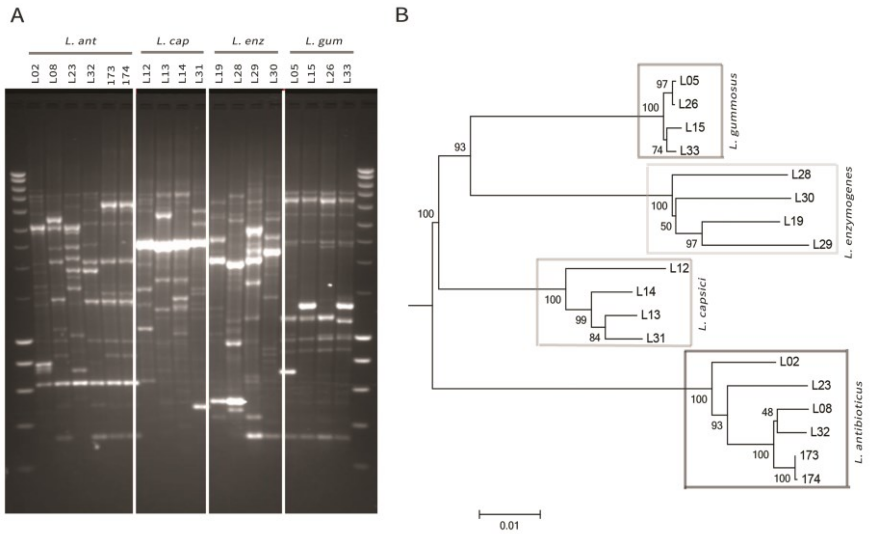

Figure 1. Genetic diversity of 18 selected *Lysobacter* strains belonging to four different species. A) Genetic profiling by BOX-PCR. Lanes on complete left and right shows Smartladder (Eurogentec) marker. B) Phylogenetic tree of the *Lysobacter* strains based on the concatenated sequences of the 16S ribosomal RNA gene (16S rRNA), a gene encoding a recombination/repair protein (*recN*) and a gene encoding the subunit C of the excinuclease ABC (*uvrC*). The evolutionary relationship of the *Lysobacter* strains was inferred by alignment with ClustalW and neighbor-joining tree construction. The numbers at the nodes indicate the level of bootstrap support of 50 or higher, based on neighbor-joining analysis of 1,000 resampled data sets. The bar indicates the relative number of substitutions per site.

The antimicrobial activity of the *Lysobacter* strains (Table 1) was tested on different media. Almost all *Lysobacter* strains showed a strong antagonistic activity against all pathogens tested (Table S1), except against the plant pathogenic bacterium *Pectobacterium atrosepticum*. The magnitude of the antagonistic activity of *Lysobacter* was media-dependent, with the strongest activity on R2A medium and the weakest activity on PDA medium (Fig. 2). *L. capsici* was the most consistent species in terms of antagonistic activity, with all *L. capsici* strains showing activity on R2A against all pathogens tested except for *X. campestris* and *L. capsici* strain L31 against *S. parasitica* (Fig. 2). On R2A, all *L. enzymogenes* and *L. gummosus* strains, with the exception of the type strains, showed activity against all pathogens tested. The type strain of *L. enzymogenes* did show activity

against *V. dahliae* JR2, *A. cochlioides* and *P. infestans,* whereas the *L. gummosus* type strain had activity against all oomycetes tested except *P. ultimum* (Fig. 2). *L. antibioticus* strains showed the highest variation in activity, with strain L23 having the broadest antimicrobial activity (Fig. 2).

A

Phenotypic characteristics		*L. antibioticus*						*L. capsici*				*L. enzymogenes*				*L. gummosus*			
		L02	L08	L23	L32	173	174	L12	L13	L14	L31	L19	L28	L29	L30	L05	L15	L26	L33
Motility		-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-
Enzymatic activity																			
Chitinase		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glucanase		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Protease		+/-	+/-	+	+	+/-	+/-	+	+	+	+	+	+	+	+	+	-	+	-
Antagonistic activity	***Medium***																		
Fungi																			
R. sol	R2A	+/-	+/-	+/-	+	+/-	-	+	+	+	+	+	+	+	-	+	+	+	-
	PDA	-	-	-	-	-	-	+	+	+	+	+	+	+	-	+	+	+	-
	1/5 PDA	-/+	-	+	+	-	-	+	+	+	+	+	+	+	-	+	+	+	-
V. dah_J	R2A	+	+	+	+	-	-/+	+	+	+	+	+	+	+	+	+	+	+	-
	PDA	+	+	+	+	NT	NT	+	+	+	+	-	NT	+	+	+	+	+	+
	1/5 PDA	+	+	+	+	-	-/+	+	+	+	+	+	+	+	+	+	+	+	+
F. sol	R2A	-	-	-/+	-	-	-	+	+	+	+	+	+	+	-	+	+	+	-
	PDA	NT	NT	-	NT	-	NT	+	+	+	+	+	+	+	-	+	+	+	-
	1/5 PDA	-	-	-	-	-	-	+	+	+	+	+	+	+	-	+	+	+	-
V. dah	R2A	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	-
	PDA	-	-	+	-	-	-	+	+	+	+	+	+	+	-	+	+	+	-
	1/5 PDA	+	-	+	-	-	+	+	+	+	+	+	+	+	-	+	+	+	-
F. oxys	R2A	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	-
	PDA	-	-	+	-	-	-	+	+	+	+	+	+	+	-	+	+	+	-
	1/5 PDA	+	+	+	-	-	-	+	+	+	+	+	+	+	-	+	+	+	-
Forl1	R2A	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-
	PDA	-	-	-	+	-	-	+	+	+	+	+	+	+	-	+	+	+	-
	1/5 PDA	-	-	+	-	-	+	+	+	+	+	-	-	+	-	+	+	+	-
A. nig	R2A	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	-
	PDA	-	-	-	-	-	-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	-	+/-	+/-	+/-	-
	1/5 PDA	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-
C. bet	R2A	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	-
	PDA	+	-/+	-/+	-	-	-	+	+	+	+	+	+	+	-	+	+	+	-
	1/5 PDA	-	-	-	-	-	-	+	+	+	+	+	+	+	-	+	+	+	-
Stem	R2A	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-
	PDA	-	-	+	-	-	-	+	+	+	+	+	+	+	-	+	+	+	-
	1/5 PDA	-/+	-/+	+	-/+	+	-/+	+	+	+	+	+	+	+	-	+	+	+	-
Oomycetes																			
A. coc	R2A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	PDA	+	+	+	+	+	+	+	+	+	+	+	+	+	+/-	+	+	+	+
	1/5 PDA	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+
P. ult	R2A	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	-
	PDA	-	-	-	-	-/+	-	+	+	+	+	+	+	+	-	+	+	+	-
	1/5 PDA	-	-/+	+	-	-	+/-	+	+	+	+	+	+	+	-	+	+	+	+
P. inf	R2A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	PDA	+	+	+	+	NT	NT	+	+	+	+	+	+	+	+	+	+	+	-/+
	1/5 PDA	+	+	+	-	NT	NT	+	+	+	+	+	+	+	-	+	+	+	-
S. par	R2A	+	+	+	-	-	+	+	+	+	+/-	+	+	+	-	+	+	+	-/+
	PDA	+/-	+/-	+/-	-	NT	NT	+	+	+	+	-/+	-/+	-/+	-	+	+	+	+
	1/5 PDA	++	+	+	-/+	NT	NT	+	+	+	+	+	+	+	-	+	+	+	-
Bacteria																			
X. cam	R2A	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-
	1/5 PDA	+	+	+	+	-	+	-	-	-	-	-	-	-	-	+	+	+	-
P. atr	R2A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1/5 PDA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	LB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

B

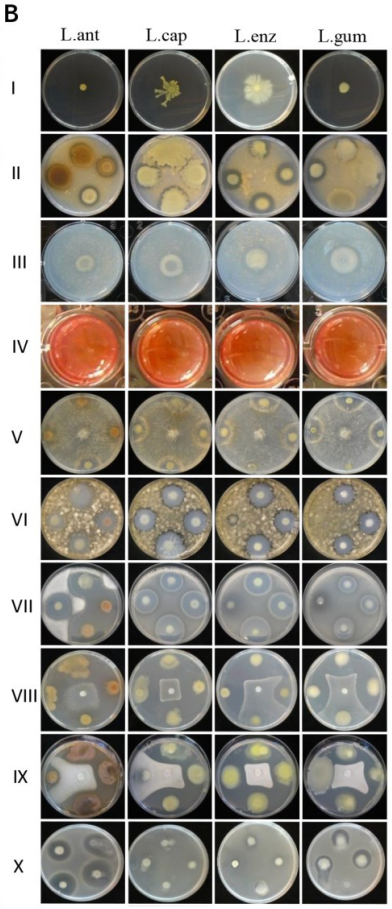

Figure 2. Phenotypic characterization of the *Lysobacter* strains, including **A)** motility, protease, chitinase and glucanase activities, and antagonistic activity against pathogenic fungi, oomycetes and bacteria. + indicates activity; - indicates no activity; +/- indicates antagonistic activity observed after 2-3 days of incubation, but the activity disappeared upon longer incubation. For the enzymatic activity, the +/- indicates weak activity; NT indicates not tested. **B)** Pictures of phenotypic characterization of *L. antibioticus* (L. ant), *L. capsici* (L. cap), *L. enzymogenes* (L. enz) and *L. gummosus* (L. gum) for I: motility on SSM medium; II: chitinase activity; III: glucanase activity, positive glucanase activity is given by the change from red to orange color (not shown); IV: protease activity; and *in vitro* antagonistic activity on R2A (except when otherwise indicated) against V: *Rhizoctonia solani*; VI: *Cercospora beticola*; VII: *Verticillium dahliae*; VIII: *Pythium ultimum*; IX: *Aphanomyces cochlioides* on PDA and X: *Xanthomonas campestris* pv *campestris* on 1/5th PDA.

In vivo activity of Lysobacter spp. against *Rhizoctonia solani*

The efficacy of the *Lysobacter* strains, several of which originate from *Rhizoctonia* suppressive soil, to control *Rhizoctonia* damping-off disease of sugar beet seedlings was tested in a sterilized (by autoclaving twice) sand-potting soil mixture and in a non-sterilized agricultural soil. Seed germination was not affected by the *Lysobacter* strains. In two bioassays, none of the strains was able to consistently suppress damping-off disease caused by *R. solani* after two weeks of plant growth (Fig. 3A). For example, strains L19 and L05 significantly reduced damping-off disease of sugar beet in bioassay 2 but not in bioassay 1 (Fig. 3A).

The results further showed that after an initial application of 10^7 CFU/g soil, *Lysobacter* strains established densities in the rhizosphere of sugar beet ranging from 10^3 to 10^8 CFU/g (Fig. 3B), with substantial variation between strains and between the two bioassays. In general, *L. gummosus* strains were better rhizosphere colonizers whereas *L. antibioticus* showed the highest variation among strains. *L. antibioticus* strains L8 and 174 were only detected in the sugar beet rhizosphere in bioassay 1. *L. antibioticus* L23 was detected at high densities (10^8 CFU/g) in bioassay 1, but at 1000-fold lower densities in bioassay 2. *L. enzymogenes* L19 was only detected in bioassay 2 (Fig. 3B).

The ability of *Lysobacter* to suppress *Rhizoctonia* damping-off disease of another host plant (cauliflower) was assessed for *Lysobacter* strains L08, L14, L15, L19 and L29 at two initial densities of 10^5 and 10^7 CFU/g of soil. Also for this crop, germination was not affected by the introduced bacterial strains and again no significant and consistent reduction in disease incidence was observed. When applied at 10^5 CFU/g of soil, strain L19 significantly reduced disease incidence but only in bioassay 2 (Fig. 3C). For bioassay 2, colonization of cauliflower rhizosphere by the *Lysobacter* strains was determined. The results showed that the densities recovered were lower (10^1 to 10^3) than initially applied except for *L. enzymogenes* L29 and *L. gummosus* L15 when applied at 10^7 CFU/g soil (Fig. S2). After an initial application of 10^5 cells/g soil, only *L. gummosus* L15 and *L enzymogenes* L19 and L29 were detected in the rhizosphere of cauliflower.

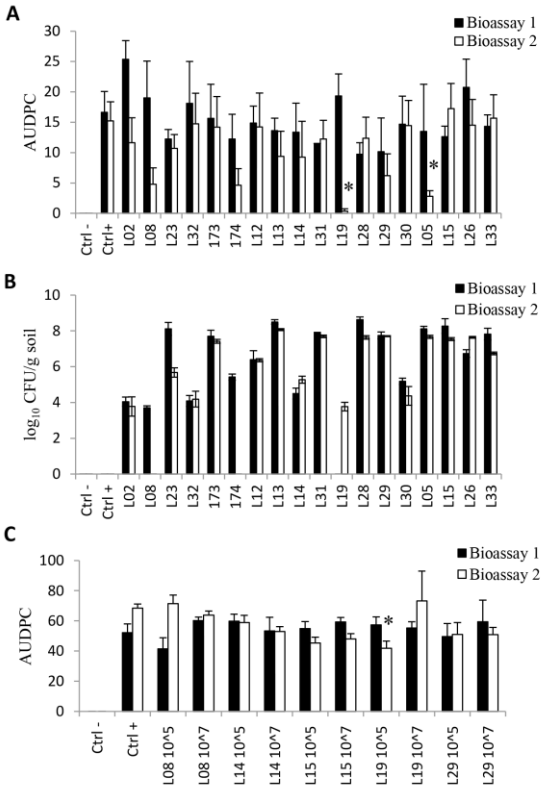

Figure 3. *In vivo Rhizoctonia* disease suppression and rhizosphere colonization ability by *Lysobacter* strains. A) Area under disease progress curve (AUDPC) of disease spread for sugar beet when *Lysobacter* strains were applied at an initial density of 10^7 CFU/g into a mixture potting soil:sand (1:9); B) Colonization of the rhizosphere of sugar beet by the *Lysobacter* strains when applied at an initial density of 10^7 CFU/g into a mixture potting soil:sand (1:9). C) AUDPC of disease spread for cauliflower when *Lysobacter* strains were applied into a conducive soil. 10^7 and 10^5 means an initial density of the inoculum at 10^7 and 10^5 cells/g soil, respectively; *L. antibioticus*: L02, L08, L23, L32, 173, 174; *L. capsici*: L12, L13, L14, L31; *L. enzymogenes*: L19, L28, L29, L30 and *L. gummosus*: L05, L15, L26, L33. For each of the two bioassays, an asterisk indicates a significant difference ($p<0.05$) with the control treatment calculated by analysis of variance and Dunnet's post-hoc analysis.

Plant growth promotion

The ability of the *Lysobacter* strains to promote plant growth *in vitro* was tested for sugar beet, cauliflower, onion and *A. thaliana*. For sugar beet, the 18 *Lysobacter* strains were applied to the seeds as well as to the root tips. For the first seed inoculation assay, almost all *L. antibioticus* strains negatively affected plant growth, decreasing plant biomass with 15-38% compared to the untreated control (Fig. 4A). One *L. capsici* and two *L. enzymogenes* strains negatively affected shoot biomass. In the second bioassay, no negative or positive effects on plant growth were observed for any of the strains (Fig. 4A), except for *L. gummosus* L26 which promoted root growth.

The ability of *Lysobacter* to colonize the surface of the seeds and the roots was determined for bioassay 2. Whilst bacteria were applied at an initial density of 10^8

cells/seed, bacterial recovery from the seed after 30 min of incubation ranged from approximately 10^3-10^4 cells/seed, with even lower numbers for *L. antibioticus* L32 (10^2 cells/seed) (Table S2). After one week of plant growth, bacteria could not be detected on sugar beet roots (Table S2). Hence, *Lysobacter* appears to be a poor root colonizer under these experimental conditions.

In the root tip inoculation assay, positive effects (ranging from 17-28% biomass increase) were observed for dry weight of shoots by two *L. antibioticus*, two *L. capsici* and one *L. enzymogenes* strains (Fig. S3). One *L. antibioticus* and one *L. gummosus* strain increased fresh (33%) and dry (38%) root biomass respectively (Fig. S3).

To determine if *Lysobacter* emits volatile compounds that promote plant growth, assays were conducted in a split Petri dish where *Lysobacter* was physically separated from sugar beet seedlings. A high variation in plant phenotypes was observed between assays. For example, *L. antibioticus* L32 increased shoot biomass with 24% and root biomass with 42% only in the first assay. *L. enzymogenes* L30 increased root biomass in the first assay whereas in the third assay it showed a negative effect on plant growth (Fig. 4A). The volatile assays were repeated in sterile potting soil:sand mixture with sugar beet, cauliflower and onion. Also in these assays, no significant and consistent results were obtained for the *Lysobacter* strains tested (data not shown). In addition, plant growth promotion was also determined by measurement of the leaf surface and no positive or negative effects of the *Lysobacter* strains were observed (data not shown).

L. antibioticus strain L08, *L. capsici* L14, *L. gummosus* L15 were also tested for volatile-mediated growth promotion of *A. thaliana* on different media. The positive control *P. fluorescens* SBW25 significantly increased shoot and root biomass (Fig. S4). However, none of the *Lysobacter* strains tested showed a plant growth promoting effect on *A. thaliana*. Furthermore, when growing on LB medium, all the three *Lysobacter* as well as *P. fluorescens* SBW25 showed a notable adverse effect on plant growth (Fig. S4).

A

Species	Strain	SUGAR BEET																							
		SEED INOC								ROOT INOC				VOLATILE											
		Assay 1				Assay 2				Assay 1				Assay 1				Assay 2				Assay 3			
		Shoot		Root		Shoot		Root		Shoot		Root		Shoot		Root		Shoot		Root		Shoot		Root	
		F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D
L. antibioticus	L02		15																					34	
L. antibioticus	L08			31											28										
L. antibioticus	L23			31											23									34	
L. antibioticus	L32													24		42	27								
L. antibioticus	173			38	24						28				45									15	
L. antibioticus	174	23	20								27		38		38									21	
L. capsici	L12										28														
L. capsici	L13										23				23		22							29	
L. capsici	L14																								
L. capsici	L31		11												29										
L. enzymogenes	L19										17				22										
L. enzymogenes	L28																							30	
L. enzymogenes	L29	21	16																						
L. enzymogenes	L30		16													46	41							24	
L. gummosus	L05																								
L. gummosus	L15																							16	
L. gummosus	L26							30									10								
L. gummosus	L33											33													

B

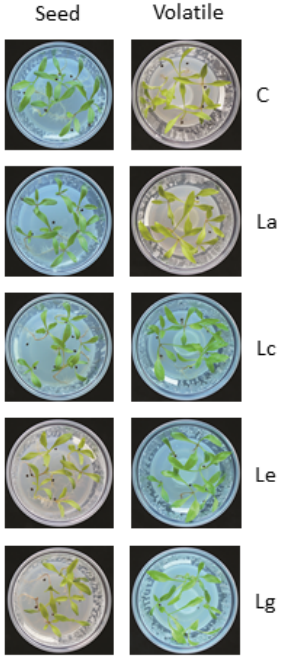

Figure 4. Sugar beet plant growth promotion by *Lysobacter* strains. **A)** Sugar beet seeds were grown on 0.5 MS medium and plant growth promotion was determined when *Lysobacter* strains were inoculated on seed or by volatiles. Each assay was performed with 3 to 5 replicates. F indicates fresh weight; D indicates dry weight. Light grey boxes indicate a statistical significant negative effect in plant growth when compared to the control and dark grey boxes indicate a statistical significant positive effect. Values within the boxes indicates the % of increase/decrease of plant weight compared to the control. **B)** Pictures of the plant growth promotion assays. C: control; La: *L. antibioticus*; Lc: *L. capsici*; Le: *L. enzymogenes*; Lg: *L. gummosus*. Significant differences ($p<0.05$) with the uninoculated control were calculated using analysis of variance and Dunnet's post-hoc analysis.

Discussion

The genus *Lysobacter* is receiving substantial ecological and biotechnological interest as producers of different exoenzymes and antibiotics (Pidot et al., 2014). During the last years, several *Lysobacter* species have been isolated from Dutch soils suppressive to the fungal root pathogen *R. solani* (Postma et al., 2008; Postma et al., 2010b). Here, we showed that 18 *Lysobacter* strains from *Rhizoctonia* suppressive showed a high genetic diversity. In a recent study, comparative genomics of seven *Lysobacter* strains (five of which are included in this study) belonging to four *Lysobacter* species showed only 55% overlap in genome content (de Bruijn et al., 2015). A high genetic diversity can confer an advantage under adverse environmental conditions as some members may exhibit phenotypes that allow them to survive and proliferate (Foster., 2005). Genome analysis also revealed the lack of flagellar genes (de Bruijn et al., 2015), which supports our findings that none of the *Lysobacter* strains tested were motile on soft agar. Nonetheless, some dispersal was observed for *L. capsici* and *L. enzymogenes* after 12 days of incubation, most likely due to gliding motility as described previously for other *Lysobacter* species (Sullivan et al., 2003; Hayward et al., 2010).

Lysobacter is known to produce a variety of bioactive compounds, including enzymes and antimicrobial compounds. Hence, they were pointed out as an untapped source of new bioactive products (Xie et al., 2012; Pidot et al., 2014). Our results showed that the *Lysobacter* strains possess chitinase and glucanase activity, confirming and extending previous research (Zhang and Yuen, 2000; Zhang et al, 2001; Palumbo et al, 2005; de Bruijn et al, 2015). Protease activity was observed for all strains belonging to *L. capsici* and *L. enzymogenes*, whereas only two out of four strains from *L. gummosus* and two out of six from *L. antibioticus* showed this activity. Chitinase, glucanase and protease activities may contribute to antimicrobial activity, since chitin, α- and β- glucans and glycoproteins are the major components of the cell walls of fungi (Barreto-Bergter and Figueiredo., 2014). Most of the *Lysobacter* strains effectively inhibited the growth of oomycetes and fungi; only *L. antibioticus* and *L. gummosus* strains showed antibacterial activity. Differences in activity were observed between *Lysobacter* species and between strains of a given species, suggesting that the genus *Lysobacter* indeed may have a large reservoir of putative novel bioactive compounds. The *in vitro* antagonistic activity was media-dependent, showing stronger activity on poor medium, confirming and extending results obtained previously for the activity of *L. enzymogenes* 3.1T8 against *Pythium aphanidermatum* (Folman et al., 2004).

Due to their broad spectrum activity, *Lysobacter* members have been proposed as promising candidates for biological control of plant diseases (Hayward et al., 2010). However, none of the *Lysobacter* strains used in this study were able to consistently reduce *R. solani* infection on sugar beet and cauliflower. These results differ from those in previous studies where several *Lysobacter* strains significantly controlled plant pathogens, including *P. aphanidermatum* on cucumber (Folman et al., 2004; Postma et al., 2009), *Bipolaris sorokiniana* on tall fescue (Kilic-Ekici and Yuen., 2003), *Uromyces appendiculatus* on bean (Yuen et al., 2001), *Xanthomonas oryzae* pv. *oryzae* on rice (Ji et al., 2008), *Phytophthora capsici* on pepper (Ko et al., 2009), *Plasmopara viticola* on grapevine (Puopolo et al., 2014), *Aphanomyces cochlioides* in sugar beet and spinach (Islam et al., 2005) and *F. oxysporum* f. sp. *radicis-lycopersici* on tomato (Puopolo et al., 2010). Furthermore, *L. capsici* YS1215 was reported to have nematicidal activity, reducing root-knot caused by *Meloidogyne incognita* by inhibiting egg hatching (Lee et al., 2014).

Most of the *Lysobacter* strains tested here poorly colonized the rhizosphere of sugar beet and cauliflower. Given the importance of root colonization for biocontrol (Bull et al., 1991; Johnson., 1994; Raaijmakers et al., 1995), this suggests that the inconsistency in disease control by the *Lysobacter* strains may be due to their lack of competitiveness in the rhizosphere of sugar beet and cauliflower. The rhizosphere differs from the bulk soil by the presence of plant root exudates that create an environment rich in nutrients. Chemotaxis and active motility towards root exudates represent the first steps in rhizosphere colonization (Benizri et al., 2001; de Weert and Bloemberg., 2006). This motility may be active, through flagellar movements, or passive, through percolating water or vectors. None of the 18 *Lysobacter* strains possess flagella, what limits the capacity of the strains to effectively compete against flagellated soil bacteria for a niche in the rhizosphere. The adherence to root tissues through biofilm formation is the next step in rhizosphere colonization (Benizri et al., 2001; Ramey et al., 2004; Danhorn and Fuqua., 2007). Several traits are involved in biofilm formation including cell wall structures and extracellular polysaccharide production (Lugtenberg et al., 2001). Biofilm production *in vitro* has been described for *L. capsici* AZ78 and appeared medium specific (Puopolo et al., 2014). Biofilm formation was observed for *Lysobacter* sp. strain SB-K88 on roots of sugar beet (Islam et al., 2005). Biofilm formation *in situ* was not tested for our 18 *Lysobacter* strains and will be subject of future studies. The root exudate composition is plant specific (Mandimba et al., 1986) and the ability to assimilate specific amino acids, vitamin B1, carbohydrates, organic acids as well as pH tolerance and competition for limiting resources also determine the rhizosphere competence (Dekkers et al., 1999; Benizri et al., 2001; Lugtenberg and

Kamilova., 2009; Ghirardi et al., 2012). In the rhizosphere there is often a limitation for soluble iron, commonly used as a cofactor in enzymes that are involved in pathways that are essential for microbial growth. Therefore, the ability to produce siderophores (small high-affinity iron chelating compounds) confers a competitive advantage. The role of competition for iron by siderophore production of *Lysobacter* sp. seems species or strain specific and not all strains, including several strains used in this study, possess iron-chelating capacity (de Bruijn et al., 2015; Ko et al., 2011; Puopolo et al., 2010).

The soil type may also influence rhizosphere colonization and biocontrol activity. For example, the colonization of *Pseudomonas* sp. strain ITRI53 and *Pantoea* sp. strain BTRH79 of Italian ryegrass was higher in loamy soils compared with sandy soils (Afzal et al., 2011). The agricultural soil used in this study is a clay soil with loam texture. Several of our *Lysobacter* strains were isolated from this agricultural soil and we expected that those conditions would provide a 'home-field advantage' for rhizosphere colonization of sugar beet and cauliflower. In a potting soil:sand mixture, we observed higher rhizosphere population densities on sugar beet seedlings as compared to the agricultural soil, with densities higher than the minimal dose of 10^5 CFU/g soil reported for other biocontrol strains (Xu and Gross, 1986; Leeman et al., 1995; Raaijmakers et al., 1995). Despite these densities, no significant and/or consistent biocontrol activity was observed for any of the *Lysobacter* strains tested.

Several biocontrol agents not only suppress disease but also promote plant growth (Johansson et al., 2003). None of the *Lysobacter* strains tested in this study, however, were able to significantly and consistently promote growth of 4 different crops when applied to seeds or root tips or when applied physically separated from the crop. Furthermore, volatiles produced by the *Lysobacter* strains when grown on LB medium even showed a negative effect on growth of *A. thaliana*. This may be due to the accumulation of toxic volatiles that are produced by *Lysobacter* spp. when growing in rich media. Weise et al. (2013) showed that *Serratia odorifera* inhibited the growth of *A. thaliana* plants due to the production of ammonia when grown on peptone-rich nutrient media. Iwata et al. (2010) reported that *Lysobacter* sp. E4 was able to fix nitrogen under free-living conditions and accumulated ammonia in the culture broth. Also hydrogen cyanide (HCN) produced by *Chromobacterium, Pseudomonas* and *Serratia* have been shown to inhibit the growth of *A. thaliana* (Blom et al., 2011). More research needs to be conducted to determine if HCN or other toxic volatiles are produced by *Lysobacter*.

Overall, our results indicate that none of the 18 *Lysobacter* strains have the potential to control *Rhizoctonia* or promote plant growth of sugar beet and cauliflower,

probably due to insufficient rhizosphere competence. However, the *Lysobacter* strains showed a high diversity in *in vitro* activity against 14 different pathogenic fungi, oomycetes and bacteria, suggesting that the genus *Lysobacter* constitutes an extensive source of (new) enzymes and antimicrobial compounds. Possibly *Lysobacter* needs to interact with a specific microbial community to become antagonistic to *Rhizoctonia* or to promote plant growth in natural environments. To better understand the potential contribution of *Lysobacter* species to the overall activities of the microbial communities responsible for soil suppressiveness against *R. solani*, in-depth metagenomic and metatranscriptomic analyses of the bacterial community compositions and functions will be needed to unravel the role of this genus in disease suppressiveness. Future work will include testing *Lysobacter* mixtures or mixtures with other bacterial genera abundant in soils suppressive to *R. solani*. Interactions of *Lysobacter* with other bacteria may stimulate the production of antimicrobial compounds as was shown recently for other bacterial genera (Tyc et al., 2014).

Conflict of interest

All authors declare no conflict of interest.

Author contributions

All authors were involved in the design of the experiments. RGE and IdB performed *in vitro* and *in vivo* activity bioassays, BOX-PCR and phylogenetic analyses. RGE performed plant growth promotion assays. All authors contributed to the writing of the manuscript and approved submission.

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Supplementary material

Figure S1. Phylogenetic trees of the 18 *Lysobacter* strains based on A) 16S ribosomal RNA gene (16S rRNA), B) recombination/reparation protein (*recN*) and C) excinuclease ABC (*uvrC*). The evolutionary relationship of the *Lysobacter* strains was inferred by alignment with ClustalW and tree construction using the neighbor-joining method in MEGA6.

Table S1. Pathogens used in this study

Organism	ID code	Species	Propagule	Strain	Media used for growth/sporulation
Fungus	R. sol	*Rhizoctonia solani*	mycelium	AG2-2 III b	PDA
Fungus	F. sol	*Fusarium solani*	mycelium	F2	PDA
Fungus	V. dah_J	*Verticillium dahliae*	mycelium	JR2	PDA
Fungus	F. oxys	*Fusarium oxysporum*	spores	gN 07-047 d1	PDA
Fungus	Forl1	*Fusarium oxysporum*	spores	Forl1	PDA
Fungus	V. dah	*Verticillium dahliae*	spores	gN 10-188	PDA
Fungus	A. nig	*Aspergillus niger*	spores	N400	PDA
Fungus	C. bet	*Cercospora beticola*	spores	BV 1133 ga1	V8
Fungus	Stem	*Stemphylium* sp.	spores	BV 10-140 a1	V8
Oomycete	A. coc	*Aphanomyces cochlioides*	mycelium	HL B-22	PDA
Oomycete	P. ult	*Pythium ultimum*	mycelium	SB	PDA
Oomycete	P. inf	*Phytophthora infestans*	mycelium	88069	PDA
Oomycete	S. par	*Saprolegnia parasitica*	mycelium	CBS223.65	PDA
Bacterium	X. cam	*Xanthomonas campestris* pv *campestris*	cells	ZTO281	LB
Bacterium	P. atr	*Pectobacterium atrosepticum*	cells	SCR1	LB

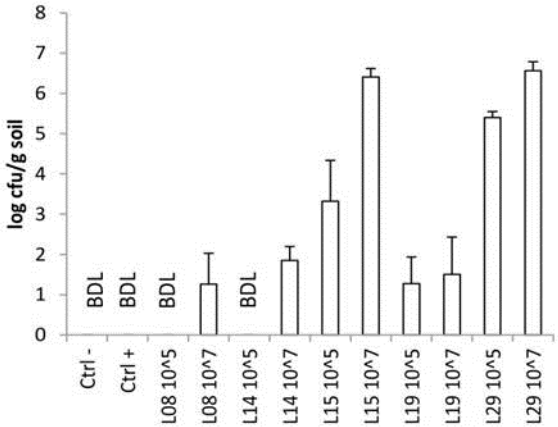

Figure S2. Colonization of *Lysobacter* strains of the rhizospheres of cauliflower plants. *L. antibioticus*: L08; *L. capsici*: L14; *L. gummosus*: L15 and *L. enzymogenes*: L19 and L29. 10^7 means an initial density of the inoculum at 10^7 cells/g soil; 10^5 means an initial density of the inoculum at 10^5 cells/g soil. BDL means below detection limit (estimated in 232 cfu/g rhizosphere).

Table S2. *In vitro* colonization of the *Lysobacter* strains of the seed and root surface of sugar beet seedlings. Colonization densities of seeds are retrieved upon pooling 6 seeds and dilution plating. Colonization densities of roots are retrieved upon pooling 4 roots per plate and dilution plating; averages of 4 plates are indicated. BDL indicates below detection limit (estimated in $2*10^2$ cfu/root).

Species	Strain	cfu/seed (x10^4)		cfu/root
	Control	BDL		BDL
L. antibioticus	L02	40.0	± 3.3	BDL
L. antibioticus	L08	37.0	± 9.5	BDL
L. antibioticus	L23	25.0	± 6.4	BDL
L. antibioticus	L32	0.3	± 0.5	BDL
L. antibioticus	173	33.0	± 2.3	BDL
L. antibioticus	174	1.5	± 0.2	BDL
L. capsici	L12	7.1	± 0.5	BDL
L. capsici	L13	35.0	± 7.1	BDL
L. capsici	L14	9.2	± 1.5	BDL
L. capsici	L31	34.0	± 6.7	BDL
L. enzymogenes	L19	20.0	± 1.2	BDL
L. enzymogenes	L28	2.6	± 0.4	BDL
L. enzymogenes	L29	4.0		BDL
L. enzymogenes	L30	1.6	± 0.2	BDL
L. gummosus	L05	20.0	± 5.3	BDL
L. gummosus	L15	29.0	± 9.6	BDL
L. gummosus	L26	5.2	± 1.4	BDL
L. gummosus	L33	1.5	± 0.1	BDL

A

Species	Strain	SUGAR BEET ROOT INOC Assay 1			
		Shoot		Root	
		F	D	F	D
L. antibioticus	L02				
L. antibioticus	L08				
L. antibioticus	L23				
L. antibioticus	L32				
L. antibioticus	173		28		
L. antibioticus	174		27		38
L. capsici	L12		28		
L. capsici	L13		23		
L. capsici	L14				
L. capsici	L31				
L. enzymogenes	L19		17		
L. enzymogenes	L28				
L. enzymogenes	L29				
L. enzymogenes	L30				
L. gummosus	L05				
L. gummosus	L15				
L. gummosus	L26				
L. gummosus	L33			33	

B

Figure S3. Sugar beet plant growth promotion by *Lysobacter* strains when inoculated on root tips. A) Sugar beet seeds were grown on 0.5 MS medium and plant growth promotion was determined when *Lysobacter* strains were inoculated on the root tip. The assay was performed once with three replicates per treatment. F indicates fresh weight; D indicates dry weight. Light grey boxes indicate a statistical significant negative effect in plant growth when compared to the control and dark grey boxes indicate a statistical significant positive effect. Values within the boxes indicates the % of increase/decrease of plant weight compared to the control. B) Pictures of the plant growth promotion assay. C: control; La: *L. antibioticus*; Lc: *L. capsici*; Le: *L. enzymogenes*; Lg: *L. gummosus*. Significant differences ($p<0.05$) with the uninoculated control were calculated using analysis of variance and Dunnet's post-hoc analysis.

A

			Arabidopsis thaliana			
			VOLATILE			
			Shoot		Root	
Species	**Strain**	**Medium**	F	D	F	D
Control	Control	NM				
Control	Control	R2A				
Control	Control	KB				
Control	Control	LB				
L. antibioticus	L08	R2A				
L. antibioticus	L08	KB				
L. antibioticus	L08	LB	95	88	91	88
L. capsici	L14	R2A				
L. capsici	L14	KB				
L. capsici	L14	LB	90	90	94	87
L. gummosus	L15	R2A				
L. gummosus	L15	KB				
L. gummosus	L15	LB	64		86	
P. fluorescens	SBW25	R2A				
P. fluorescens	SBW25	KB	177	308	147	176
P. fluorescens	SBW25	LB	91	92	74	

B

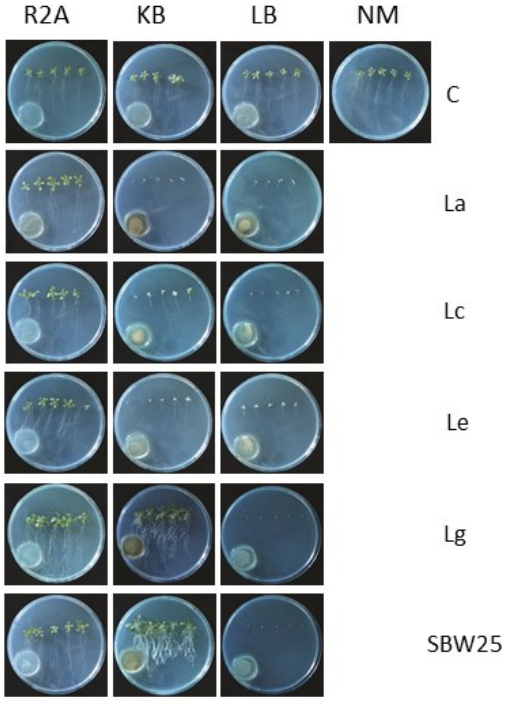

Figure S4. *Arabidopsis thaliana* plant growth promotion by *Lysobacter* volatiles. **A)** Effect of the volatiles of three *Lysobacter* species and the positive control *Pseudomonas fluorescens* SBW25 when grown on different media: R2A, KB and LB. NM means no media added (control). The assay was performed once with 5 replicates. F indicates fresh weight; D indicates dry weight. Light grey boxes indicate a statistical significant negative effect in plant growth compared to the control and dark grey boxes indicate a statistical significant positive effect. Values within the boxes indicates the % of increase/decrease of plant weight compared to the control. **B)** Pictures of the plant growth promotion assay. C: control; La: *L. antibioticus*; Lc: *L. capsici*; Le*: L. enzymogenes*; Lg: *L. gummosus*. Significant differences ($p<0.05$) with the uninoculated control were calculated using analysis of variance and Dunnet's post-hoc analysis.

Chapter 3

Evaluation of bacterial consortia to enhance plant disease control

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(To be submitted)

Abstract

Isolation and characterization of soil and rhizosphere microbes for plant growth promotion and biocontrol of plant diseases has been the subject of numerous studies over the past three decades. Although a variety of microbial strains have shown promising activities and are applied in agriculture or horticulture, most microbial strains tested to date have failed to provide consistent effects in plant disease control under diverse field conditions. Application of microbial consortia with complementary or synergistic activities, also referred to as synthetic communities or 'SynComs', has been proposed to resolve inconsistent effects observed for single microbial strains. Here we evaluated the biocontrol activity of consortia of different bacterial genera against damping-off disease of sugar beet caused by the fungal pathogen *Rhizoctonia solani*. The bacterial genera tested were isolated from a soil naturally suppressive to *R. solani* and included three *Lysobacter*, six *Pseudomonas* and six *Streptomyces* strains. *In vitro* assays showed that the *in vitro* antifungal activity of these bacterial genera was growth medium dependent and that most combinations did not lead to an enhanced activity against *R. solani*. Using the combination of the three *Lysobacter* strains as the baseline, no consistent control of damping-off disease of sugar beet seedlings was obtained in four independent experiments for any of the bacterial consortia tested. Therefore, the design of effective synthetic microbial communities should focus on identifying other criteria or microbial traits associated with effective partnerships rather than randomly combining microbial genera that individually exhibit antagonistic properties.

Introduction

The plant microbiome has a major impact on plant health and productivity (Turner et al., 2013). Several members of the plant microbiome (pathogens) can negatively affect plant growth, whereas other microbes may exert beneficial effects on plants. Among the plant growth-promoting rhizobacteria (PGPR) and biological control agents (BCA), numerous genera, species and strains have been tested over the past three decades (Turner et al., 2013; Fahad et al., 2015). Natural disease suppressive soils have been and still are an important resource for the isolation of microbes with plant protective traits (Weller et al., 2002). However, most of the microbial strains with promising biocontrol activities under controlled conditions have failed under commercial greenhouse or field conditions (Mazzola and Freilich, 2016). This lack of consistent activity has been attributed, in part, to poor root colonization of the introduced microbial strain, being outcompeted by the resident microbial community, or to interference with the regulation and production of the bioactive compounds (Alabouvette et al., 2009; Lugtenberg and Kamilova, 2009). Hence, strategies to improve the consistency of antagonistic microorganisms in biocontrol have focused on: i) enhancing survival, plant colonization and/or antagonistic activity of single strains, and ii) combining microbial strains with different and complementary modes of action (Alabouvette et al., 2009; Sharma et al., 2009; Wei et al., 2015; Mazzola and Freilich, 2016; Szczech and Maciorowski, 2016).

The natural control of soil-borne pathogens observed in disease suppressive soils has been attributed to the combined activity of multiple microbial genera rather than to the activity of a single microbial genus or species (Lemanceau and Alabouvette, 1991; Raaijmakers et al., 1995; Mendes et al., 2011; Chapelle et al., 2015; van der Voort et al., 2016). Several studies have indeed shown enhanced biocontrol activity and enhanced production of antimicrobial compounds when two or more strains were combined (Szczech and Shoda, 2004; Roberts et al., 2005; de Boer et al., 2007; Garbeva and de Boer, 2009). Furthermore, Tyc et al. (2014) showed that the production of so-called cryptic antimicrobial compounds may occur only when microorganisms interact. Hence, co-culturing of different microbial strains has been proposed as a promising strategy to stimulate silent gene clusters, to enhance antimicrobial activity and to improve the consistency of biological control of plant pathogens (Garbeva et al., 2011; Seyedsayamdost et al., 2012; Tyc et al., 2014). Collectively, these studies have led to the design of so-called 'SynComs', i.e. synthetic microbial communities, to promote plant growth and enhance crop productivity (Großkopf and Soyer, 2014; Lebeis et al., 2015; Mazzola and Freilich, 2016). Combining antagonistic

microbial genera may result in different outcomes: i) increased biocontrol activity towards a pathogen due to the combination of different modes of action (Guetsky et al., 2001; Szczech and Shoda, 2004; de Boer et al., 2007), ii) reduced biocontrol activity due to incompatible biocontrol mechanisms or interspecific competition leading to poor survival or reduced activity of one or more of the members of the SynCom (Bora et al., 2004; Stockwell et al., 2010; 2011), or iii) similar level of protection as provided by the single strains (Janousek et al., 2009; Xu et al., 2010).

In this study, we evaluated the *in vitro* and *in vivo* activities of synthetic communities of different bacterial genera isolated from a soil naturally suppressive to the plant pathogen *Rhizoctonia solani.* The strains represented the genera *Lysobacter*, *Pseudomonas* and *Streptomyces,* each of which was previously shown to have growth-inhibitory activity *in vitro* against *R. solani* (Postma et al., 2010). *Lysobacter* species were found more abundant in *Rhizoctonia* disease suppressive soils (Postma et al., 2010) but were not able to consistently reduce damping-off disease of sugar beet or cauliflower when applied as single strains (Gómez Expósito et al., 2015). Based on these initial results, we hypothesized that the *Lysobacter* species and strains may provide more consistent biocontrol activity when they are part of a consortium with strains from other bacterial genera, in particular *Pseudomonas* and *Streptomyces*. Also these latter two genera were enriched in soils suppressive to damping-off disease of sugar beet caused by *R. solani* (Mendes et al., 2011; Cordovez et al., 2015; van der Voort et al., 2016). First, we characterized the isolates by phylogenetic analyses and then tested monocultures and mixtures *in vitro* on different growth media for their activity against *R. solani.* Finally, we tested several combinations of these three bacterial genera for their biocontrol activity *in vivo*.

Materials and methods

Strains, culture and storage conditions

The bacterial strains used in this study (Table 1) were isolated from Dutch agricultural soils suppressive to *R. solani* (Postma et al., 2008; Postma et al., 2010). The fungal strains used in this study were *R. solani* AG2-2IIIB, provided by the Institute of Sugar Beet Research (IRS), and *R. solani* AG2-1/21. The *Pseudomonas* and *Lysobacter* strains were pre-cultured in 10 ml of tryptone soy broth (TSB, Oxoid) and incubated for 2-3 days at 25°C. The *Streptomyces* strains were pre-cultured in 10 ml TSB supplemented with 10% sucrose (w/v) (TSBS) and incubated for 2-3 days at 30°C. All bacterial cultures were grown on a rotary shaker at 150

rpm and then washed three times with 0.9% NaCl. The bacterial strains were kept in 40% (v/v) glycerol at -80°C and *R. solani* was kept as mycelial plugs in mineral oil at 10 °C.

Soil collection and storage

The agricultural soil used in the bioassays was collected in June 2012 from a pear orchard located in Zwaagdijk, The Netherlands (52°41´53.549´´N, 5°6´58.643´´E) at a depth of 10-40 cm. The soil, classified as clay soil with loam texture (29.9% of the particles are >50 µm, 26.4% of the particles are <2 µm) was air-dried, sieved (0.5 cm mesh) to remove plant/root material, and stored at 8°C until use.

Phylogenetic characterization of the bacterial isolates

Genetic identification of the *Lysobacter* strains was described previously in Gómez Expósito et al. (2015). The 16S ribosomal RNA gene of the *Pseudomonas* and *Streptomyces* isolates was amplified by PCR with primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1392R (5'-ACGGGCGGTGTGTACA-3') (DeAngelis et al., 2009). Amplification reactions were conducted in 25 µl volume composed of 1 µl each of forward and reverse primer (10 µM), 1 µl dNTPs (5 mM each), 1.5 µl $MgCl_2$ (25 mM), 5 µl 5x GoTaq Flexibuffer, 0.125 µl GoTaq Polymerase (5U/µl) (Promega) and 15.375 µl miliQ water. DNA was added by toothpick inoculation of bacterial cells in the reaction mix. The reaction volume was heated to 95°C for 3 min, followed by 35 cycles of 1 minute at 95°C, 1 minute at 58°C, 1.4 minutes at 72°C and 1 cycle at 72°C for 5 minutes. PCR products were sequenced by Macrogen Inc. (Amsterdam, The Netherlands). Phylogenetic trees were constructed for the *Pseudomonas* and *Streptomyces* isolates by aligning their 16S sequences with those of the type strains retrieved from the Greengenes database (McDonald et al., 2012) (http://greengenes.lbl.gov/). Alignments were performed with Muscle (Edgar, 2004) and neighbor joining in MEGA6 (Tamura et al., 2013). The 16S rRNA sequences of our isolates were also aligned by Muscle with the *Lysobacter*, *Pseudomonas* and *Streptomyces* sequences obtained from the high-density 16S ribosomal DNA oligonucleotide microarray (PhyloChip) (Mendes et al., 2011). In addition, for the *Streptomyces*, the 16 rRNA sequences of 173 *Streptomyces* strains isolated from another *R. solani* suppressive soil were included (Cordovez et al., 2015). Phylogenetic trees for all three bacterial genera were constructed using the neighbor joining method in iTOL (http://itol.embl.de/) (Letunic and Bork, 2007) to determine whether our isolates were closely related to the ones detected at higher abundance in another soil suppressive to *R. solani* (Mendes et al., 2011).

To amplify the *thaC*2 gene encoding the antifungal lipopeptide thanamycin (Mendes et al., 2011), primers *thaC*2qF (5' CGGTTTCATCGGTCCTTTC 3') and *thaC*2qR (5' TCCAGGTGGCGATCATAGTT 3') were used. Amplification reactions were conducted in 25 µl volume composed of 1 µl of each primer (10 µM), 1 µl dNTPs (10 mM each), 5 µl of GoTaq Flexibuffer, 0.25 µl GoTaq polymerase (5U/µl), 14.75 µl MilliQ water and 2 µl DNA (obtained by resuspending a single fresh *Pseudomonas* colony in 400 µl of MilliQ water, heating it at 100°C for 10 min and briefly centrifuging to remove most of the cell wall content). The reaction volume was heated to 95°C for 3 min, followed by 30 times a cycle of 30 seconds at 95°C, 55°C for 15 seconds and 72°C for 1 min. PCR reactions were finished with an incubation at 72°C for 5 minutes and then kept at 12°C. Five µl of the PCR product was visualized on 1.5% agarose gel. DNA from *Pseudomonas* SH-C52, containing the *thaC*2 gene (Mendes et al., 2011), was used as a positive control.

In vitro activity of monocultures and synthetic communities

Bacterial suspensions were adjusted to 10^9 CFU/ml and mixtures were prepared in equal ratios. Three 2 µl droplets of each bacterial suspension were spotted at the edges of 90-mm-diameter Petri dishes containing R2A medium (Oxoid), $1/10^{th}$ strength tryptone soy agar (TSA, Oxoid), water agar (WA-N) (Garbeva and de Boer, 2009) or M9 agar medium [200 ml l^{-1} of M9 salts (64 g l^{-1} Na_2HPO_4 . $7H_2O$, 15 g l^{-1} KH_2PO_4, 2.5 g l^{-1} NaCl, 5.0 g l^{-1} NH_4Cl), 2 ml l^{-1} of 1M $MgSO_4$, 20 ml l^{-1} of 20% glucose, 100 µl l^{-1} of 1M $CaCl_2$ and 15 g l^{-1} agar] (Harwood and Cutting, 1990) (the agar used was Bacto agar, BD). A single fresh *R. solani* AG2-2IIIB $1/5^{th}$ potato dextrose agar (PDA, Oxoid) agar plug (5 mm) was placed in the center of the plates. Non-bacteria-inoculated Petri dishes were used as controls. Petri dishes were incubated at 25°C for one week and subsequently inhibitory halos were monitored. The experiment was performed once, with three replicates for each treatment.

In vivo activity of synthetic communities

The activity of the combination of the *Lysobacter* , *Pseudomonas* and *Streptomyces* strains was tested *in vivo* on sugar beet. The bacteria were pre-grown in 10 ml of TSB (TSBS for the *Streptomyces* strains) for 2-3 days at 25°C. The bacterial strain mixtures were prepared in equal ratios and mixed in Zwaagdijk conducive soil at an initial density of 10^7 CFU/g soil for each bacterial strain and approximately 20% (v/w) soil water content. Rectangle shaped trays (19.5 x 6 x 3 cm) were filled with 250 g of soil (8 replicates per treatment) and sixteen sugar beet seeds coated with the fungicides thyram and hymexazol, and with the insecticide poncho beta were sown in a row, 1 cm apart. Non-bacteria-inoculated soil was

used as a control. Trays were placed in boxes with transparent lids in a growth chamber at 25°C with 16 h photoperiod and a light intensity of 150 micromol photons m^{-2} s^{-1}. After 7 days, when the seeds were germinated, an agar plug (5 mm, 1/5th potato dextrose (PDA, Oxoid)) fully grown with *R. solani* AG2-2IIIB was was placed at 1 cm depth with the mycelium touching the base of the stem of the first seedling. *R. solani* disease symptoms were monitored at regular intervals during 3 weeks by scoring the number of diseased plants suffering from damping-off. The experiment was repeated 4 times. Statistically significant differences were determined by analyses of variance (ANOVA) and post hoc Dunnet's analyses ($P<0.05$) performed in SPSS 23.0.

Table 1. Origin of the *Lysobacter*, *Pseudomonas* and *Streptomyces* strains used in this study.

Code	Species	Soil type	Crop	Origin	Location	Year	Reference
L08	*Lysobacter antibioticus*	clay	cauliflower	Suppressive soil	Zwaagdijk, NL	2003	Postma *et al.,* 2010b
L14	*Lysobacter capsici*	clay	cauliflower	Suppressive soil	Zwaagdijk, NL	2003	Postma *et al.,* 2010a
L15	*Lysobacter gummosus*	clay	grass/clover	Suppressive soil	Pietersbierum, NL	2003	Postma *et al.,* 2008
P29	*Pseudomonas japonica*	clay	cauliflower	Suppressive soil	Zwaagdijk, NL	2003	(this study)
P33	*Pseudomonas frederiksbergensis*	clay	cauliflower	Suppressive soil	Zwaagdijk, NL	2003	(this study)
P48A	*Pseudomonas corrugata*	clay	cauliflower	Suppressive soil	Zwaagdijk, NL	2003	(this study)
P58	*Pseudomonas oryzihabitans*	clay	cauliflower	Suppressive soil	Zwaagdijk, NL	2003	(this study)
P62	*Pseudomonas helmanticensis*	clay	cauliflower	Suppressive soil	Zwaagdijk, NL	2003	(this study)
P63	*Pseudomonas baetica*	clay	cauliflower	Suppressive soil	Zwaagdijk, NL	2003	(this study)
S36	*Streptomyces griseus*	clay	cauliflower	Suppressive soil	Zwaagdijk, NL	2003	(this study)
S37	*Streptomyces sporoverrucosus*	clay	cauliflower	Suppressive soil	Zwaagdijk, NL	2003	(this study)
S45	*Streptomyces clavifer*	clay	cauliflower	Suppressive soil	Zwaagdijk, NL	2003	(this study)
S64	*Streptomyces clavifer*	clay	cauliflower	Suppressive soil	Zwaagdijk, NL	2003	(this study)
S65	*Streptomyces omiyaensis*	clay	cauliflower	Suppressive soil	Zwaagdijk, NL	2003	(this study)
S72	*Streptomyces enissocaesilis*	clay	cauliflower	Suppressive soil	Zwaagdijk, NL	2003	(this study)

Results

Phylogenetic characterization of the bacterial strains

We tested three *Lysobacter*, six *Pseudomonas* and six *Streptomyces* strains, all isolated from a *Rhizoctonia* suppressive soil (Postma et al., 2010). The *Lysobacter* strains were previously identified as *L. antibioticus* (L08), *L. capsici* (L14) and *L. gummosus* (L15) (Gómez Expósito et al., 2015). Phylogenetic analysis revealed that the *Pseudomonas* strains clustered with type strains of six different *Pseudomonas* species, including *P. japonica* (P29), *P. frederiksbergensis* (P33), *P. corrugata* (P48A), *P. oryzihabitans* (P58), *P. helmanticensis* (P62) and *P. baetica* (P63) (Fig. 1). The six *Streptomyces* strains clustered with type strains of five different *Streptomyces* species, including *S. griseus* (S36), *S. sporoverrucosus* (S37), *S. clavifer* (S45 and S64), *S. omiyaensis* (S65) and *S. enissocaesilis* (S72) (Fig. 2). Alignment of the 16S rRNA sequences of the selected strains with the *Lysobacter*, *Pseudomonas* and *Streptomyces* sequences detected previously by PhyloChip in the rhizosphere of sugar beet seedlings grown in a *R. solani* suppressive soil (Mendes et al., 2011) revealed that: i) *Lysobacter* strains L08, L14 and L15 clustered together with *Lysobacter* sp. strain XL1 by PhyloChip, detected in higher abundance in suppressive than in conducive soil (Fig. S1); ii) four of the six *Pseudomonas* strains (P29, P58, P62 and P63) were phylogenetically related to *Pseudomonas* operational taxonomic units (OTUs) detected by PhyloChip (Fig. S2); the six *Streptomyces* strains were phylogenetically related with either *Streptomyces* detected by PhyloChip (Mendes et al., 2011) or with *Streptomyces* isolates obtained from the same suppressive soil (Cordovez et al., 2015) (Fig. S3).

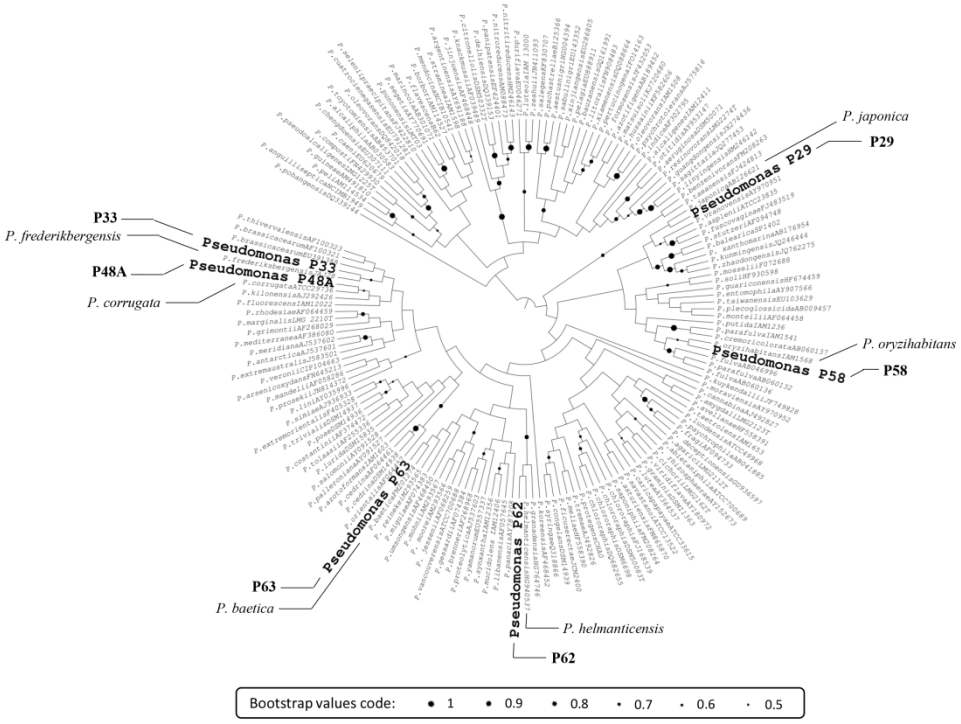

Figure 1. Phylogenetic delineation of the six *Pseudomonas* strains used in this study. The phylogenetic relationship of the 16S rRNA sequences of the six *Pseudomonas* isolates (in bold) and the 16S rRNA sequences of the type strains of different *Pseudomonas* species (in italics) obtained from the Ribosomal Database Project (RDP) was inferred by alignment with Muscle and neighbor-joining tree construction. The bootstrap values indicated at the nodes are based on 1000 bootstrap replicates. The black dots at the nodes indicate the level of bootstrap support higher than 0.5. The *Pseudomonas* type strains are indicated by the species name followed by the ID number.

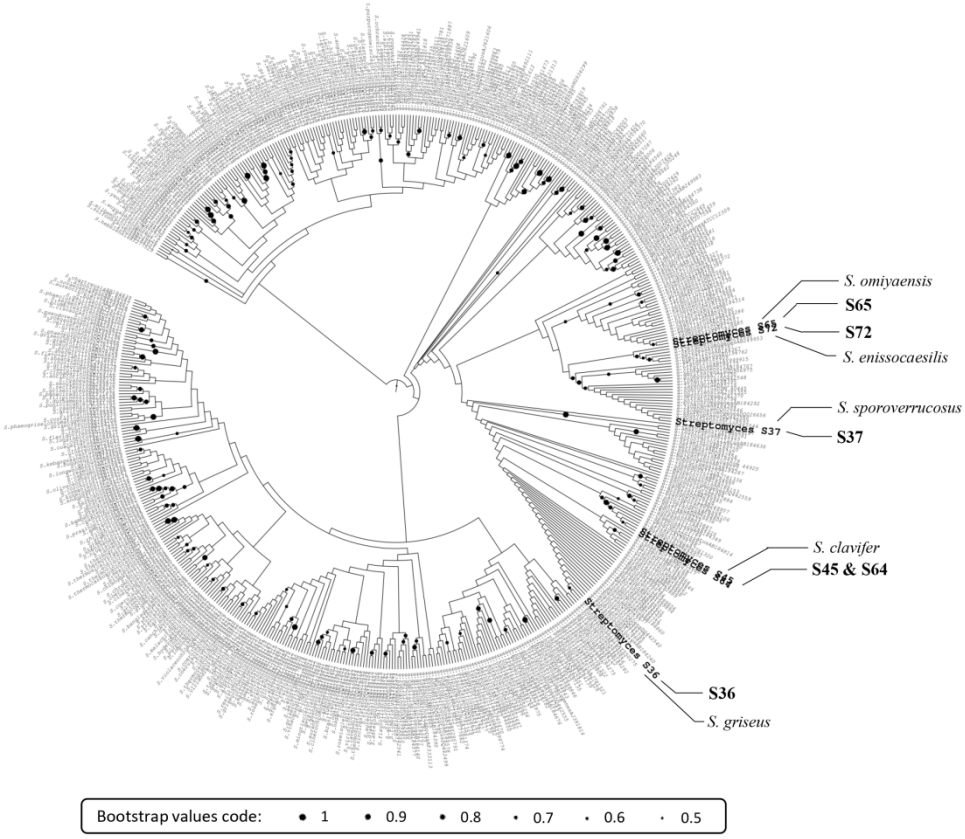

Figure 2. Phylogenetic delineation of the six *Streptomyces* isolates used in this study. The phylogenetic relationship of the 16S rRNA sequences of the six *Streptomyces* isolates (in bold) and the 16S rRNA sequences from the *Streptomyces* type strains (in italics) obtained from the Ribosomal Database Project (RDP) was inferred by alignment with Muscle and neighbor-joining tree construction. The bootstrap values indicated at the nodes are based on 1000 bootstrap replicates. The black dots at the nodes indicate the level of bootstrap support higher than 0.5. The *Streptomyces* type strains are indicated by the species name followed by the ID number.

In vitro activity of bacterial consortia

The *in vitro* antifungal activity of single strains of *Lysobacter*, *Pseudomonas* and *Streptomyces* and mixtures (Table 1) was tested on four different media with a total of 15 strains and 39 pairwise combinations. All strains showed antifungal activity on at least one of the growth media tested (Table S1). Most strains (11 out of 15) showed inhibition of *R. solani* hyphal growth on the nutrient-rich media R2A and 1/10 TSA, whereas 9 out of 15 strains showed activity against *R. solani* on the nutrient-poor media M9 and WA-N (Table S1). For most strains, inhibition of hyphal growth of *R. solani* was observed both in monoculture and in combination with another bacterial strain: antagonistic activity was observed for 25-29 out of the total of 39 combinations (Fig. 3). 10, 10, 6 and 4 combinations showed antifungal activity only in co-culture on R2A, 1/10 TSA, M9 and WA-N, respectively (Fig. 3, Table S1). Positive, negative and neutral interactions were assigned to combinations in which the antifungal activity in the co-culture increased, decreased or remained similar as compared to the monoculture, respectively. On the growth media 1/10 TSA, M9 and WA-N, most combinations did not change the antifungal activity observed for the individual strains (Fig. S4, Table S1). The majority of the combinations with a positive effect for both of the strains (pos-pos interactions) occurred on the nutrient-rich media R2A (10) and 1/10 TSA (9) as compared to the nutrient-poor media M9 (1) and WA-N (3) (Fig S4, Table S1). Conversely, most of the combinations with a negative effect on activity for each of the individual strains were observed on the nutrient-poor medium WA-N (9 neg-neg interactions) (Fig S4, Table S1). The number of combinations with a positive effect for one of the strains but a negative effect for the other (pos-neg interactions) decreased with a decrease in nutrient content of the growth medium, ranging from 12 to 1 on R2A and WA-N, respectively (Fig S4, Table S1). Interestingly, the majority of positive interactions occurred when *L. gummosus* L15 was combined with *Pseudomonas* and *Streptomyces* strains on the nutrient-rich media, but the opposite effect was observed on the nutrient-poor media (Table S1). *L. antibioticus* L08 showed the opposite with a decreased antifungal activity in co-culture with *Streptomyces* strains on R2A medium (Table S1). Combinations of *L. capsici* L14 with *Pseudomonas* and *Streptomyces* strains led to more random outcomes. Consortia of all three *Lysobacter* (3L), all six *Pseudomonas* (6P), all six *Streptomyces* strains (6S), all *Lysobacter* with all *Pseudomonas* strains (3L+6P), all three *Lysobacter* with all *Streptomyces* strains (3L+6S) and all 15 strains (3L+6P+6S) did not have a differential effect on fungal growth inhibition on any of the four media (Table S1). These results indicate that

the number of positive or negative interactions is limited and that the activity is combination-specific and growth-medium dependent.

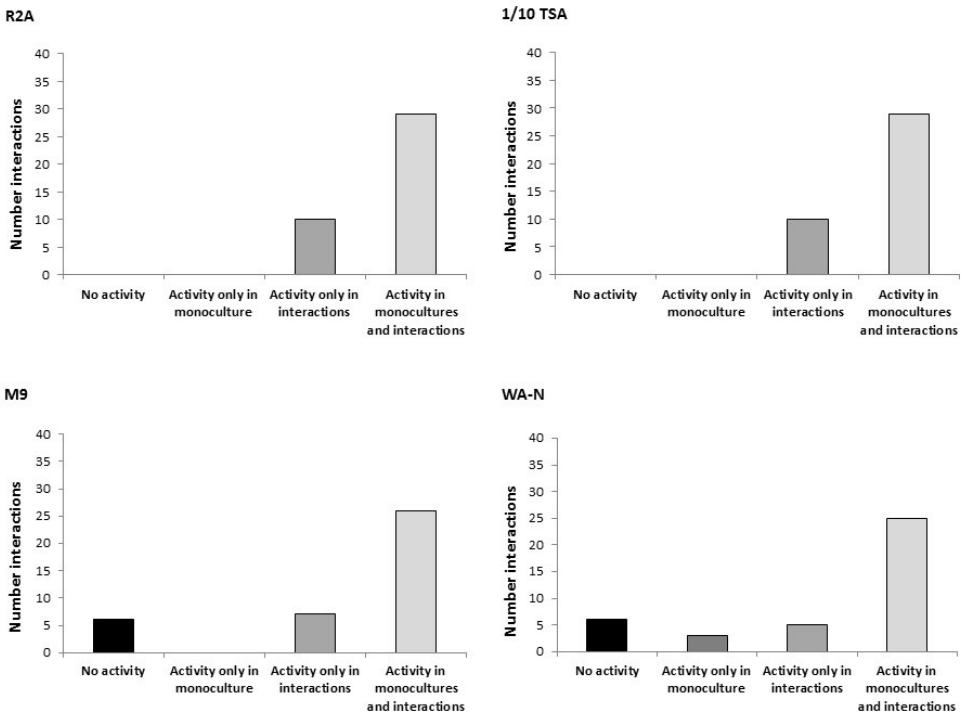

Figure 3. Effect of co-culturing different *Lysobacter* (N=3), *Pseudomonas* (N=6) and *Streptomyces* (N=6) strains on the *in vitro* antifungal activity against the fungal plant pathogen *Rhizoctonia solani*. A total of 39 dual interactions were tested on four different growth media, including two nutrient-rich media (R2A, 1/10 TSA) and two nutrient-poor media (M9, WA-N). Shown are the number of interactions with no antifungal activity observed (neither for the interaction nor for the monocultures) (No activity); number of interactions for which antifungal activity was only observed for the individual strains but not for the interactions (Activity only in monoculture); number of interactions for which antifungal activity was only observed when the strains were combined but not individually (Activity only in interactions); number of interactions for which antifungal activity was observed both for the strains in monocultures as during interaction (Activity in monocultures and interactions).

In vivo activity of bacterial consortia

The biocontrol efficacy of each of the three *Lysobacter* strains L08, L14 and L15, representing three species, was previously assessed and revealed that none of the three strains was effective individually in controlling *R. solani* damping-off disease (Gómez Expósito et al., 2015). Results presented here show that also combining the three *Lysobacter* strains did not lead to a more consistent biocontrol efficacy: in none of the four independent experiments, the consortium of the three *Lysobacter* strains significantly controlled damping-off disease relative to the untreated control (Fig. 4A). For the *Pseudomonas* strains, two different consortia were evaluated: 6P, containing all six *Pseudomonas* strains; and 4P, containing strains P29, P58, P62 and P63 (all four phylogenetically related to the *Pseudomonas* OTUs detected by the PhyloChip in the *Rhizoctonia* suppressive soil (Mendes et al., 2011)) (Fig. S2). The *Streptomyces* consortium tested consisted of all six strains (6S) (Fig. S3).

None of the bacterial consortia adversely affected sugar beet seed germination (data not shown). In four independent experiments, however, none of the bacterial consortia was able to consistently suppress damping-off disease caused by *R. solani* (Fig. 4). For example, combination of the three *Lysobacter* strains (used as baseline) with the six *Pseudomonas* or six *Streptomyces* strains did significantly reduce damping-off disease of sugar beet in bioassay 2 but not in the other independent bioassays (Fig. 4F and G). Similarly, the mixture of six *Pseudomonas* strains significantly reduced damping-off disease in bioassay 3, but not in the other bioassays (Fig. 4C). Collectively, these results showed that none of the bacterial consortia tested led to a more consistent biocontrol of damping-off disease of sugar beet.

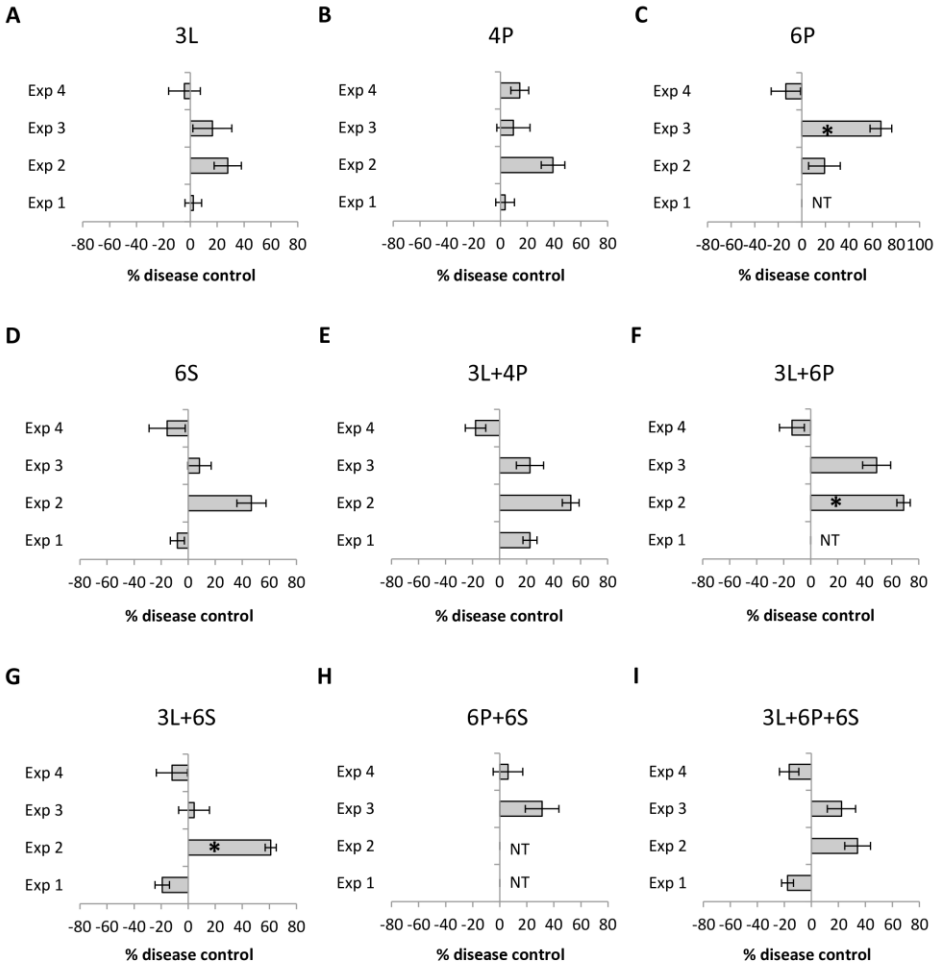

Figure 4. Effect of consortia of *Lysobacter* (L), *Pseudomonas* (P) and *Streptomyces* (S) strains on the efficacy of biocontrol of damping-off disease of sugar beet caused by *Rhizoctonia solani*. In four independent experiments, the level of disease control of the bacterial consortia was expressed as a percentage relative to the control (pathogen only, no bacteria introduced). The negative control (no bacteria, no pathogen inoculated) did not show any disease symptoms in any of the four experiments. Bacterial strains were mixed in equal ratios, each at an initial density of 10^7 CFU/g soil. The mixtures used were 3L: all three *Lysobacter* strains; 4P: *Pseudomonas* mixture with strains P29, P58, P62 and P63; 6P: all six *Pseudomonas* strains; 6S: all 6 *Streptomyces* strains. NT means not tested. The effects of the different bacterial consortia on disease incidence for the four independent experiments are shown in panels A-I: A) 3L; B) 4P; C) 6P; D) 6S; E) 3L+4P; F) 3L+6P; G) 3L+6S; H) 6P+6S; I) 3L+6P+6S. The error bar indicates the standard error of the mean. For each experiment, data were analysed by analysis of variance and Dunnet's post-hoc test. An asterisk indicates a statistically significant difference ($p<0.05$) between the bacterial treatment and the control.

Discussion

The bacterial genus *Lysobacter* has been proposed as an interesting source of new biocontrol agents (BCAs) and new bioactive compounds (Hayward et al., 2010). Previous work indeed demonstrated that application of single *Lysobacter* strains as BCAs significantly reduced disease severity in several crops (Yuen et al., 2001; Kilic-Ekici and Yuen, 2003; Folman et al., 2004; Ji et al., 2008; Ko et al., 2009; Postma et al., 2009; Puopolo et al., 2010; Puopolo et al., 2014). Furthermore, several *Lysobacter* species were found more abundant in *Rhizoctonia* suppressive soils (Postma et al., 2008; Postma et al., 2010; Postma et al., 2011). However, our former bioassays with 18 *Lysobacter* strains from soils suppressive to *R. solani*, showed no significant effects of each of the individual strains on *R. solani* damping-off disease in sugar beet and cauliflower (Gómez Expósito et al., 2015). As disease suppressiveness of soils is often attributed to the combined activity of multiple microbial genera (Lemanceau and Alabouvette, 1991; Raaijmakers et al., 1995; Weller et al., 2002; Mazzola, 2004), we hypothesized that combinations of the *Lysobacter* strains with other bacterial genera may trigger their antifungal activity leading to a more consistent biocontrol activity against the fungal root pathogen *R. solani*.

In vitro assays showed that combinations of *Lysobacter* with *Pseudomonas* and *Streptomyces* strains did, in general, not result in increased nor decreased antifungal activity. Only a few pairwise combinations led to an increased antifungal activity, whereas other combinations led to a decreased antifungal activity. Microbial competitive interactions can lead to an increased bacterial motility or to an enhancement in the production of antimicrobial compounds (Garbeva and de Boer, 2009; Tyc et al., 2014). This enhancement of antimicrobial compounds production can be due to cooperation, where the strains provide catalytic components, leading to new products when combined, or due to competition for trace elements, carbon or other energy sources (Slattery et al., 2001; Hibbing et al., 2010; Foster and Bell, 2012; Shong et al., 2012). The production of secondary metabolites may also depend on nutrient availability (Valanarasu et al., 2010). Our results indeed show that the majority of enhanced antifungal activities were observed on nutrient-rich media. Nevertheless, the majority of bacterial combinations did not result in a decrease or increase of antifungal activity as compared to the monocultures, supporting and extending the findings of Tyc et al. (2014). An increase in antagonistic activity by bacterial interactions may be due to synergistic effects or to competition for nutrients (de Boer et al., 2007). A reduced antagonistic activity by microbial interactions may be due to incompatible modes of action, for example if one strain produces enzymes that inactivate

the bioactive compounds produced by the other strain (Stockwell et al., 2010). The modes of action of the *Pseudomonas* and *Streptomyces* strains tested in this study are still unclear. The *Pseudomonas* strains included in this study were negative for *thaC2*, one of the genes involved in the biosynthesis of the antifungal lipopeptide thanamycin (Mendes et al., 2011). Initial inoculum ratios of the consortia and differences in growth dynamics (Szczech and Shoda, 2004; Xu et al., 2011) may also have influenced the antagonistic performance, but in this study only consortia with equal ratios were tested.

Natural environments such as soil and rhizosphere harbor an enormous diversity of microorganisms with complex webs of interactions. From this immense diversity, plant roots select for a subset to create their 'own' root-associated microbiome (Mendes et al., 2011; Bulgarelli et al., 2013; Edwards et al., 2015; Lebeis et al., 2015). The use of synthetic communities has been proposed to mimic this subset of root-associated microbes and complex networks of interactions (Kim et al., 2008; Großkopf and Soyer, 2014; Mazzola and Freilich, 2016). Recent studies on root colonization of synthetic communities showed that only certain microbial groups within the synthetic community applied could indeed colonize plants roots and leaves. For example, Lebeis et al. (2015) observed that only 14 out of 38 strains composing the synthetic community applied to soil were robust colonizers of *Arabidopsis thaliana* roots. Bai et al. (2015) observed that several isolates in the synthetic community that were derived from soil and roots showed higher colonization rates of soil and roots compared to leaves, although colonization of the leaves by these isolates was observed. The same was observed for leaf-derived isolates, suggesting that microbes are better adapted to their original niche but can relocate and adjust, to some extent, to new niches.

None of our synthetic communities composed of *Lysobacter*, *Pseudomonas* and/or *Streptomyces* showed an improved level of disease control in a reproducible manner. These results oppose to the earlier results by Roberts et al. (2005) and Szczech and Schoda (2004) who showed that biocontrol of *R. solani* was improved by a combination of *Burkholderia cepacia* or *B. ambifaria* with *Trichoderma virens* on cucumber or by a combination of *B. cepacia* with *Bacillus subtilis* on tomato.

In a previous study, we showed that the *Lysobacter* strains were poor colonizers of sugar beet and also cauliflower rhizospheres (Gómez Expósito et al., 2015). In the study presented here, root colonization of each of the strains in the synthetic community tested was not determined due to a lack of proper markers to monitor their densities in the microbially diverse rhizosphere. Rhizosphere colonization is important for biocontrol (Bull et al., 1991; Johnson, 1994; Raaijmakers et al., 1995; Haas and Defago, 2005) and the

minimum population density reported to be needed for biocontrol by *Pseudomonas* strains ranges from 10^5-10^6 CFU (colony forming units) per g of root (Raaijmakers et al., 1995). Hence, poor colonization of the rhizosphere may have been one of the reasons for a lack of consistent biocontrol activity of the bacterial consortia tested here.

Biodiversity strongly influences ecosystem functioning in complex environments that provide different niches. Positive interactions may occur when increasing biodiversity, especially between different taxa that are more prone to use resources in a different way, making the use of available resources more efficient (Jousset et al., 2011). However, while an agar plate constitutes a more homogeneous environment, soils establish a highly heterogeneous habitat. Therefore, whereas in the *in vitro* assay bacteria are forced to interact with each other, in soil environments there is a strong spatial distribution where microbes can form aggregates thereby excluding other microbes (Kim et al., 2008; Vos et al., 2013). Thus, only microbes that are in close proximity (like in a biofilm) can sense signal molecules necessary for the production of certain secondary metabolites (Xavier, 2011). On the other hand, in natural environments the different niches often overlap, which may lead to competition and antagonistic interactions among the resident microbiota. Therefore, the global performance of microbial mixtures can be also negatively affected, if the activity of "key species" is inhibited (Jousset et al., 2011). Due to the enormous diversity of microbes in soil environments and due to the limitations in addressing the functionality of each microbe in natural environments, the design of effective synthetic communities is very challenging. Recently, the use of network analyses of microbiomes has been proposed as a method to design microbial assemblages based on relative abundance and co-occurrence in order to better select taxa associated with plant health (Poudel et al., 2016).

Overall, our results showed that combining several *Lysobacter*, *Pseudomonas* and *Streptomyces* species and strains led to a differential antifungal activity against *R. solani in vitro* on different agar media, and that antifungal activity was affected only in specific bacterial interactions. Even though the synthetic communities used in this study showed antifungal activity *in vitro,* their net effect was not enhanced compared with the activity of the monocultures. Despite the fact that the strains applied in the disease assays were closely related to strains detected in higher abundance in *Rhizoctonia* suppressive soil, application of these synthetic communities did not result in consistent levels of disease suppression. This is presumably due to either a lack of colonization ability of the strains, a lack of production of secondary metabolites in rhizosphere environments or incompatibility in modes of action. Although the genus *Lysobacter* has been previously associated with disease suppressive soils (Postma et al., 2008; Postma et al., 2010; Postma

et al., 2011) and it has been proposed as a candidate genus to be used for biological control (Hayward et al., 2010), our findings could not support their active role in disease suppression against *R. solani in vivo*, neither alone (Gómez Expósito et al., 2015) nor in combination with other bacterial species. To summarize, we observed that enhancement in antifungal activity by bacterial interactions is strain and media dependent and that an increase in species richness does not necessarily improve performance in disease suppression *in situ*. Therefore, the design of effective synthetic microbial communities should focus on identifying other criteria or microbial traits associated with effective partnerships rather than randomly combining microbial genera that individually exhibit strong antagonistic properties.

Author contributions

All authors were involved in the design of the experiments. RGE performed the experiments, analyzed the data, created the figures and drafted the manuscript. All authors supervised the experimental design and contributed to the revision and writing of the manuscript.

Acknowledgments

We thank Olaf Tyc for his valuable advices on the statistical analysis of the *in vitro* activity of monocultures and synthetic communities. This research was funded by the Dutch Technology Foundation (STW).

Supplementary material

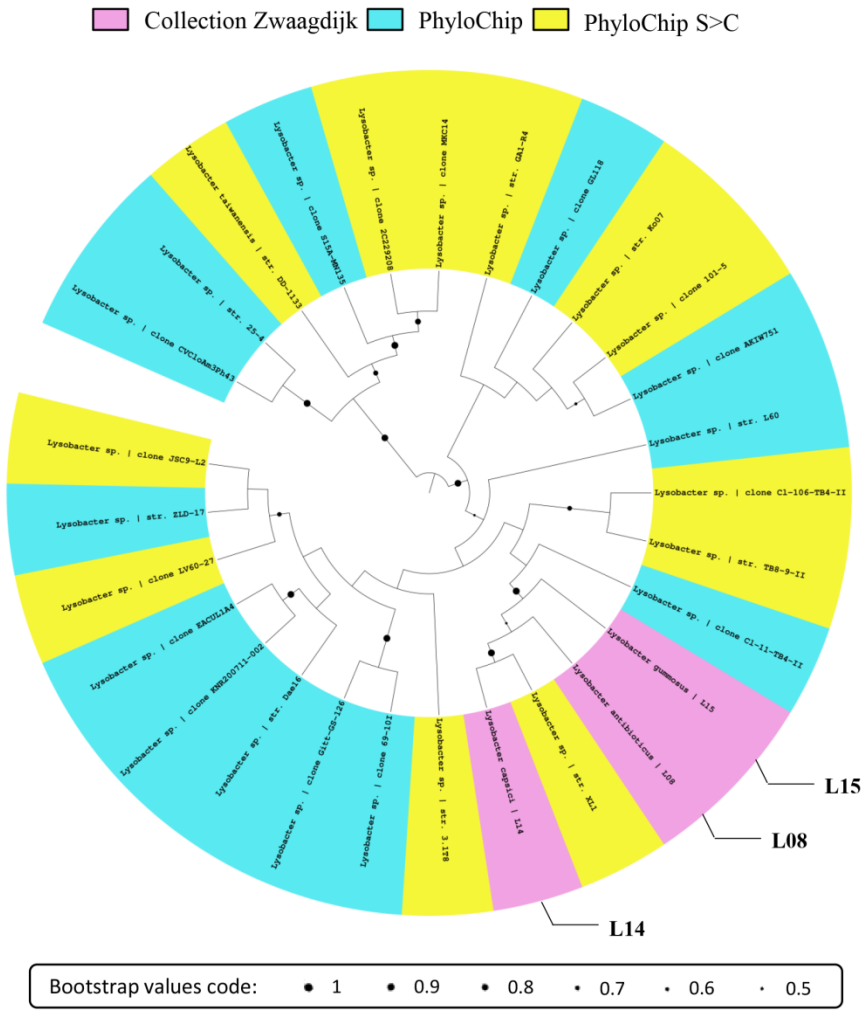

Figure S1. Phylogenetic delineation of the three *Lysobacter* strains used in this study and the *Lysobacter* operational taxonomic units (OTUs) detected by the PhyloChip of Hoeven *Rhizoctonia* suppressive soil. The evolutionary relationship of the 16S rRNA sequences was inferred by alignment with Muscle and neighbor-joining tree construction. The bootstrap values indicated at the nodes are based on 1000 bootstrap replicates. The black dots at the nodes indicate the level of bootstrap support higher than 0.5. Collection Zwaagdijk: strains used in this study; PhyloChip: OTUs detected with the PhyloChip; PhyloChip S>C: OTUs detected with the PhyloChip in higher abundance in suppressive versus conducive soil. Highlighted are the strains used in this study

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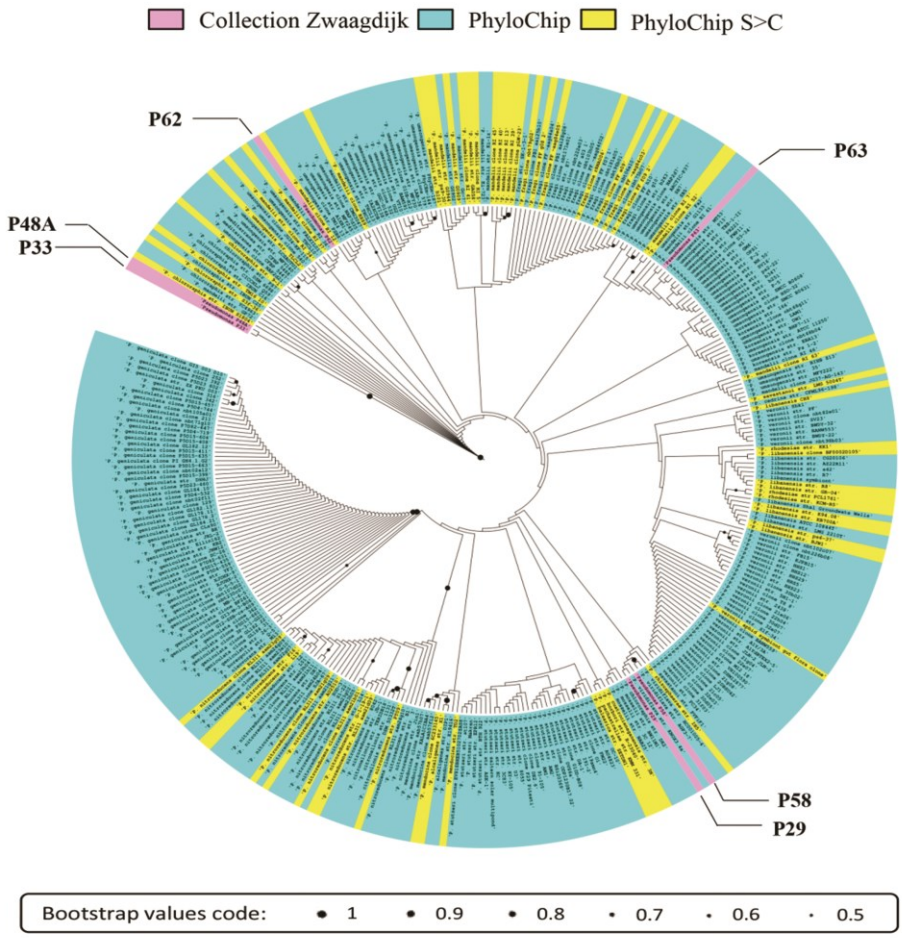

Figure S2. Phylogenetic delineation of the three *Pseudomonas* strains used in this study and the *Pseudomonas* operational taxonomic units (OTUs) detected by the PhyloChip of Hoeven *Rhizoctonia* suppressive soil. The evolutionary relationship of the 16S rRNA sequences was inferred by alignment with Muscle and neighbor-joining tree construction. The bootstrap values indicated at the nodes are based on 1000 bootstrap replicates. The black dots at the nodes indicate the level of bootstrap support higher than 0.5. Collection Zwaagdijk: strains used in this study; PhyloChip: OTUs detected with the PhyloChip; PhyloChip S>C: OTUs detected with the PhyloChip in higher abundance in suppressive versus conducive soil. Highlighted are the strains used in this study.

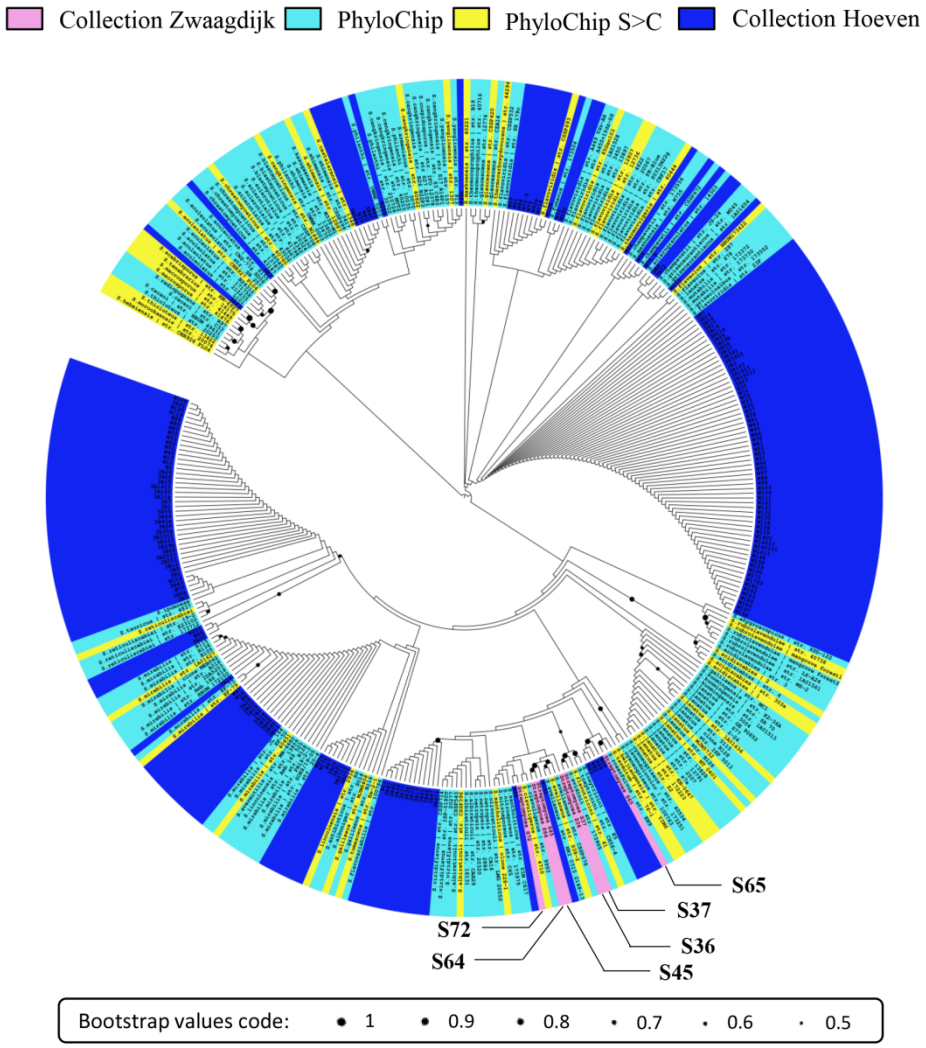

Figure S3. Phylogenetic delineation of the three *Streptomyces* strains used in this study, the *Streptomyces* operational taxonomic units (OTUs) detected by the PhyloChip of Hoeven *Rhizoctonia* suppressive soil and the *Streptomyces* strains isolated from Hoeven *Rhizoctonia* suppressive soil. The evolutionary relationship of the 16S rRNA sequences was inferred by alignment with Muscle and neighbor-joining tree construction. The bootstrap values indicated at the nodes are based on 1000 bootstrap replicates. The black dots at the nodes indicate the level of bootstrap support higher than 0.5. Collection Zwaagdijk: strains used in this study; PhyloChip: OTUs detected with the PhyloChip; PhyloChip S>C: OTUs detected with the PhyloChip in higher abundance in suppressive versus conducive soil; Collection Hoeven: strains isolated from Hoeven Rhizoctonia suppressive soil. Highlighted are the strains used in this study

Table S1. *In vitro* inhibition of hyphal growth of *Rhizoctonia solani* by monocultures and mixtures of different *Lysobacter*, *Pseudomonas* and *Streptomyces* strains. The bacterial strains were spot-inoculated at the edge of four different agar media that differ in nutrient content (R2A, 1/10 TSA, M9, WA-N) and a mycelial plug of *R. solani* was placed in the middle of the plate and allowed to grow outwards. After 7 days of incubation, hyphal growth inhibition was assessed by measuring the size of the inhibition zone surrounding the bacterial colonies. For each bacterial strain or mixture of strains, three replicates were used and the average sizes of the inhibition zone with standard deviations (±) are shown. Data were statistically analysed by analysis of variance and LSD post-hoc test. Coloured boxes indicate a statistically significant effect of the bacterial strain mixtures as compared to each of the individual strain. Dark grey boxes indicate a net positive effect of the mixture for each of the individual strains, light grey boxes indicate a net negative effect, and black boxes indicate those cases for which the effect of the interaction is positive for one strain but negative for the other strain that is part of the mixture. L: *Lysobacter*; P: *Pseudomonas*; S: *Streptomyces*, followed by the strain number.

Interaction Type	Isolate/Mix	Fungal inhibition (cm)			
		Nutrient level			
		R2A	1/10 TSA	M9	WA-N
	Ctrl	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Monocultures	L8	0.2 ± 0.0	0.6 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
	L14	1.0 ± 0.0	0.8 ± 0.0	0.4 ± 0.1	0.2 ± 0.0
	L15	0.3 ± 0.0	0.6 ± 0.1	0.2 ± 0.0	0.2 ± 0.0
Dual interactions	L8+L14	0.0 ± 0.0	0.5 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
	L8+L15	0.7 ± 0.0	0.8 ± 0.0	0.0 ± 0.0	0.2 ± 0.0
	L14+L15	0.7 ± 0.0	0.6 ± 0.0	0.1 ± 0.0	0.1 ± 0.1
Monocultures	P29	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
	P33	0.3 ± 0.0	0.3 ± 0.1	0.0 ± 0.0	0.1 ± 0.0
	P48A	0.4 ± 0.0	0.4 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
	P58	0.3 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0
	P62	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	P63	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Dual interactions	L8 + P29	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
	L8 + P33	0.1 ± 0.0	0.5 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	L8 + P48A	0.1 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
	L8 + P58	0.3 ± 0.0	0.0 ± 0.0	0.3 ± 0.0	0.0 ± 0.0
	L8 + P62	0.1 ± 0.0	0.4 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	L8 + P63	0.1 ± 0.0	0.5 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
	L14 + P29	0.3 ± 0.0	0.8 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
	L14 + P33	0.8 ± 0.0	0.8 ± 0.0	0.3 ± 0.1	0.1 ± 0.0
	L14 + P48A	0.7 ± 0.0	0.8 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
	L14 + P58	0.2 ± 0.0	1.1 ± 0.1	0.3 ± 0.0	0.0 ± 0.0
	L14 + P62	0.7 ± 0.0	0.8 ± 0.0	0.0 ± 0.0	0.2 ± 0.0
	L14 + P63	0.7 ± 0.0	0.9 ± 0.1	0.0 ± 0.0	0.2 ± 0.0
	L15 + P29	0.4 ± 0.2	0.3 ± 0.0	0.2 ± 0.0	0.0 ± 0.0
	L15 + P33	0.5 ± 0.0	0.8 ± 0.1	0.1 ± 0.0	0.0 ± 0.0
	L15 + P48A	0.6 ± 0.1	0.6 ± 0.1	0.1 ± 0.0	0.0 ± 0.0
	L15 + P58	0.2 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0
	L15 + P62	0.5 ± 0.0	0.6 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	L15 + P63	0.4 ± 0.1	0.7 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Interaction type	Isolate/Mix	Fungal inhibition (cm)							
		Nutrient level							
		R2A		1/10 TSA		M9		WA-N	
Monocultures	S36	0.1	± 0.0	0.0	± 0.0	0.0	± 0.0	0.0	± 0.0
	S37	0.2	± 0.0	0.2	± 0.0	0.2	± 0.0	0.2	± 0.0
	S45	0.2	± 0.0	0.2	± 0.1	0.2	± 0.0	0.2	± 0.0
	S64	0.0	± 0.0	0.0	± 0.0	0.0	± 0.0	0.2	± 0.0
	S65	0.0	± 0.0	0.0	± 0.0	0.2	± 0.0	0.1	± 0.0
	S72	0.4	± 0.0	0.4	± 0.0	1.7	± 0.0	0.0	± 0.0
Dual interactions	L8 + S36	0.0	± 0.0	0.5	± 0.0	0.1	± 0.0	0.0	± 0.0
	L8 + S37	0.0	± 0.0	0.4	± 0.1	0.2	± 0.0	0.0	± 0.0
	L8 + S45	0.0	± 0.0	0.5	± 0.0	0.3	± 0.1	0.2	± 0.0
	L8 + S64	0.0	± 0.0	0.5	± 0.0	0.0	± 0.0	0.2	± 0.0
	L8 + S65	0.0	± 0.0	0.6	± 0.0	0.2	± 0.0	0.2	± 0.0
	L8 + S72	0.0	± 0.0	0.5	± 0.0	0.0	± 0.0	0.0	± 0.0
	L14 + S36	0.7	± 0.0	0.8	± 0.0	0.3	± 0.0	0.0	± 0.0
	L14 + S37	0.7	± 0.0	0.8	± 0.0	0.1	± 0.0	0.0	± 0.0
	L14 + S45	0.7	± 0.0	0.8	± 0.0	0.4	± 0.1	0.3	± 0.0
	L14 + S64	0.7	± 0.0	0.7	± 0.1	0.4	± 0.0	0.4	± 0.0
	L14 + S65	0.7	± 0.0	0.8	± 0.0	0.2	± 0.0	0.1	± 0.0
	L14 + S72	0.7	± 0.0	0.8	± 0.0	0.1	± 0.0	0.1	± 0.0
	L15 + S36	0.6	± 0.0	0.8	± 0.0	0.0	± 0.0	0.0	± 0.0
	L15 + S37	0.6	± 0.0	0.6	± 0.0	0.2	± 0.0	0.0	± 0.0
	L15 + S45	0.6	± 0.0	0.7	± 0.1	0.1	± 0.1	0.0	± 0.0
	L15 + S64	0.5	± 0.0	0.7	± 0.0	0.0	± 0.0	0.0	± 0.0
	L15 + S65	0.5	± 0.0	0.7	± 0.0	0.2	± 0.0	0.0	± 0.0
	L15 + S72	0.5	± 0.0	0.7	± 0.0	0.0	± 0.0	0.0	± 0.0
Multiple interactions	3L	0.7	± 0.0	0.6	± 0.0	0.2	± 0.1	0.2	± 0.0
	6P	0.2	± 0.0	0.3	± 0.0	0.2	± 0.0	0.1	± 0.0
	6S	0.2	± 0.0	0.2	± 0.0	0.1	± 0.0	0.0	± 0.0
	3L + 6P	0.4	± 0.1	0.4	± 0.0	0.0	± 0.0	0.0	± 0.0
	3L + 6S	0.3	± 0.0	0.2	± 0.0	0.0	± 0.0	0.0	± 0.0
	3L + 6P + 6S	0.3	± 0.0	0.2	± 0.0	0.2	± 0.0	0.2	± 0.1

Numbers preceding the letters (L, P, S) refer to the number of strains used in that mixture.

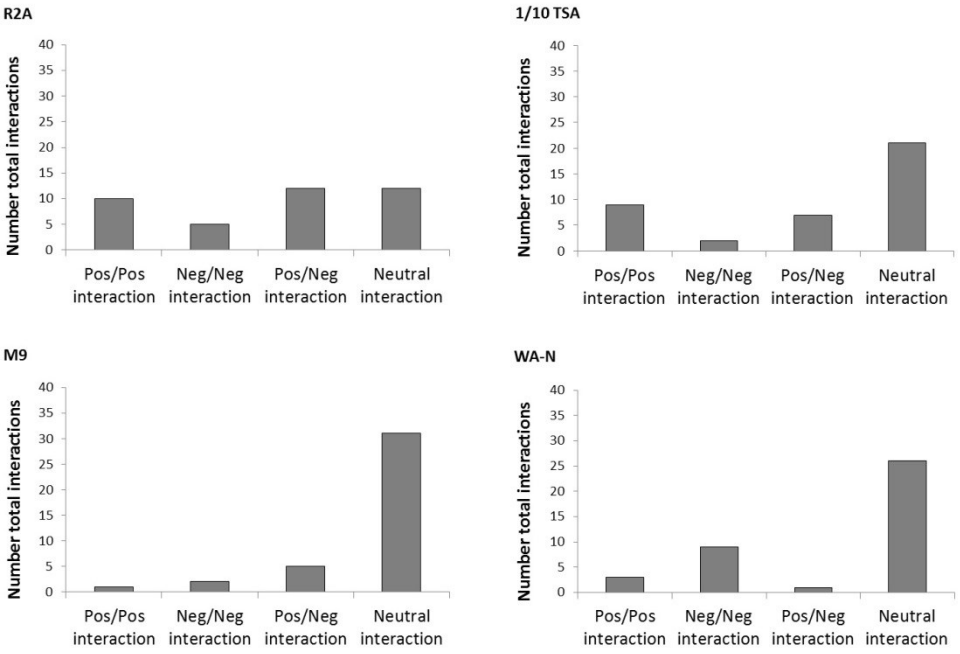

Figure S4. Effect of dual interactions of *Lysobacter*, *Pseudomonas* and *Streptomyces* strains compared to the activity of the monocultures in inhibiting the mycelial growth of *R. solani*. Pos/Pos indicate an increase in antifungal activity for the dual interaction compared with the activity of each of the individual strains; Neg/Neg indicate a decrease in antifungal activity for the dual interaction compared with the activity of each of the individual strains; Pos/Neg indicate that the effect of the bacterial interaction is positive for one of the two strains but negative for the other; Neutral interactions indicate interactions that have no significant differences when compared with the activity of the monocultures. A total of 39 pairwise interactions (n=3) were tested for each of the media tested (R2A, 1/10 TSA, M9 and WA-N) and differences in activity were calculated by analysis of variance and LSD post-hoc analysis.

Chapter 4

Successional changes in rhizosphere microbiome composition and functions during induction of the soil immune response to a fungal root pathogen

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(To be submitted)

Abstract

In disease suppressive soils, soil-borne plant pathogens cause little or no disease due to specific microbial activities in the plant rhizosphere. Disease suppressiveness to several fungal root pathogens is typically induced in field soils during repeated infections of susceptible plants by the virulent pathogen, a phenomenon that resembles the adaptive immune response in animals. For most disease suppressive soils, however, the microbial communities and mechanisms involved in the induction of disease suppression are largely unknown. In this study, we show that soil suppressiveness against damping-off disease caused by the fungal root pathogen *Rhizoctonia solani* can be induced in a small-scale, short-term bioassay by growing the host plant (sugar beet) repeatedly in presence of the pathogen. We investigated the dynamics of the rhizobacterial community composition as well as their gene expression *in situ* during the transition of the soil from the disease-conducive to the disease-suppressive state, a process referred to as rebiosis. Taxonomic analyses revealed only minor changes in the rhizobacterial community composition whereas metatranscriptome analysis showed substantial changes in the expression of specific bacterial genes during rebiosis. Among the genes found up-regulated in suppressive soil were genes involved in stress response (mainly oxidative stress), production of lytic enzymes, hydrogen cyanide, siderophores and indole acetic acid. The majority of these upregulated genes were assigned to the bacterial families *Rhizobiaceae*, *Bradyrhizobiaceae*, *Phyllobacteraceae*, *Erythrobacteraceae*, *Sphingomonadaceae*, *Brucellaceae*, *Caulobacteraceae*, *Opitutaceae* and *Comamonadaceae*. In conclusion, our results indicate that the soil immune response against the fungal root pathogen *R. solani* is associated with changes in the activity of specific members of the microbial communities rather than with substantial alterations in the rhizobacterial community composition. This study highlights the importance of combining multiple 'omics approaches to obtain a more comprehensive insight into the successional changes in microbiome composition and functions during rebiosis.

Introduction

Soil and plant-associated microorganisms play a crucial role in plant health and productivity and have been proposed as an integral component of novel sustainable strategies to minimize crop losses due to abiotic and biotic stresses (Berendsen et al., 2012; Mendes et al., 2013; Mueller and Sachs, 2015; Bender et al., 2016). The composition and activities of the plant microbiome are greatly influenced by the plant through deposition of root exudates (Doornbos et al., 2012). Some exudate constituents can be detrimental to soil-borne plant pathogens whereas others act as cues for pathogen germination, root colonization and infection. Plant exudates also attract and accommodate numerous commensal and mutualistic microorganisms that promote plant growth and provide protection against invading plant pathogens, either directly *via* competition and antibiosis or indirectly *via* induction of local or systemic plant defenses (Alabouvette et al., 2006; Raaijmakers et al., 2009; Bulgarelli et al., 2013; Ciancio et al., 2016; Rellán-Álvarez et al., 2016).

The best examples of microbiome-mediated protection of plants against root pathogens are disease suppressive soils. These are 'soils in which a pathogen does not establish or persist, establishes but causes little or no disease, or establishes and causes disease at first but then the disease declines' (Weller et al., 2002). Suppressive soils occur world-wide and have been described for various plant diseases, including fungal diseases caused by *Rhizoctonia solani (*Postma et al., 2010; Mendes et al., 2011), *Gaeumannomyces graminis* var *tritici* (Raaijmakers and Weller, 1998; de Souza et al., 2003), and *Fusarium oxysporum* (Scher and Baker, 1980; Klein et al., 2013), bacterial diseases caused by *Ralstonia solanacearum* (Shiomi et al., 1999) and *Streptomyces scabies* (Weinhold et al., 1964), or diseases caused by nematodes such as *Meloidogyne incognita* (Pyrowolakis et al., 2002; Becker and Borneman, 2004).

Two types of disease suppressiveness of soils can be distinguished: i) general suppression which is universal to soils and based on competitive activities of the overall micro- and macroflora, providing a basal level of defense, and ii) specific suppression which is superimposed over general suppression and attributed to the enrichment of subsets of microbial genera with specific, concerted activities towards the invading pathogen (Weller et al., 2002). General and specific suppression can be eliminated by soil pasteurization or biocides, but only specific suppression can be transferred to non-suppressive (conducive) soils *via* soil transplantations (van der Voort et al., 2016). Specific suppression of soils against several fungal root pathogens is typically induced in field soils during continuous

cultivation of a susceptible host plant followed by a disease outbreak (Raaijmakers and Mazzola, 2016). While soil physical-chemical properties can modulate the onset and intensity of their disease-suppressive state, functionality of the response is in most cases microbiological in nature. Once established, specific suppression against fungal pathogens can dissipate if non-host plants are grown or when other root diseases emerge, but is rapidly regained in presence of the original host plant and inducing pathogen (Raaijmakers and Mazzola, 2016). These characteristics of general and specific disease suppression of soils are strikingly comparable to those described for innate and adaptive immunity in animals, where the innate immune response provides a first basal, non-specific line of defense and where the adaptive immune response requires time to react to the invading pathogen and has a memory (Lapsansky et al., 2016; Raaijmakers and Mazzola, 2016).

Most of the mechanistic studies to date typically use end-point measurements comparing the composition of soils and plant-associated microbiomes of suppressive soils with those of non-suppressive (conducive) soils (Mendes et al., 2011; Li et al., 2015; Shen et al., 2015; Giné et al., 2016). However, these studies do not consider the temporal changes in plant microbiome composition and functions expressed during the trajectory from the conducive to the disease suppressive state, a process also referred to as rebiosis (Petersen and Round, 2014). In this study, we established a reproducible, small-scale and short-term bioassay to induce soil suppressiveness to the fungal root pathogen *R. solani* by growing the host plant (sugar beet) repeatedly in presence of the pathogen. Subsequently, we investigated the successional changes in the rhizosphere microbiome composition and functions during the transition of the soil from the conducive to the disease-suppressive state. Based on these microbiome and metatranscriptome analyses, we identified specific bacterial taxa and putative functions associated with the initial stages of soil suppressiveness.

Materials and methods

Soil collection, storage and physicochemical analysis

The agricultural soil used in this study was collected from an orchard located in Zwaagdijk, The Netherlands (52°41'53.549" N, 5°6'58.643" E) in June 2012 at a depth of 10-40 cm. The soil was air-dried, sieved (0.5 cm mesh) to remove plant material and stored at 8°C in the dark until further use. The field soil was subjected to physical and chemical analyses at the Chemical Biological Soil Laboratory (CBLB, Wageningen University, The Netherlands).

Growth and storage conditions of the fungal pathogen

The fungal isolate *R. solani* AG2-2IIIB was kindly provided by the Institute of Sugar Beet Research (IRS, Bergen op Zoom, Netherlands) and kept in mineral oil at 10°C. Agar plugs with fungal hyphae used as inoculum in the soil bioassays were prepared by growing *R. solani* on 1/5th potato dextrose agar (PDA, Oxoid) medium for one week at 25°C.

Induction of soil suppressiveness against *R. solani*

Sugar beet seedlings (*Beta vulgaris* cv. Rhino) were cultivated for successive growth cycles of 3 weeks each in the agricultural soil in absence and in presence of *R. solani* inoculum. Rectangle trays (22 x 36 x 5.5 cm) were filled with 3 kg of the field soil, hydrated to 20% (v/w) and sown with sixty sugar beet seeds coated with thiram, hymexazol and poncho-beta to control seed-borne diseases caused by *Pythium* and *Aphanomyces* species. Five replicates were used for each of the following three treatments; i) soil not inoculated with *R. solani* and grown with sugar beet seedlings only (referred to as treatment B (Beet)), ii) soil grown with sugar beet and inoculated with *R. solani* (referred to as treatment BR (Beet+*Rhizoctonia*)) and, iii) soil not grown to sugar beet and not inoculated with *R. solani* (referred to as treatment NC (Not Cultivated)). For growth cycles 1 to 4, sugar beet seeds were pre-germinated in sterile river sand for 5 days and transplanted in the field soil. For growth cycles 5 to 8, seeds were germinated directly in the field soil. *R. solani* was not inoculated during cycle 5 for the treatment BR in order to avoid an overload of the pathogen in the system and therefore that cycle was not included in the analyses. Sugar beet seedlings were grown for 3 weeks in trays with transparent lids in a growth chamber at 24°C with 70% relative humidity and a 16h photoperiod and a light intensity of 150 micromol photons m^{-2} s^{-1}. *R. solani* was inoculated seven days after sowing by placing 6 fresh *R. solani* AG2-2IIIB agar plugs (5 mm) at the outer rows of germinated seedlings, with the mycelial side touching the seedlings. The number of *R. solani* diseased plants showing the typical damping-off symptoms was scored at regular intervals during a period of two weeks (Fig. 1). Hyponex nutrient solution was added to the soil once per week during growth cycles 1 to 4. From cycle 5 onwards, no extra nutrition was added to avoid accumulation of salts on the soil surface. The time period between the growth cycles was 2-3 days.

A trial to investigate if disease suppression towards *R. solani* can be transplanted to a conducive soil was performed as above indicated with minor differences. In brief, suppressiveness was induced by repeatedly growing twelve sugar beet seedlings in pots (7

x 7 x 8 cm) filled with 250 g of field soil under the same conditions as above indicated. Sugar beet seedlings were grown for six growth cycles in presence (BR) and in absence (B) of *R. solani*, with five replicates per treatment. For cycle 6, an additional treatment was included in which 10% (w/w) of the induced suppressive soil from cycle 5 was transplanted into 90% (w/w) non-cycled conducive soil (NC) and challenged with *R. solani* (TR) (Transplanted+*Rhizoctonia*).

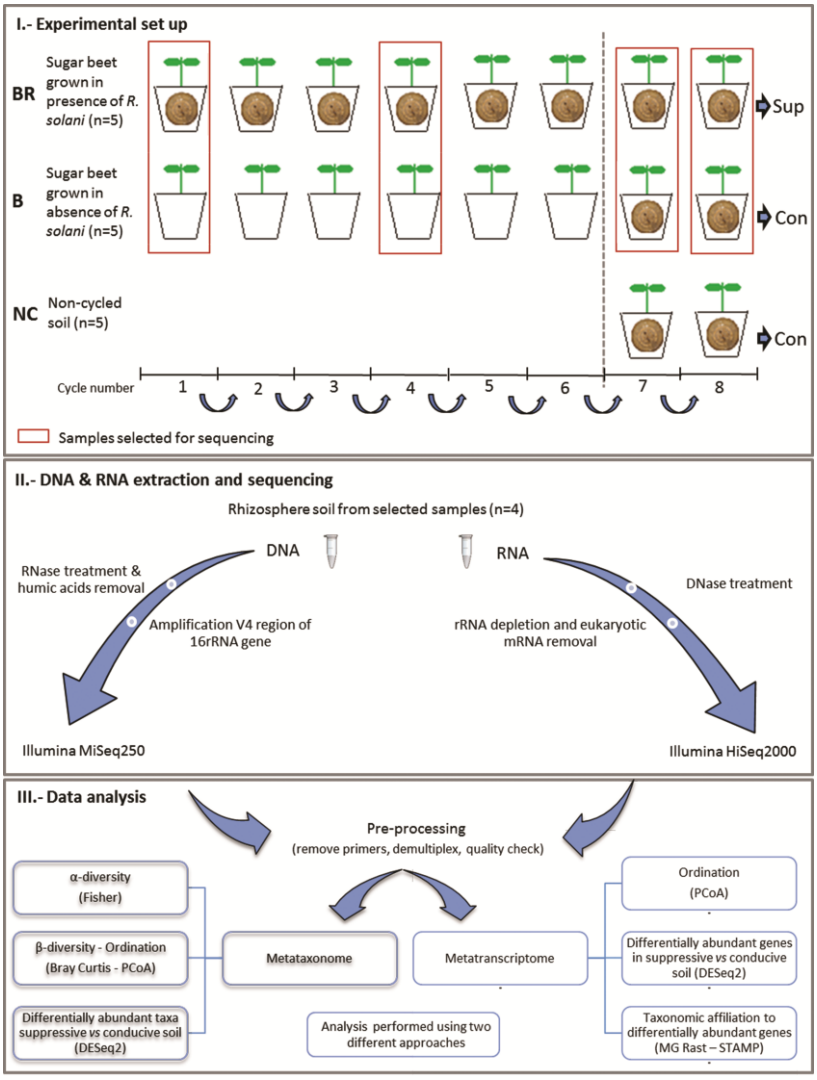

Figure 1. Schematic workflow of the experimental design to determine the changes in bacterial community composition and functions during induction of *Rhizoctonia* disease suppression. Sugar beet seedlings were grown in presence (BR) and in absence (B) of the fungal pathogen *Rhizoctonia solani* by recycling the soil for up to 6 cycles of growth, followed by two extra cycles in the presence of *R. solani* (n=5). In addition, at cycle 7, fresh conducive soil that did not undergo the cycling process (NC) was introduced and subjected to two cycles of growth with sugar beet in presence the of *R. solani*. The soil subjected to treatment BR became suppressive (Sup) whereas treatments B and NC remained conducive (Con). Four different cycles (boxed in red) were selected for DNA and RNA isolation of rhizosphere soil samples (n=4). DNA was purified and the V4 region of 16rRNA gene was sequenced by Illumina MiSeq250. RNA was purified, depleted for rRNA and eukaryotic mRNA and sequenced by Illumina HiSeq2000. The resulted metataxonome (DNA) and metatranscriptome (RNA) were subjected to statistical analyses to determine the changes in bacterial community composition and functionsduring *Rhizoctonia* disease decline.

Isolation and characterization of chitin-degrading bacteria from disease-suppressive soil

The rhizosphere soil of sugar beet plants grown in the induced suppressive soil was resuspended in 25 ml of 10 mM $MgSO_4.7H_2O$, vortexed 1 minute, sonicated 1 minute and vortexed once more for 15 seconds. The rhizosphere suspensions were dilution plated onto Petri dishes with a bottom layer of 10 ml of either R2A or WA-N (Garbeva and de Boer, 2009) with an overlay of 5 ml of R2A or WA-N, respectively, containing 1% (w/v) chitin and 25 mg/L delvocid (to suppress fungal growth). Plates were incubated for one week at 25°C and colonies showing a chitin-degradation halo were isolated, purified and stored in 40% (v/v) glycerol at -80°C. Plate counting showed that on R2A + 1% chitin, 3.5 x 10^8 cfu/g rhizospheric suppressive soil were retrieved. The ability of the selected bacterial isolates to degrade chitin *in vitro* was tested. The bacterial isolates were pre-cultured on R2A at 25°C as indicated above. Four droplets of 5 µl washed bacterial cells were spotted on R2A with a top layer R2A with 1% (w/v) chitin. Plates were incubated at 25°C for up to 6 weeks and plates were regularly monitored for chitin-degradation halos around the bacterial colonies.

For phylogenetic characterization, a 650-bp fragment of the 16S ribosomal RNA gene of the chitin-degrading bacterial isolates was sequenced (Baseclear, Leiden, The Netherlands). The isolates were identified based on the highest hit of the 16S rRNA sequences with sequences in the SILVA reference database (Quast et al., 2013). Phylogenetic trees were constructed by aligning the 16S-sequences of the chitin degraders and the type strains for each of the identified bacterial families (Cole et al., 2009) using Muscle (Edgar, 2004) and neighbor joining in MEGA6 (Tamura et al., 2013).

To test the *in vivo* activity of the chitinolytic bacterial isolates against *R. solani*, isolates were grown on R2A plates at 25°C for five days to ensure enough biomass. Cells grown on the agar surface were resuspended in 5 ml of 0.9% (w/v) NaCl. Collected cells were washed 2 times with 0.9% NaCl at 7,000 X *g* for 10 min. The bacterial density was measured with a spectrophotometer at OD_{600} and adjusted to 10^8 cells/ml (OD_{600}=1 is used here as an equivalent to 10^9 cells/ml). Pots (19.5 x 6 x 3 cm) were filled with 250 g of conducive field soil with 20% (v/w) hydration with a total of 8 replicates per treatment. Fifteen sugar beet seeds were sown per pot and 100 µl of a suspension containing 10^8 cells/ml of bacterial suspension were inoculated on top of each seed. Non-inoculated soil was used as a control. Pots were placed in boxes with transparent lids in a growth chamber at 24°C with 70% humidity and a 16 h photoperiod. One week after sowing, *R. solani* was added by placing a fresh *R. solani* AG2-2IIIB agar plug (5 mm) grown on 1/5th PDA placed

with the mycelial side touching the first seedling. *R. solani* disease symptoms were scored at regular intervals during three weeks by scoring the number of diseased plants suffering from damping-off. The bioassay was done once. Statistical analysis applied were performed by analysis of variance with Dunnet post hoc analyses, performed with SPSS version 23.0.

Metagenomic DNA and RNA isolation

At the end of each growth cycle, the rhizospheric soil (i.e. soil firmly attached to the roots) was collected from the sugar beet seedlings that were not suffering from infection by *R. solani*. More specifically, roots with firmly attached soil were directly suspended in RNA*later*® (Ambion, Carlsbad, CA, USA), vortexed for 1 min, sonicated for 1 min, vortexed for 15 seconds and stored at 4°C overnight. The next day, the roots were vortexed for 1 min and the RNA*later*® was collected. The roots were placed in fresh RNA*later*® solution and vortexed again for 1 min and then removed. The two RNA*later*® washes were pooled and stored at -80 until nucleic acid (DNA, RNA) extractions were performed. For DNA and RNA isolations, 0.5 g rhizospheric soil samples embedded in RNA*later* were centrifuged at 15,871 X *g* and 4°C for 5 minutes. RNA*later* was discarded and soil pellets were washed three times with diethlylpyrocarbonate (DEPC) water containing 0.5% β-mercaptoethanol and centrifuged at 13,000 rpm and 4°C for 5 minutes to discard any RNA*later* residues. RNA was isolated with the RNA Power Soil Total RNA Isolation Kit (MOBIO Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions with slight modifications. In brief, all centrifugations were done at 4°C instead of room temperature and the tubes were also kept on ice during RNA elution. The incubation of the eluted RNA with SR4 solution was done at -20°C for 30 min instead of 10 min and the final elution was done in 50 µl of SR7 solution. After RNA elution, DNA was eluted from the same sample with the RNA Power Soil DNA Elution Accessory Kit (MOBIO Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions with the same modifications as indicated above for the RNA isolation. DNA and RNA were visualized by electrophoresis on a 1.5% (w/v) agarose 1X TAE gel with ethidium bromide staining and the total DNA concentration was quantified with a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Both DNA and RNA samples were stored at -80°C.

Microbiome analysis: 16S library preparation, sequencing and data analysis

Libraries were constructed from the extracted DNA samples by amplifying the V4 region of the 16S rRNA gene using the following primers: Forward: 5'-GTGCCAGCMGCCGCGGTAA-3' and Reverse: 5'-GGACTACHVGGGTWTCTAAT-3'. A total of 40 libraries were created (4 biological replicates of growth cycles B1, 4, 7, 8; BR1, 4, 7, 8, and NC1, 2. The libraries were sequenced in 2 lanes by using the Illumina MiSeq250 platform (Fig. 1) (BGI Tech Solutions, Hongkong, Co., Ltd.). Sequences were analyzed using a pipeline implemented using Snakemake (Koster and Rahmann, 2012) in Mothur (Schloss et al., 2009) as follows: primer sequences were removed from each FASTQC file using Flexbar version 2.5 (Dodt et al., 2012). FastQC (Andrews, 2010) was used for quality check of the sequences and PANDAseq (Masella et al., 2012) was used to merge paired-end reads with a minimum overlap of 40bp and at least a PHRED score of 25. Sequences were trimmed to a length of 253 bp using AlienTrimmer 0.4.0 (Criscuolo and Brisse, 2013) and converted to FASTA format and concatenated into a single file. An OTU table was constructed using the UPARSE (Edgar, 2013) strategy by first dereplicating the reads,, sorting them by abundance with at least two sequences and finally clustering by the HPC-CLUST algorithm (Matias Rodrigues and von Mering, 2014). Taxonomic information for each OTU clustering data was added to the BIOM file by the SINA classifier provided by the SILVA database (Pruesse et al., 2012). Rarefaction curves were constructed with the Vegan package (Oksanen et al., 2015) and the species richness (alpha diversity) was calculated using the Phyloseq package (McMurdie and Holmes, 2013) in Rstudio. Further downstream statistical analyses were performed on filtered data (single and doubletons were removed) and on normalized data (relative abundance was calculated by dividing the number of counts of one OTU by the total number of OTU counts of that sample). The beta diversity and PERMANOVA analysis were determined with the Vegan package (Oksanen et al., 2015) and visualized by GeneMaths XT version 2.11 (Applied Maths, Belgium). Bacterial diversity was analyzed by merging the taxa at different taxonomic levels with the Phyloseq package (McMurdie and Holmes, 2013) and visualized by the ggplot2 package (Wickham, 2009). Differential abundance of OTUs (with a cutoff at 2-fold or higher) was calculated with the DeSeq2 package (Love et al., 2014). Differentially abundant OTUs were visualized by volcano plots using the package Calibrate (Graffelman, 2010).

Metatranscriptomic analysis: cDNA library preparation, sequencing and bioinformatics

Preparation of the cDNA libraries from the extracted rhizospheric RNA and subsequent sequencing was performed by Vertis_Biotech AG (Freising, Germany). Total RNA was examined by capillary electrophoresis on a Shimadzu MultiNA microchip electrophoresis system to ensure sufficient quality and integrity. RNA samples were treated with TURBO DNase (Ambion) in order to remove possible traces of DNA. Samples were then purified and concentrated using the RNeasy Micro Kit (Qiagen) and examined again by capillary electrophoresis. The ribosomal RNA (rRNA) was depleted using the RiboZero rRNA Removal Kit (Epicentre) (3:2 mixture of bacteria and plant reagents). Directly after rRNA depletion, the RNAs were treated with oligo-dT magnetic beads (Dynal/life technologies) to remove the poly(A) and eukaryotic mRNA molecules. The depleted RNA samples were then fragmented with ultrasound (2 pulses of 30 sec at 4°C). First-strand cDNA synthesis was primed with a N6 randomized primer. The Illumina TruSeq sequencing adapters were ligated to the 5' and 3' ends of the cDNAs. The cDNAs were finally amplified with PCR with a proofreading enzyme and purified. Aliquots of the PCR amplified libraries were examined by capillary electrophoresis.

For the sequencing, samples were pooled in approximately equimolar amounts. The pools were size-fractionated in the range of 300–500 bp using a differential clean-up with the Agencourt AMPure kit. Aliquots of the fractionated library pools were examined by capillary electrophoresis. The primers used for PCR amplification were designed for TruSeq sequencing for Illumina. The sequences of the adapters (122 bp length) flanking the cDNA insert were: TrueSeq_Sense_primer 5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-3', and TrueSeq_Antisense_NNNNNN_primer 5'-CAAGCAGAAGACGGCATACGAGAT-NNNNNN-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3' (where NNNNNN is the sequence of the barcode). The cDNA pools (4 pools of 8 samples per pool) were single read sequenced on an Illumina HiSeq 2000 with 100 bp read length, each in a separate lane (Fig. 1). Quality check of the obtained sequences was assessed by FastQC (Andrews, 2010) and trimming of the reads was done by AlienTrimmer 0.4.0. (Criscuolo and Brisse, 2013) with a minimum length of 60 bp and a minimum Phred quality score of 25. Trimmed reads were submitted to MG-Rast version 3.6 (Meyer et al., 2008). The resulting RNA transcripts were annotated with the SEED subsystems database (Overbeek et al., 2014) in MG-RAST and exported into a BIOM file to be analyzed in Rstudio. Data was converted into a suitable BIOM file using the biom (https://CRAN.R-project.org/package=biom) and the Phyloseq (McMurdie and

Holmes, 2013) packages. Rarefaction curves were constructed with the Vegan package (Oksanen et al., 2015). Further downstream statistical analyses were done on normalized data (calculated by dividing the number of counts of one transcript by the total number of counts of that sample, and referred as relative abundance). The beta diversity and PERMANOVA analysis were estimated with the Vegan package (Oksanen et al., 2015) and visualized by GeneMaths XT version 2.11 (Applied Maths, Belgium). Differential expression of transcripts was calculated with DeSeq2 (Love et al., 2014). Differentially expressed transcripts (>2-fold change) were visualized by constructing volcano plots using the package Calibrate (Graffelman, 2010). In depth analysis of relevant functions were performed in MG-RAST (Meyer et al., 2008) using hierarchical classification with the SEED subsystems database with an e-value ≤ 10e-3, % identity > 40%, and match length > 23 aa. Taxonomic affiliation of relevant functions was assessed based on protein similarity using the best hit classification method in MG-RAST, with the M5NR database, e-value ≤ 10e-3, % identity > 40%, and match length > 23 aa. Statistical analyses were done in STAMP (Parks et al., 2014), using the 2-groups comparison with the two-sided Welch's t-test, Welch's inverted as CI method, Storey's FDR correction and a p-value <0.05 as threshold and a minimum of 10 reads per transcript.

Results

The soil immune response: pathogen-mediated induction of disease suppression

For several fungal root pathogens, including *R. solani*, specific disease suppression is typically induced in the field during continuous cultivation of the susceptible host plant followed by a disease outbreak (Postma et al., 2010; Raaijmakers and Mazzola, 2016). To try to mimic these conditions, sugar beet seedlings were grown for successive cycles of 3 weeks each under controlled conditions in a soil collected from an agricultural field in the Netherlands. The sugar beet seedlings were grown in absence or in presence of *R. solani* inoculum to determine if the induction of disease suppressiveness requires the presence of the fungal pathogen (Fig. 1). The first bioassay showed that suppressiveness to *Rhizoctonia* damping-off disease of sugar beet can be induced in six successive growth cycles, but only in presence of *R. solani* inoculum. When sugar beet seedlings were grown successively in absence of *R. solani* inoculum, the soil remained conducive (Fig. S1). When the induced suppressive soil (BR6) was mixed with the conducive soil (B6) in a 1:9 ratio (w/w), suppressiveness to *Rhizoctonia* damping-off disease could be transferred to similar levels

as observed in the BR6 soil (Fig. S1). These results show that suppressiveness can be induced in bioassays under controlled conditions and can be transplanted to conducive soil. They confirm and extend results of previous studies conducted with *Rhizoctonia*-suppressive field soil (Mendes et al., 2011; van der Voort et al., 2016). Physical-chemical analyses of the rhizospheric soils further revealed that the B6 and BR6 soils were similar but different from the soil not planted with sugar beet (non-cycled soil, B0) (Table 1). The concentrations of the major nutrients N, P and K were lower in the cycled soils than in the non-cycled soil, whereas pH increased (Table 1). Collectively, these results indicate that the difference in disease suppressiveness between B6 and BR6 is most likely (micro)biological in nature and not due to differences in physical-chemical properties of the soil.

Results from the second bioassay confirmed that disease suppressiveness is induced after six successive growth cycles of sugar beet in presence of *R. solani* (BR): disease incidence increased from approximately 43% in growth cycle 1 to 60% in cycle 2 and then declined after growth cycle 4 to approximately 15-20% in cycles 6-8 (Fig. 2A). In contrast, disease suppressiveness could not be induced when sugar beet was grown in absence of *R. solani* (B) (Fig. 2B). In soil not cycled with sugar beet (NC), disease incidence was 35% and 85% when *R. solani* was introduced for 2 successive times.

Isolation and characterization of chitin-degrading bacteria from suppressive soil

Previous research has shown that soil amendments with chitin, a major component of the cell wall of fungi such as *R. solani*, could induce disease suppressiveness. In this context, chitinolytic bacteria have been suggested as potential key players (Sneh, 1981; Singh et al., 1999; Hjort et al., 2014; Postma and Schilder, 2015). To study their potential role, we isolated chitinolytic bacteria from roots of sugar beet grown in the induced suppressive soil and tested their ability to suppress *Rhizoctonia* disease *in vivo*. Hundred thirty isolates were identified, belonging to four different phyla: Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria (Table S1). Isolates belonging to bacterial families previously associated with disease suppressive soils (i.e. *Sphingomonadaceae*, *Comamonadaceae*, *Micromonosporaceae*, *Rhizobiaceae* and *Bradyrhizobiaceae* (Hunter et al., 2006; Kyselkova et al., 2009; Chapelle et al., 2015; Xue et al., 2015)) were selected for further analysis. Based on their phylogenetic relationship (Fig. S2 to S6), one isolate from each of these five families was selected and their ability to degrade chitin *in vitro* was confirmed on plates containing chitin (Fig. S7). When tested in soil bioassays, however, none of these isolates were able to control *Rhizoctonia* damping-off disease on sugar beet (Fig.3). These results suggest that

chitinolytic rhizobacteria do not play a major role in the soil suppressiveness induced by successive cycling of sugar beet seedlings in presence of the pathogen.

Table 1. Chemical analysis of the macro and micronutrients of the soil used in this study at different stages of disease suppression.

Chemical	Unit	Det. lim.	B0	B6	BR6
$CaCO_3$	(%)		0.87	3.77	3.53
Ca	(mg/kg)	40	7914	19722	20821
Cu	(mg/kg)	3	44	48	49
Fe	(mg/kg)	30	17113	15964	15538
K	(mg/kg)	70	3296	2263	2295
Mg	(mg/kg)	10	3645	4341	4341
Mn	(mg/kg)	1	667	441	478
Na	(mg/kg)	100	111	68	78
P	(mg/kg)	3	971	1099	1098
S	(mg/kg)	30	694	857	922
Zn	(mg/kg)	5	73	74	74
Co	(mg/kg)	0,05	6.07	5.12	5.18
Mo	(mg/kg)	0,3	0.5	0.4	0.5
EC	(µS/cm)		199	172	189
$N\text{-}NH_4$	(mg/kg)	1	10.2	3.0	3.6
$N\text{-}(NO_3+NO_2)$	(mg/kg)	0.5	15.8	4.0	3.5
$P\text{-}PO_4$	(mg/kg)	0.4	14.9	8.8	9.4
Cl	(mg/kg)		60.9	21.7	23.0
C-elementary	(g/kg)		46.4	47.3	45.2
N-elementary	(g/kg)		4.24	3.94	3.87
pH	20±1˚C		6.47	7.15	7.14

B0: non-cycled control soil; B6: soil cycled 6 times with sugar beet in absence of *R. solani* (conducive); BR6: soil cycled 6 times with sugar beet in presence of *R. solani* (suppressive). Det. lim. means detection limit.

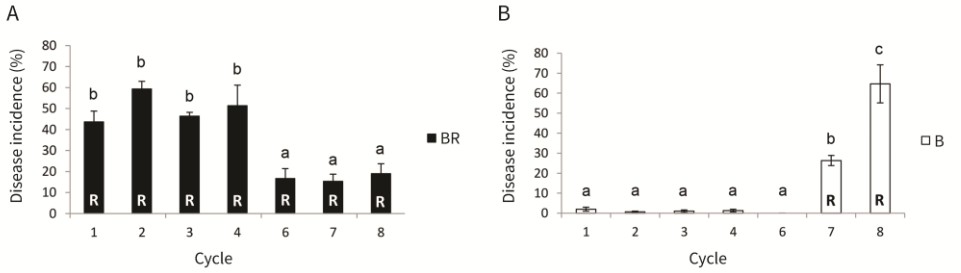

Figure 2. *Rhizoctonia* disease incidence of sugar beet seedlings during the induction of disease suppression. The percentage of sugar beet seedlings showing damping-off symptoms along the different cycles of growth for **A)** soil cycled with sugar beet in presence of *R. solani* (BR), and **B)** soil cycled with sugar beet only (B) was determined. Bars with a different letter indicate a significant difference ($p<0.05$) between the samples analyzed with analysis of variance and Hochsberg post hoc analysis. A letter R within the bars indicate treatments in which *R. solani* was inoculated.

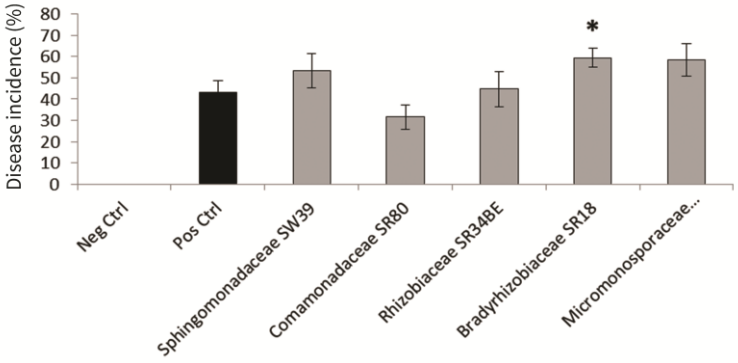

Figure 3. *In vivo Rhizoctonia* disease control on sugar beet seedlings by application of chitin-degrading isolates retrieved from suppressive soil. Percentage of diseased sugar beet seedlings due to *R. solani* after an initial application of 10^7 cells/seed of bacterial suspensions was compared with the positive control (Pos Ctrl) (no bacteria addition and *R. solani* inoculation), measured 21 days after *R. solani* inoculation. An asterisk indicates a significant difference ($p < 0.05$) with the control treatment calculated by analysis of variance and Dunnet's post-hoc analyses.

Temporal changes in rhizobacterial community composition during rebiosis

To identify other bacterial genera and mechanisms involved in disease suppressiveness, we investigated the compositional changes in the rhizobacterial community during the transition from the conducive to the disease suppressive state. Rhizospheric soil was collected from roots of 3-week-old sugar beet seedlings in growth cycles 1, 4, 7 and 8, and subjected to total DNA extractions followed by 16S rRNA Illumina Miseq sequencing (Fig. 1). After quality checks and trimming, 57.56 ± 0.93% of the reads were of sufficient quality and kept for further analyses (Table S2). Rarefaction curves showed that the sequencing coverage was acceptable up to genus level (Fig. S8). A total of 51,628 OTUs were detected. No significant differences were found in species richness (alpha diversity) between the rhizobacterial communities from the different growth cycles and from the two different treatments B and BR (Fig. S9). Analysis of the beta diversity revealed 37 different phyla, with the Acidobacteria, Proteobacteria, Bacteroidetes, Verrucomicrobia and Actinobacteria as the most prevalent (Fig. S10). Principal component analysis further showed that successive cycling of sugar beet seedlings had a significant impact on the rhizobacterial community composition, for both B and BR (Fig. 4, Fig. S11). The most significant shifts in rhizobacterial community composition were found between cycles 1 and 4 and between cycles 4 and 7. No significant differences were observed in rhizobacterial community composition between B7 and BR7 (p=0,442) and between B8 and BR8 (p=0,599) (Fig. 4, Table S3) despite the significant differences in phenotype (i.e. disease suppressiveness) between B and BR (Fig. 2). These results indicate that the taxonomic differences at family level between rhizobacterial communities of sugar beet seedlings grown in *Rhizoctonia* conducive or suppressive soil conditions were minor to insignificant.

To further investigate this at lower taxonomic levels, we zoomed in on differences at OTU level using two complementary approaches: i) identify taxonomic differences between suppressive and conducive soil at the same time points (comparing B vs BR), and ii) identify changes in the abundance of rhizobacterial OTUs during the transition from the conducive to the suppressive state, i.e. comparing different growth cycles within the BR treatment. With the first approach, we compared the rhizobacterial community composition between growth cycles B7 and BR7, and between B8 and BR8. In total, 7 OTUs were found at least two-fold more abundant in BR7 than in B7, and 7 OTUs more abundant in BR8 than in B8. These 14 OTUs belong to Bacteroidetes, Gemmatimonadetes, Proteobacteria, unclassified at phylum level and Candidate division OD1 (Fig. 5A, B). Three of these 14 OTUs were common between both time points and belonged to the

Bacteroidetes phylum: 2 OTUs were classified as *Sphingobacteriaceae* and 1 OTU as *Cryomorphaceae*. Looking more in detail into the dynamics of these 3 OTUs along the trajectory from the conducive to the suppressive state showed that their abundance was low in cycles BR1 and BR4 and then increased as the soil became suppressive (BR7 and BR8) (Fig. 5C-E). The abundance of these 3 OTUs showed no change in the conducive soil cycled with sugar beet only (B1 to B8) (Fig. 5C-E).

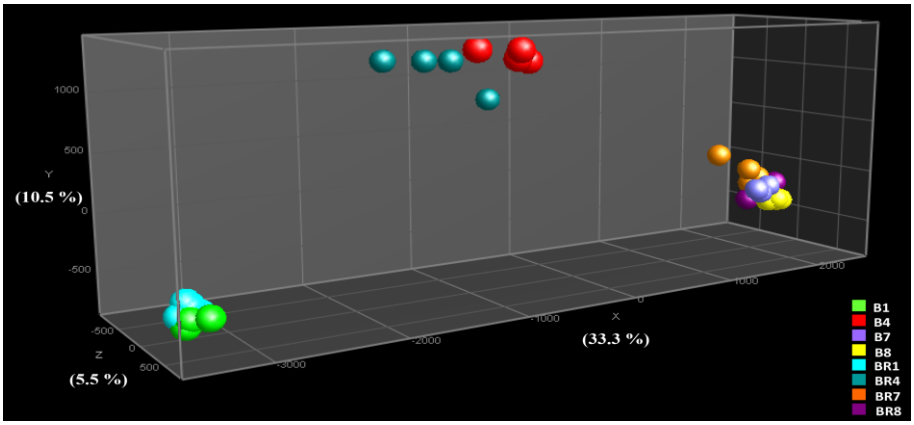

Figure 4. PCoA of the beta diversity of 16S amplicon data of the rhizosphere bacterial community collected at different cycles during induction of disease suppression. Principal component analysis (PCA) was calculated using the Bray-Curtis' coefficient. B: soil cycled with sugar beet in the absence of *R. solani* (remained conducive); BR: soil cycled with sugar beet in the presence of *R. solani* (became suppressive). The number following the treatment represents the cycle number. At the axes, the percentage of variation explained is indicated.

With the second approach, we investigated which bacterial taxa became more abundant during the transition from the conducive to the suppressive state in the BR treatment. No significant differences were observed in composition and abundance between the two cycles where the soil had become suppressive (BR7, BR8) (Fig. 4, Table S3). Therefore, we pooled the data of BR7 and BR8 and compared their rhizobacterial community composition to that of either BR1 or BR4. This comparison resulted in a total of 427 OTUs found in significantly higher abundance for both comparisons (Fig. S12). To exclude OTUs becoming more abundant due to successive growth of sugar beet only, similar analyses were adopted for the samples of the B treatment. Here, 395 OTUs were found in significantly higher abundance in B7+B8 vs B1 and B4. Matching the 395 OTUs that were more abundant in B7+B8 to the 427 OTUs more abundant in BR7+BR8 resulted in a total of 158 OTUs that were uniquely associated with the disease suppressive state of BR7+BR8. Most of these 158 OTUs belonged to Acidobacteria (23.9%), Proteobacteria (16.4%), Planctomycetes (10.7%), Bacteroidetes (10.1%) and Actinobaceria (6.9%) (Fig. S13, Table S4). We looked in more detail into the dynamics of the 10 OTUs (classified at family level) found most differentially abundant in suppressive soil compared to the conducive soil during rebiosis (Fig. S14), and found that the abundance of several of these OTUs increased as the soil became suppressive (BR7, BR8) whereas the abundance of these OTUs showed no or minor changes in abundance in the conducive soil cycled with sugar beet only (B1 to B8) (Fig. S14 A, D, E and I).

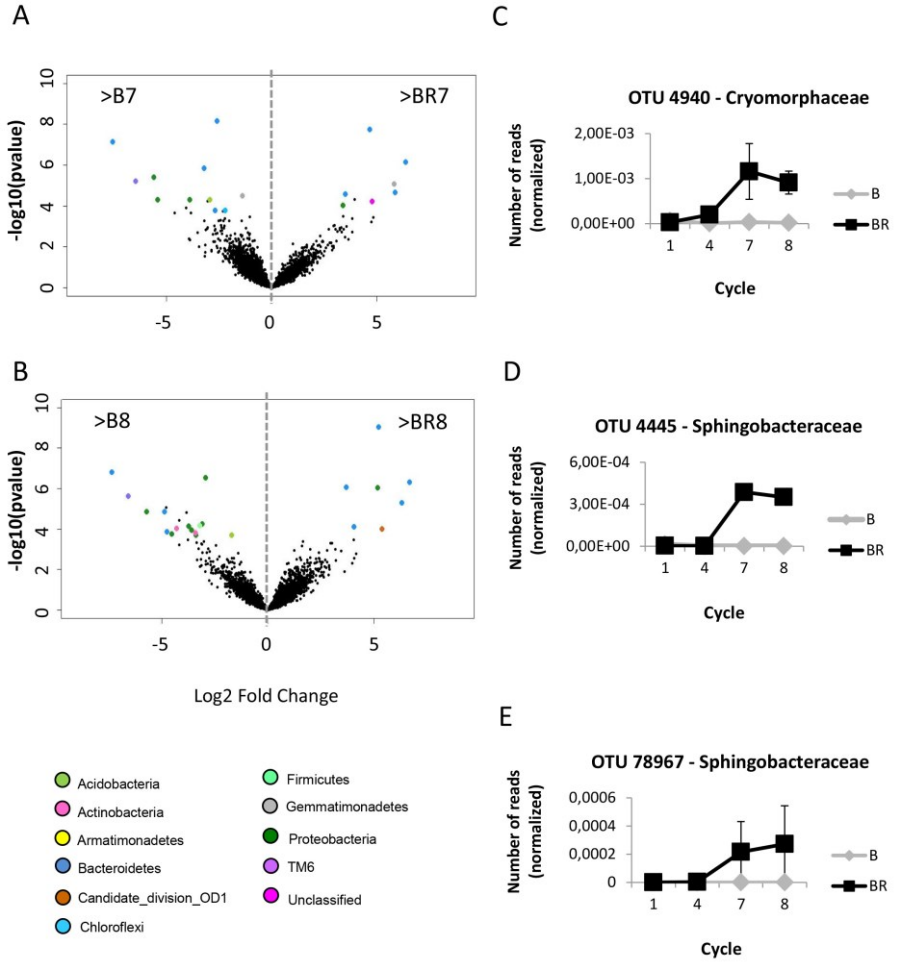

Figure 5. Differentially abundant OTUs between conducive and suppressive soil at the same cycling time points (approach A) and dynamics of the OTUs found in higher abundance in suppressive soil. A) Differential abundance of OTUs between conducive and suppressive soil at cycle 7, where >B7: OTUs found in higher abundance in treatment B (conducive) and >BR7: OTUs found in higher abundance in treatment BR (suppressive); B) Differential abundance of OTUs between conducive and suppressive soil at cycle 8, where >B8: OTUs found in higher abundance in treatment B (conducive) and >BR8: OTUs found in higher abundance in treatment BR (suppressive). Dots represent the different OTUs and those OTUs that appear in higher abundance (log2 fold change ≥ 1 and p-value < 0.05 calculated with Wald test) were colored based on the phylum. Only three OTUs were in common and in higher abundance in both suppressive time points (BR7 and BR8) compared with conducive time points (B7 and B8) and their dynamics are shown in panels C-E). C) OTU 4940, belonging to the *Cryomorphaceae* family (unclassified at genus level); D) OTU 4445, belonging to the *Sphingobacteraceae* family (unclassified at genus level); E) OTU 78967, belonging to the *Sphingobacteraceae* (genus *Fluviicola*).

Temporal changes in rhizobacterial functions during rebiosis

To get insight into the functions expressed in the rhizobacterial community members during transition from the conducive to the suppressive state, total RNA of rhizosphere soils was extracted, depleted of eukaryotic RNA and 16S rRNA followed by Illumina HiSeq sequencing (Fig. 1). After quality checks and trimming, 84.80 ± 0.64% of the reads with a length of 60-101 nucleotides remained (Table S5). After de-replication to remove artificial duplicate reads (ADRs), the putative and classified rRNA reads represented 17.10 ± 1.07% of the sequences. Non rRNA sequences were submitted to pORF prediction and 78.65 ± 0.83% were predicted as protein coding regions of which 19.46 ± 0.37% of the reads had a predicted protein feature, and 74.03 ± 0.84% of those predicted proteins were successfully assigned to functional categories. Among all the reads with predicted proteins and rRNA, 22.27 ± 1.44% were assigned to Eukarya (Table S5).

The obtained RNA transcripts were annotated with the SEED Subsystem database in MG-RAST and exported into a BIOM file to be analyzed in Rstudio. Rarefaction curves showed that the coverage depth was acceptable until level 3 of classification (Fig. S15). The successive cycling had a significant impact on the abundance levels of the functions (Fig. 6). In contrast to the taxonomic analyses (Fig. 4), a statistically significant separation was observed for the RNA transcripts between the conducive and suppressive soils (B7 *vs* BR7 and B8 *vs* BR8) (Table S3). To study these functional changes in more depth, the two approaches used for the taxonomic analyses were also used here: i) identify functional differences between suppressive and conducive soil at the same time points, i.e. comparing B vs BR, and ii) identify changes in the abundance of specific functions during the transition from the conducive to the suppressive state, i.e. comparing growth cycles within the BR treatment. Numerous functions covering multiple categories were identified (Fig. S16-S19). We observed an increase in general microbial activity in the suppressive soil, reflected by an increase in functions associated with primary metabolism (metabolism of amino acids, carbohydrates, fatty acids, proteins, DNA and RNA, nucleosides and nucleotides, aromatic compounds, nitrogen and phosphorous) and transcripts involved in cell division, cell cycle, cell wall generation and respiration (Table S6). We also observed an increase in functions associated with colonization, including motility, quorum sensing and biofilm formation (Table S6). Next to this, we observed an increase of transcripts previously reported for mechanisms associated with disease suppression (Kloepper et al., 1980; Cook et al., 1995; Howell, 2003; Haas and Defago, 2005; Lemanceau et al., 2006; Junaid et al., 2013; Jambhulkar et al., 2015), including acquisition and metabolism of iron (siderophore

production), secondary metabolism (including lytic enzymes, auxin metabolism), membrane transport, sulfur metabolism, and virulence and resistance to antibiotics and toxic compounds (including genes involved in HCN production) (Table S6). Also several transcripts associated with stress responses, including osmotic, acidic and oxidative stress, were up-regulated in the suppressive soil (Table S6). A limited number of functional categories comprising transcripts were found more abundant in suppressive soil using both approaches (Table S6). One of these functional categories included stress responses, a finding that is consistent with earlier results of Chapelle et al. (2015).

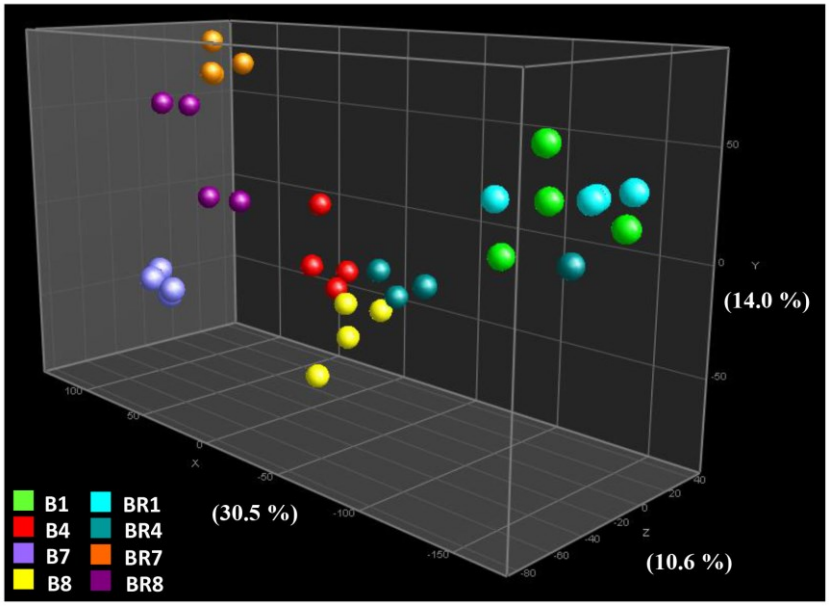

Figure 6. PCoA of the beta diversity of transcriptome data of the rhizosphere bacterial community collected at different cycles during induction of disease suppression. Principal component analysis (PCA) was calculated using the Bray-Curtis' coefficient. B: soil cycled with sugar beet in absence of *R. solani* (remained conducive); BR: soil cycled with sugar beet in presence of *R. solani* (became suppressive). The number following the treatment represents the cycle number. At the axes, the percentage of variation explained is indicated.

Involvement of oxidative and acidic stress responses in disease suppressiveness

Since oxidative and acid stress responses were also identified as potential mechanisms in another *Rhizoctonia* suppressive soil (Chapelle et al., 2015), we focused on these functions in more detail. Up-regulated transcripts within the stress response were mainly associated with regulation of oxidative stress, and to a lesser extent with acid resistance mechanisms (Fig. 7A) and RNA polymerase sigma factors (Fig. 7B, Table S6). The dynamics of transcripts associated with the regulation of oxidative stress revealed that their levels increased as the soil became suppressive (BR7, BR8) compared with the time points where the soil was conducive (BR1, BR4); no changes in their transcript levels were found for the conducive soil cycled with sugar beet only (B) (Fig. 7C). The dynamics of the transcripts involved in acid resistance mechanisms were relatively high in the conducive soil and then decreased as the infection progressed (Fig. 7D). In the soil that became suppressive, however, there was a decrease of these transcripts until cycle 7, followed by an increase in cycle 8 (Fig. 7D). Chapelle et al. (2015) proposed that the oxidative and acid stress responses are triggered by *R. solani via* the production of oxalic acid. Looking more into this mechanism, we found a higher number of transcripts of the alpha subunit of the formate dehydrogenase (Fig S20A and B, Table S6), an enzyme involved in metabolism of formate, a degradation product from oxalate (Kersters and Vancanneyt, 1984; Svedružić et al., 2005). We further observed an increase of transcripts of the formate dehydrogenase gene as the soil became suppressive, whereas no increase was observed for the soil that remained conducive (Fig. S20C). This suggests that degradation of oxalate produced by *R. solani* may be one of the first steps of the bacterial community response to invasion of the rhizosphere by the pathogen (Fig. S20).

We observed a higher number of transcripts associated with dispersal through flagella (like FlhD and RpoD), production of exopolysaccharides and peptidoglycans, and biofilm formation (Table S6). We also detected an increase of transcripts involved in the acquisition of carbohydrates and iron (Table S6). For iron, we observed an increase in transcripts associated with iron acquisition and transport (Fig. S21A), more specifically in TonB receptors and protein B (Fig. S21B). The dynamics of the transcripts involved in transport of iron in the soil that became suppressive were relatively constant and higher than in the conducive soil. Only in cycle 4, transcripts involved in transport of iron were similar to the levels detected in the conducive soil (Fig. S21C).

Other genes up-regulated in the suppressive soil were those involved in the production of HCN, lytic enzymes such as endoglucanases, enzymes involved in the metabolism of sulfur compounds, and multidrug efflux pumps involved in the secretion of

secondary metabolites (Table S6). Moreover, we found that transcripts associated with indole-3-acetic acid (IAA) were also increased. IAA is a plant growth hormone but also a signal molecule involved in the regulation of several processes indicated above, such as exopolysaccharide production, biofilm formation, motility, resistance and iron acquisition (Mueller et al., 2009) (Table S6).

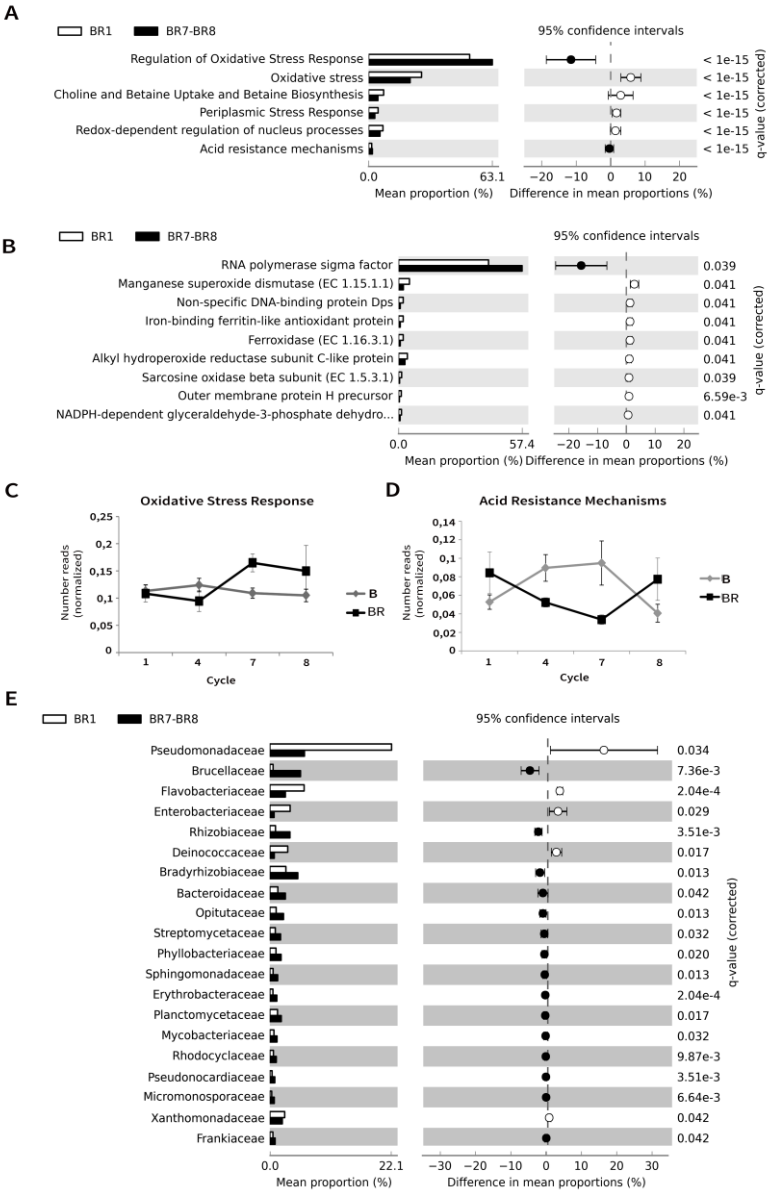

Figure 7. Abundance of transcripts involved in stress response, their dynamics, and assigned bacterial taxa in suppressive soil during suppressiveness induction (BR7+BR8 vs BR1; approach B). **A)** transcripts differentially expressed at level 3; **B)** transcripts differentially expressed at level 4; **C)** dynamics of the transcripts involved in oxidative stress response during cycling; **D)** dynamics of the transcripts involved in acid resistance mechanismsduring cycling; **E)** top 20 most active bacterial taxa associated with the transcripts of stress response that were more abundant in suppressive (black) and in conducive (white) soil.

Merging taxonomy and functions

To unravel which bacterial groups are connected to the up-regulated functions in the suppressive soil, we performed taxonomic assignments (based on highest blast hit in the M5NR database) of the transcripts for each of the different functional categories. Transcripts associated with stress responses were predominantly assigned to the families *Bradyrhizobiaceae*, *Rhizobiaceae*, *Brucellaceae*, *Phyllobacteriaceae*, *Sphingomonadaceae*, *Erythrobacteraceae*, *Rhodocyclaceae*, *Frankiaceae*, *Micromonosporaceae*, *Mycobacteriaceae*, *Pseudonocardiaceae Streptomycetaceae*, *Bacteroidaceae*, *Planctomycetaceae,* and *Opitutaceae* (Fig. 7E). Several of these bacterial families (i.e. *Rhizobiaceae*, *Bradyrhizobiaceae*, *Phyllobacteriaceae*) were also found associated with the upregulated transcripts for the formate dehydrogenase and iron acquisition (Fig. S20D and S21D).

Taxonomic affiliations at functional level 1 of all transcripts of 26 functional categories found in higher abundance in the suppressive soil revealed that mainly Alphaproteobacteria (*Rhizobiaceae*, *Bradyrhizobiaceae*, *Phyllobacteriaceae*, *Sphingomonadaceae*, *Brucellaceae*, *Erythrobacteraceae*, *Caulobacteraceae*), Betaproteobacteria (*Comamonadaceae*, *Rhodocyclaceae*), Deltaproteobacteria (*Polyangiaceae*), Actinobacteria (*Frankiaceae*, *Micromonosporaceae*, *Mycobacteriaceae*, *Nocardioidaceae*, *Pseudonocardiaceae*, *Streptomycetaceae*) and Verrucomicrobia (*Opitutaceae*) were the most active taxa for the comparison BR1 vs BR7+BR8 (Fig. 8). For the comparison BR4 vs BR7+BR8, *Oxalobacteraceae* were found as the most active bacterial family (Fig. S22). Furthermore, several of these bacterial families, in particular *Phyllobacteriaceae*, *Sphingomonadaceae*, *Caulobacteraceae*, *Opitutaceae*, *Micromonosporaceae*, *Rhodocyclaceae*, *Nocardioidaceae*, *Streptomycetaceae*, *Polyangiaceae*, *Pseudonocardiaceae*, and *Planctomycetaceae,* were not only more active, but also more abundant based on the 16S-based analysis (Fig. 5, Fig. S13, Table S4).

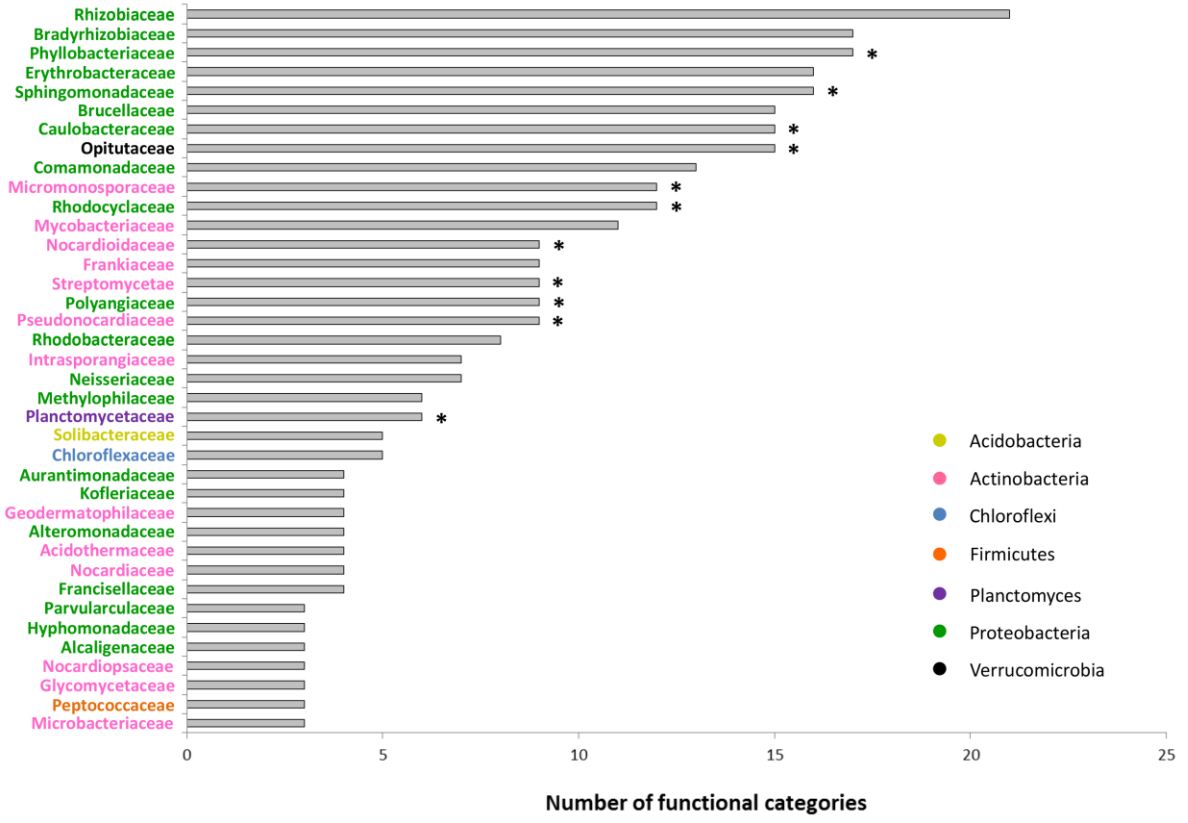

Figure 8. Summary of bacterial families activated in suppressive soil during suppressiveness induction for the functions found up-regulated in suppressive soil. Bacterial taxa were assigned to all transcripts found in higher abundance in suppressive than conducive soil for each functional category and for both approaches. The total number of functions (level 3) was counted per bacterial family for the suppressive soil (BR7+BR8) compared with conducive soil (BR1). An asterisk indicates those families that are not only more active but also more abundant in suppressive soil, based on 16S amplicon data analyses (approaches A and B).

Discussion

For several fungal root pathogens, specific disease suppression is typically induced in the field during continuous cultivation of the susceptible host plant followed by a disease outbreak (Postma et al., 2010; Raaijmakers and Mazzola, 2016). Here we showed that the induction of disease suppressiveness against *R. solani* can be mimicked under controlled conditions by repeated cropping of sugar beet in presence of the pathogen. This suggests that the fungal pathogen, its cell wall constituents or metabolites released in interactions with the plant root trigger responses in the indigenous bacterial community leading to suppression of infection. Suppressiveness induction upon chitin amendments has been previously reported (Henis et al., 1967; Cretoiu et al., 2014; Postma and Schilder, 2015). It was postulated that chitin stimulates the activities of chitinolytic microbes leading to degradation of the fungal cell wall (Hjort et al., 2007). To test the relative importance of this general defense mechanism, we isolated and characterized various chitinolytic bacteria from the induced suppressive soil. However, none of the five strains tested here was able to control *Rhizoctonia* damping-off disease. These results oppose to results of previous studies that have shown that addition of chitinolytic bacteria to soil could reduce disease caused by *F. oxysporum* (Hariprasad et al., 2011; Abdallah et al., 2016), *M. incognita* (Yang et al., 2014; Lee et al., 2015) or *R. solani* (Park et al., 1995). In these studies, they proposed that additional mechanisms acted synergistically with the chitinolytic activity of the isolates. These additional mechanisms included proteolytic, pectinolytic or gelatinolytic activities or hydrogen cyanide (HCN) production.

Most dynamic bacterial taxa

Our study showed that the species richness did not differ significantly between conducive and suppressive soil conditions, that is, the total number of bacterial taxa as well as their presence/absence was consistent between both soil phenotypes and among the different plant growth cycles. The abundance levels of specific taxa were mainly affected by successive cycling, supporting and extending results shown previously for soils suppressive against the take-all pathogen *G. graminis* (Schreiner et al., 2010). Exudates released by plant roots act as microbial chemoattractant and have been proposed as a mechanism of plants to recruit beneficial bacteria for protection against soil-borne pathogens (Lakshmanan et al., 2014; Cha et al., 2015). Assessment of changes in the rhizobacterial community revealed that only few OTUs were more abundant in suppressive than conducive soil. These OTUs belonged to Acidobacteria, Proteobacteria, Planctomyces,

Bacteroidetes and Actinobacteria. Especially members of the Bacteriodetes, i.e. *Sphingobacteriaceae* and *Cryomorphaceae,* stood out as their dynamics correlated well with the dynamics of disease suppressiveness. These results extend previous studies that suggested a role of *Sphingobacteraceae* in suppressiveness to *Rhizoctonia solani* (Chapelle et al., 2015; Huang et al., 2016).

Most dynamic bacterial functions

We observed a distinct separation in RNA transcript levels between the conducive and the suppressive soil, suggesting that the induced suppressiveness is mainly due to changes in functions rather than changes in community composition. Transcripts up-regulated in suppressive soil were grouped in two main categories. The first group contains transcripts involved in primary metabolism, i.e. metabolism of amino acids, carbohydrates, fatty acids, proteins, DNA and RNA, nucleosides and nucleotides, aromatic compounds, nitrogen and phosphorous as well as transcripts involved in cell division, cell cycle, cell wall generation and respiration. The second group harbors transcripts of genes and functions putatively associated with disease suppression.

When analyzing in more detail the transcripts up-regulated in suppressive soil, we observed a high similarity with the model proposed by Chapelle et al. (2015). We observed that the successive cycling of the host plant had a strong effect in shaping the rhizobacterial community, most probably driven by the root exudates. Secondly, we found an increase of transcripts involved in stress responses, including oxidative, acidic, osmotic and periplasmic stress. Stress responses can be considered as the first step activated in microbial populations upon pathogen invasion of the rhizosphere environment (Casadevall, 2007). These changes in turn lead to changes in microbial community composition and/or activities. Oxalic acid produced by *R. solani* (Nagarajkumar et al., 2005) is involved in virulence (Maxwell, 1970; Stone and Armentrout, 1985; Dutton and Evans, 1996) and proposed here as one of the fungal metabolites that drives the observed changes in gene expression in the rhizobacterial community. This hypothesis is supported by an increased number of transcripts in the suppressive soil encoding enzymes involved in the catabolism of oxalate. Interesting to mention is that Bravo et al. (2013) showed that oxalotrophic bacteria were able to disperse by using *Trichoderma* fungal hyphae to reach oxalate sources. Oxalic acid is also used as a signal molecule by different mycophagous bacteria to sense and feed on fungi (Rudnick et al., 2015). Transcripts associated with the acid stress response were also found increased in suppressive soil, although their dynamics

was not consistent with the dynamics of disease suppressiveness. Microbes can protect themselves against acid stress by glutamate-dependent, arginine-dependent or oxidative mechanisms (Castanié-Cornet et al., 2010). Among the up-regulated transcripts in suppressive soil were enzymes involved in both the arginine-dependent and the glutamate-dependent systems, suggesting that different acid resistance systems are taking place during suppressiveness induction.

Niche exclusion by root colonization has been described as a mechanism to suppress fungal invasion of the rhizosphere and plant disease (Bull et al., 1991). A number of functions associated with competitive root colonization were indeed upregulated in the suppressive soil, including motility and biofilm formation. Biofilm formation on the root surface will allow a stronger and more stable symbiotic association between the microbes and the plant (Fujishige et al., 2006). Bacteria being part of a biofilm will communicate more effectively with each other and will be more resistant to predators, chemicals and antibiotics than planktonic cells (Gilbert et al., 1997; Decho, 2000;Soto, 2013). Related to the antimicrobial resistance of biofilms, we observed an increase of transcripts in multidrug efflux pumps that can expel a broad range of antibiotics (Soto, 2013; Sun et al., 2014). Combined with the finding that transcripts involved in iron acquisition were found in higher abundance in the suppressive soil, we postulate that niche exclusion and siderophore-mediated competition for iron contribute to soil suppressiveness to *R. solani*. Other potential mechanisms of *R. solani* suppression include production of antimicrobial secondary metabolites, including hydrogen cyanide, endoglucanases and sulfur-containing metabolites. For this latter group of metabolites, the sulfur-containing volatile compounds such as dimethyl disulphide and dimethyl trisulfide are possible candidates as they are known to have strong antifungal activities (Schmidt et al., 2015).

Transcripts involved in auxin (indole-3-aceic acid (IAA)) biosynthesis were also enhanced in the suppressive soil. Auxin produced by bacteria can act as signal molecules (Spaepen and Vanderleyden, 2011; Sukumar et al., 2013), activating genes involved in biofilm formation, motility, protozoan grazing resistance and iron utilization and transport (Mueller et al., 2009; Spaepen and Vanderleyden, 2011). Thus, the increased motility, biofilm formation and iron acquisition may be triggered by the enhanced auxin levels.

Most important bacterial families: 'meta'-analysis of *Rhizoctonia* suppressive soils

In an attempt to identify specific bacterial families playing a key role in suppression of *R. solani*, we did a comparative analysis of bacterial families more abundant and more active

in the *Rhizoctonia* suppressive soil used in this study and in a different soil also suppressive against *R. solani* (Mendes et al., 2011; Chapelle et al., 2015). *Sphingomonadaceae* appeared as the sole bacterial family that was both more abundant and more active in both suppressive soils. The bacterial families *Micromonosporaceae*, *Nocardioidaceae*, *Pseudonocardioidaceae*, *Streptomycetaceae*, *Planctomycetaceae*, *Caulobacteraceae*, *Phyllobacteriaceae*, *Rhodocyclaceae*, *Polyangiaceae* and *Opitutaceae* were found as more abundant in both suppressive soils and more active in this study but not in Chapelle et al. (2015). Important to mention is that several bacterial families detected more active for the majority of functions up-regulated in this study were not found as more active for the majority of functions up-regulated in (Chapelle et al., 2015). However, certain of these bacterial families were found more active only for specific functions suggested to be involved in disease suppression, and these included *Caulobacteraceae, Rhodocyclaceae*, *Phyllobacteraceae*, *Brucellaceae* and *Micromonosporaceae* (Chapelle et al., 2015; see Fig. S4) (Table S7).

Conclusions

In this study, we showed that disease suppressiveness can be induced reproducibly in small-scale bioassays under controlled conditions and that the presence of the pathogen is essential in this process. We showed that the disease suppressive state correlates with an enhanced rhizobacterial activity rather than with significant shifts in bacterial community composition. Identifying functional biomarkers provides more complete information about the interactions occurring between microbes, which are, in turn, essential to understand microbial ecology (Gilbert et al., 2016). Thus, research on disease suppressiveness of soils should combine taxonomic and functional analyses to unravel the complexity of the underlying mechanisms. In future experiments, we will isolate several of the identified active bacterial families to validate if the proposed genes and functions are indeed involved in the early stages of soil suppressiveness to damping-off disease caused by *R. solani*.

Author contributions

RGE, IdB, JP and JMR were involved in the design of the experiments. RGE performed the experiments, analyzed the data and created the figures and drafted the manuscript. AJ performed the bioassay on the transferability of disease suppression. IdB, JP and JMR contributed to the writing of the manuscript. All authors revised the manuscript.

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Supplementary material

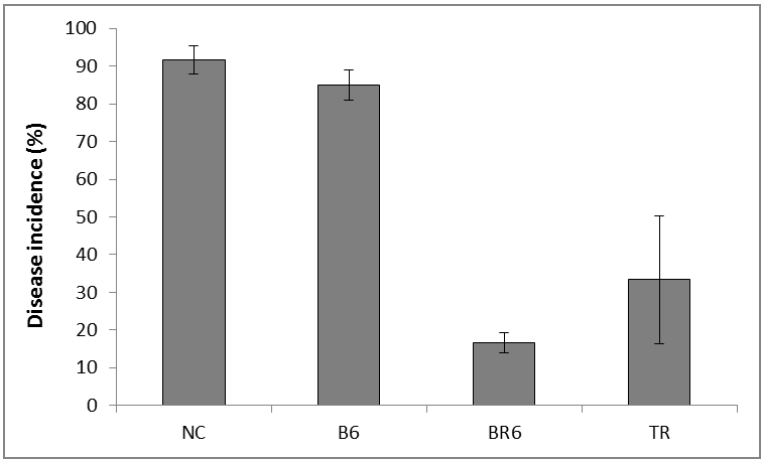

Figure S1. *Rhizoctonia* disease incidence in a preliminary bioassay of sugar beet seedlings grown in soil for the 6th cycle of growth. Percentage of sugar beet seedlings suffering from damping-off symptoms for B6: soil cycled with sugar beet in absence of *R. solani* for 5 cycles of growth and in presence of *R. solani* during cycle 6 (conducive); BR6: soil cycled with sugar beet beet in presence of *R. solani* for six cycles of growth (suppressive); NC: non-cycled control during the first 5 cycles of growth and introduced for the first time at cycle six by growing sugar beet in presence of *R. solani*; TR: 90% of non-cycled conducive soil amended with 10% of induced suppressive soil used to growth sugar beet in presence of *R. solani*.

Table S1. Taxonomy of the chitinolytic bacterial isolates obtained from the induced suppressive soil.

Phylum	Class	Order	Family	Genus	# isolates
Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	*Microbacterium*	5
				unclassified	1
			Micrococcaceae	*Arthrobacter*	2
			Promicromonosporaceae	*Cellulosimicrobium*	3
		Micromonosporales	Micromonosporaceae	unclassified	2
		Streptomycetales	Streptomycetaceae	*Streptomyces*	20
Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	*Dyadobacter*	1
	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	*Chryseobacterium*	1
Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	26
			Paenibacillaceae	*Paenibacillus*	4
			Planococcaceae	*Lysinibacillus*	2
				Psychrobacillus	1
				Solibacillus	1
Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	unclassified	2
			Hyphomicrobiaceae	*Devosia*	1
			Rhizobiaceae	*Shinella*	1
		Sphingomonadales	Sphingomonadaceae	*Novosphingobium*	1
				Sphingobium	12
				Sphingomonas	1
				Sphingopyxis	3
	Betaproteobacteria	Burkholderiales	Comamonadaceae	*Delftia*	3
				Variovorax	2
				unclassified	6
	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	9
		Xanthomonadales	Xanthomonadaceae	*Pseudoxanthomonas*	12
				Stenotrophomonas	8

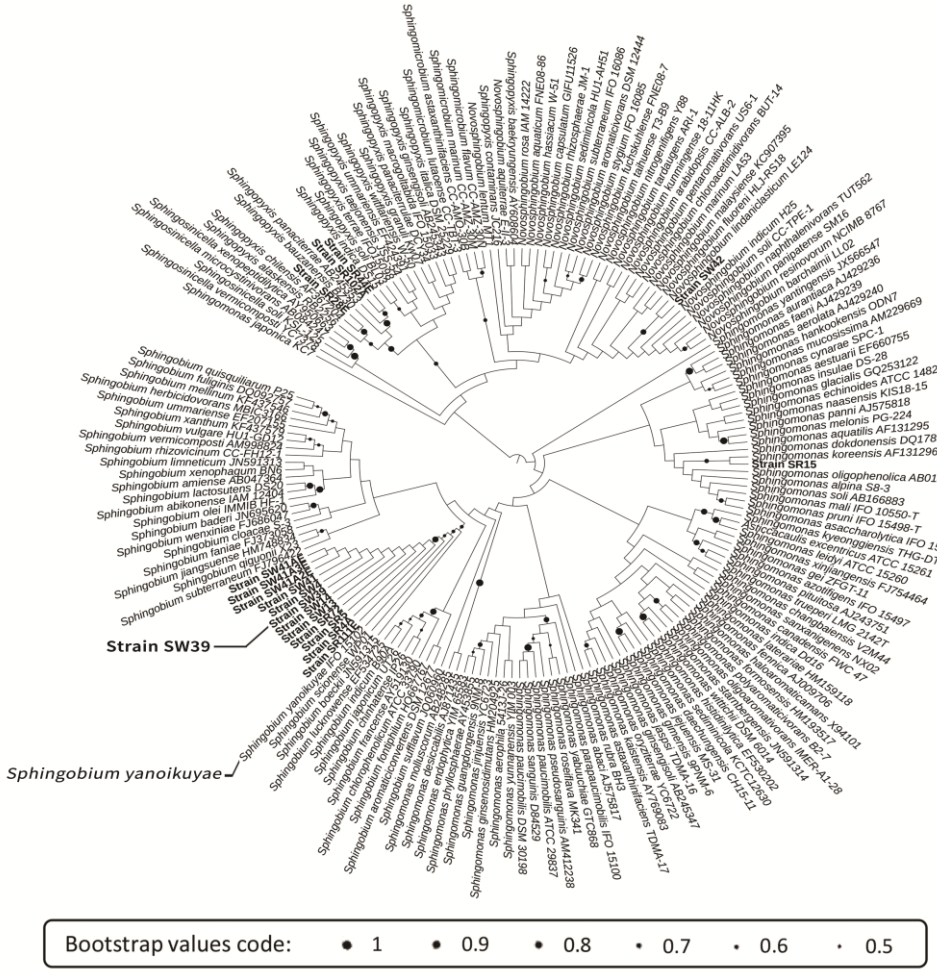

Figure S2. Phylogenetic tree of the 16S ribosomal gene (16S rRNA) of the chitin-degrading isolates retrieved from suppressive soil belonging to the *Sphingomonadaceae* and the type strains within the family *Sphingomonadaceae*. The phylogenetic relationship of the 16S rRNA sequences of the isolates within the *Sphingomonadaceae* (in bold) and the 16S rRNA sequences of the type strains (in italics) obtained from the Ribosomal Database Project (RDP) was inferred by alignment with Muscle and neighbor-joining tree construction with 1,000 resampled data sets. The black dots at the nodes indicate the level of bootstrap support higher than 0.5. The type strains are indicated with the strain name followed by the ID number. Highlighted are the strain selected for the *in vivo* assay (SW39) and its closest type strain (*Sphingobium yanoikuyae*).

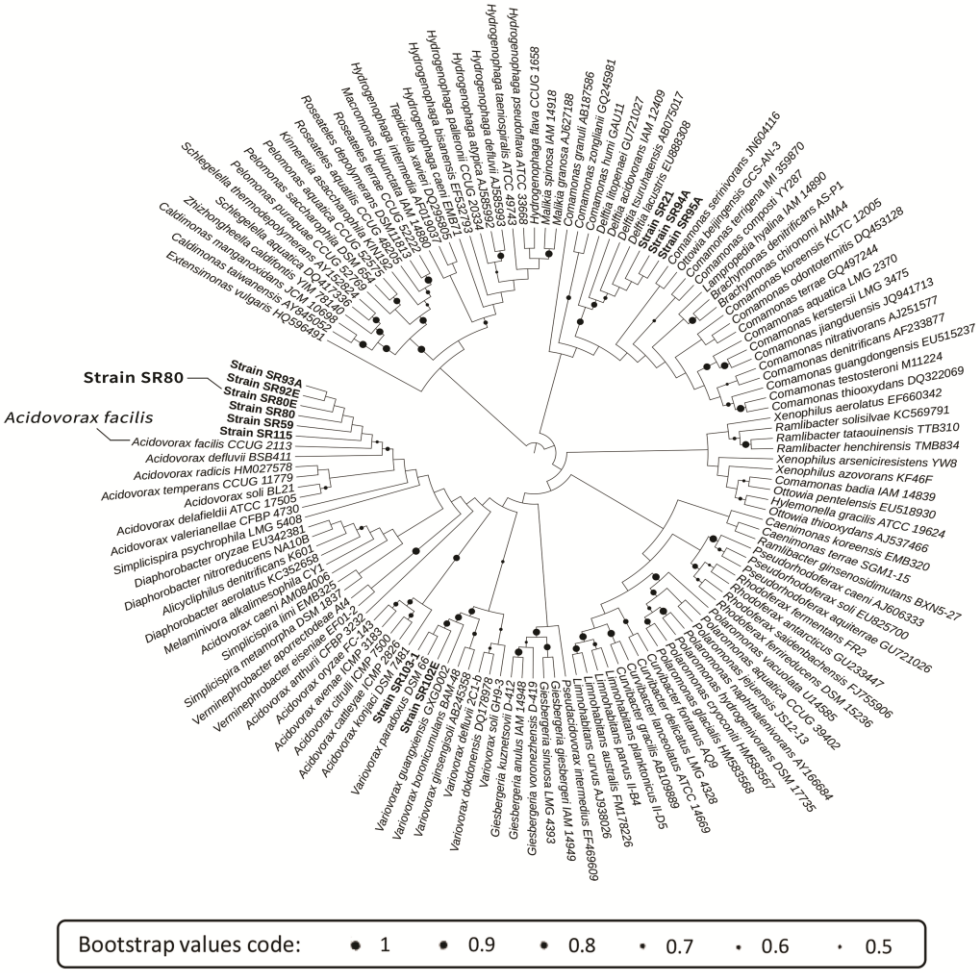

Figure S3. Phylogenetic tree of the 16S ribosomal gene (16S rRNA) of the chitin-degrading isolates retrieved from suppressive soil belonging to the *Comamonadaceae* and the type strains within the family *Comamonadaceae*. The phylogenetic relationship of the 16S rRNA sequences of the isolates within the *Comamonadaceae* (in bold) and the 16S rRNA sequences of the type strains (in italics) obtained from the Ribosomal Database Project (RDP) was inferred by alignment with Muscle and neighbor-joining tree construction with 1,000 resampled data sets. The black dots at the nodes indicate the level of bootstrap support higher than 0.5. The type strains are indicated with the strain name followed by the ID number. Highlighted are the strain selected for the *in vivo* assay (SR80) and its closest type strain (*Acidovorax facilis*).

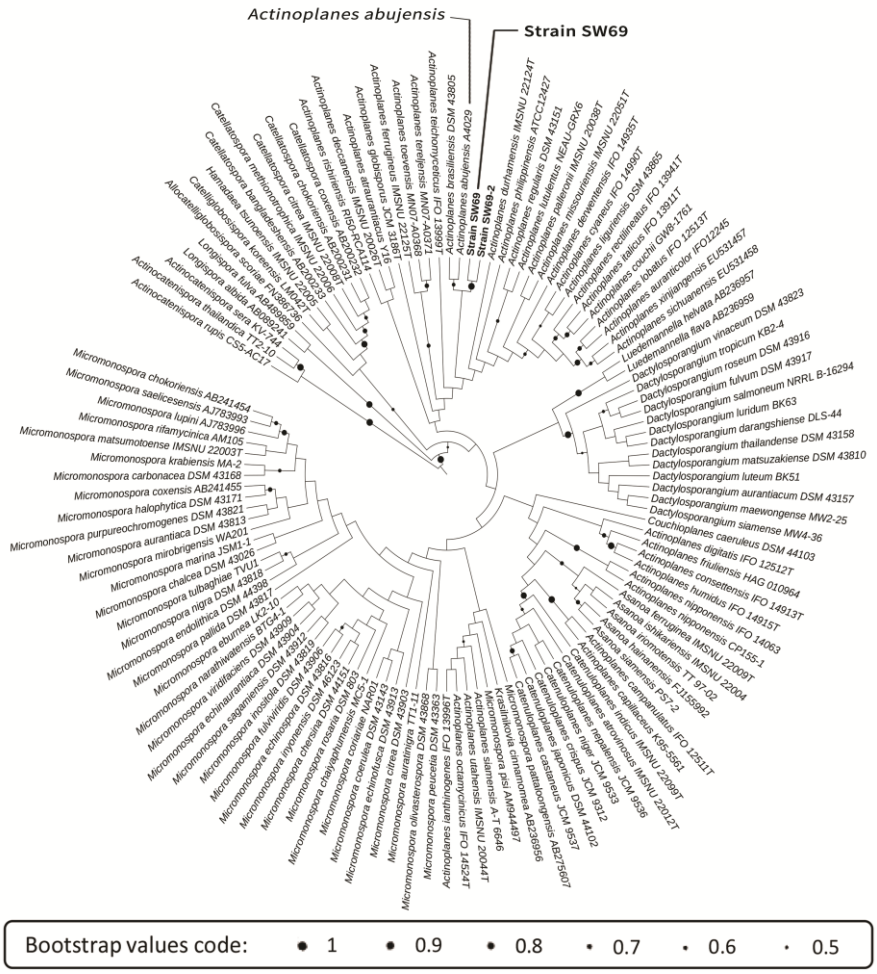

Figure S4. Phylogenetic tree of the 16S ribosomal gene (16S rRNA) of the chitin-degrading isolates retrieved from suppressive soil belonging to the *Micromonosporaceae* and the type strains within the family *Micromonosporaceae e*. The phylogenetic relationship of the 16S rRNA sequences of the isolates within the *Micromonosporaceae* (in bold) and the 16S rRNA sequences of the type strains (in italics) obtained from the Ribosomal Database Project (RDP) was inferred by alignment with Muscle and neighbor-joining tree construction with 1,000 resampled data sets. The black dots at the nodes indicate the level of bootstrap support higher than 0.5. The type strains are indicated with the strain name followed by the ID number. Highlighted are the strain selected for the *in vivo* assay (SW69) and its closest type strain (*Actinoplanes abujensis*).

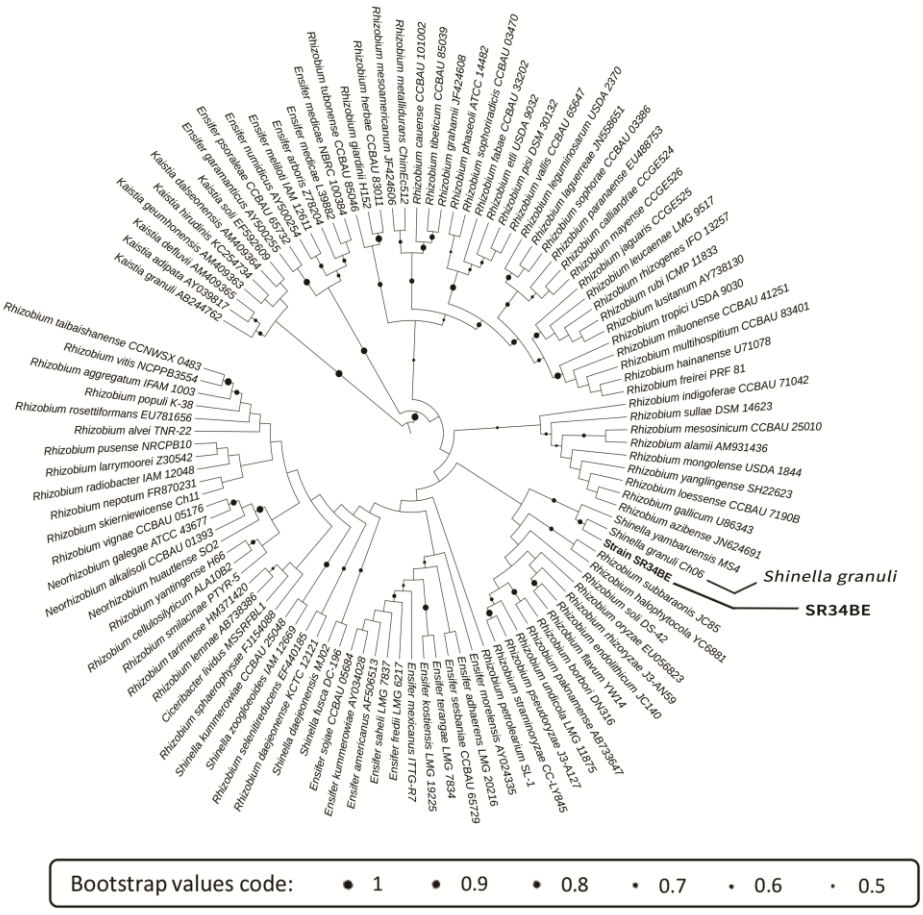

Figure S5. Phylogenetic tree of the 16S ribosomal gene (16S rRNA) of the chitin-degrading isolates retrieved from suppressive soil belonging to the *Rhizobiaceae* and the type strains within the family *Rhizobiaceae*. The phylogenetic relationship of the 16S rRNA sequences of the isolates within the *Rhizobiaceae* (in bold) and the 16S rRNA sequences of the type strains (in italics) obtained from the Ribosomal Database Project (RDP) was inferred by alignment with Muscle and neighbor-joining tree construction with 1,000 resampled data sets. The black dots at the nodes indicate the level of bootstrap support higher than 0.5. The type strains are indicated with the strain name followed by the ID number. Highlighted are the strain selected for the *in vivo* assay (SR34BE) and its closest type strain (*Shinella granuli*).

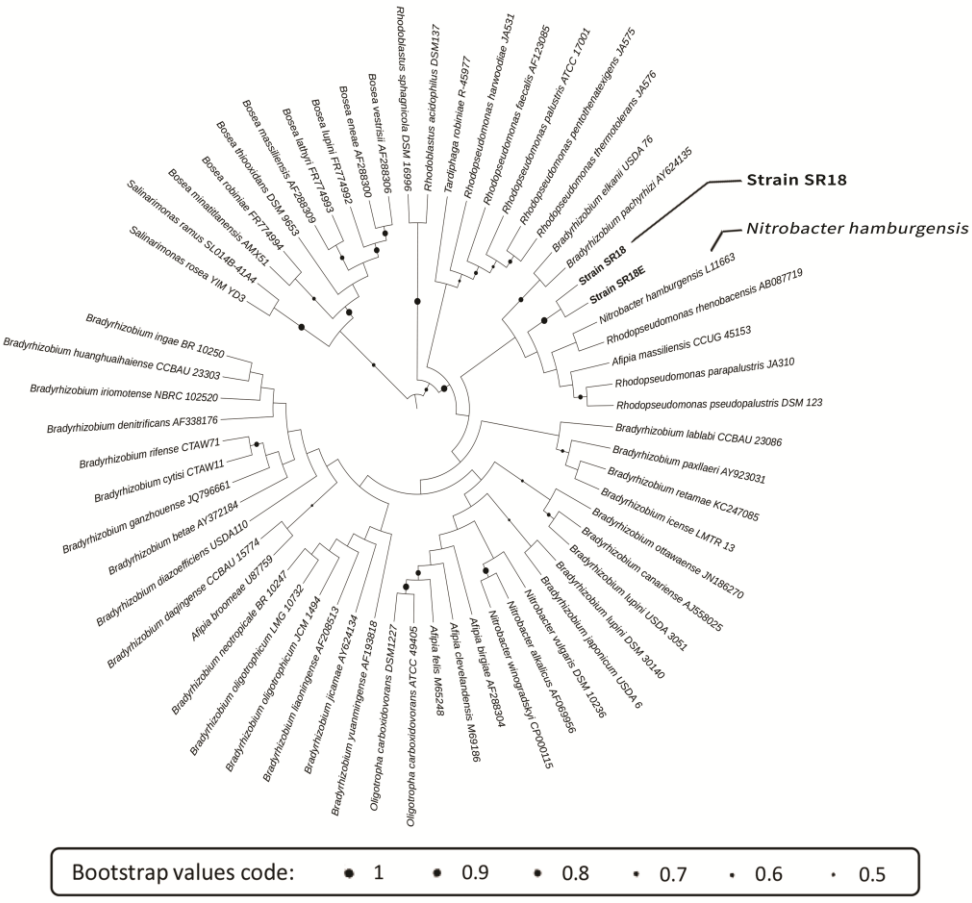

Figure S6. Phylogenetic tree of the 16S ribosomal gene (16S rRNA) of the chitin-degrading isolates retrieved from suppressive soil belonging to the *Bradyrhizobiaceae* and the type strains within the family *Bradyrhizobiaceae*. The phylogenetic relationship of the 16S rRNA sequences of the isolates within the *Bradyrhizobiaceae* (in bold) and the 16S rRNA sequences of the type strains (in italics) obtained from the Ribosomal Database Project (RDP) was inferred by alignment with Muscle and neighbor-joining tree construction with 1,000 resampled data sets. The black dots at the nodes indicate the level of bootstrap support higher than 0.5. The type strains are indicated with the strain name followed by the ID number. Highlighted are the strain selected for the *in vivo* assay (SR18) and its closest type strain (*Nitrobacter hamburgensis*).

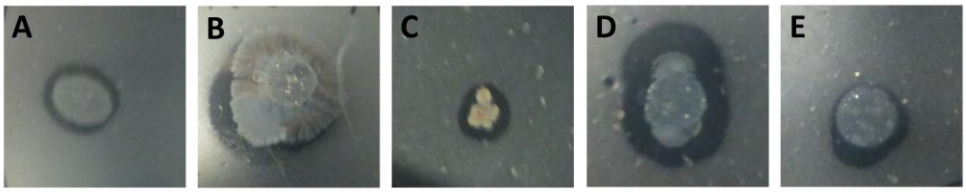

Figure S7. *In vitro* degradation of chitin by the selected bacterial isolates retrieved from suppressive soil. Halos around the bacterial colonies represent degradation of chitin, after 6 weeks of incubation at 25˚C for A) *Sphingomonadaceae* SW39; B) *Comamonadaceae* SR80; C) *Micromonosporaceae* SW69; D) *Rhizobiaceae* SR34BE; E) *Bradyrhizobiaceae* SR18.

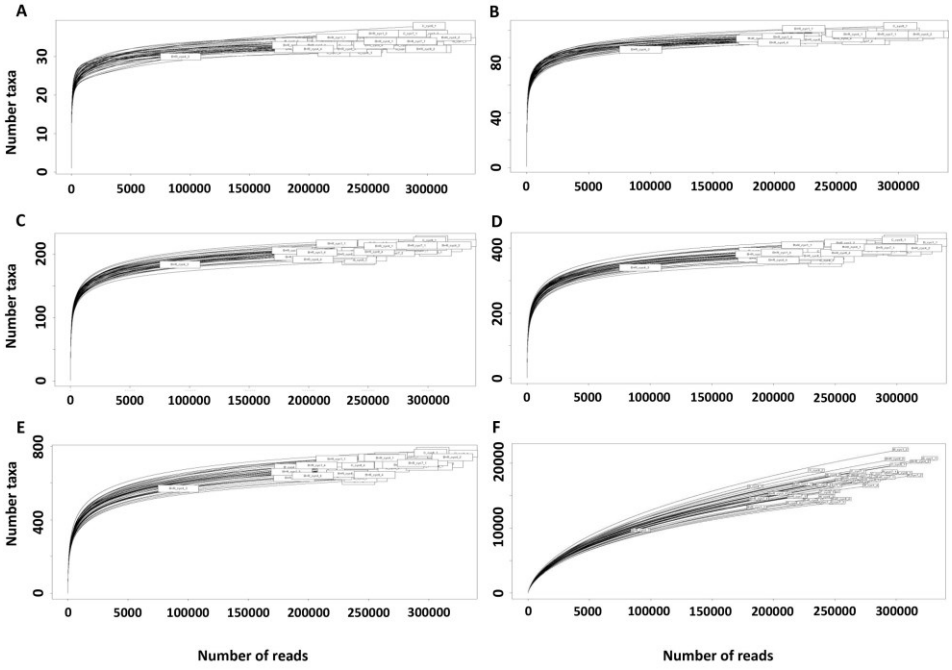

Figure S8. Rarefaction curves representing the depth in coverage of the 16S amplicon sequencing at different taxonomic levels. A) phylum level; B) class level; C) order level; D) family level; E) genus level; F) OTU level.

Table S2. Summary of the number of reads obtained from the 16S amplicon sequencing and after the processing and filtration steps.

Sample	Num raw reads ($x10^5$)	Num reads after quality check ($x10^5$)	Num reads after processing ($x10^5$)
B1_1	5.21	3.27	3.25
B1_2	4.87	3.03	3.00
B1_3	4.67	2.49	2.47
B1_4	5.13	2.79	2.77
B4_1	4.16	2.65	2.64
B4_2	2.84	1.85	1.84
B4_3	4.80	2.47	2.46
B4_4	5.22	2.70	2.68
B7_1	4.79	2.94	2.92
B7_2	4.90	3.15	3.13
B7_3	4.81	2.51	2.50
B7_4	4.37	2.41	2.40
B8_1	4.79	3.06	3.04
B8_2	3.88	2.48	2.47
B8_3	4.73	2.43	2.42
B8_4	4.53	2.16	2.15
BR1_1	3.44	2.23	2.22
BR1_2	3.97	2.58	2.57
BR1_3	3.49	1.86	1.85
BR1_4	3.72	2.06	2.05
BR4_1	4.09	2.63	2.62
BR4_2	5.03	3.19	3.17
BR4_3	1.80	0.91	0.91
BR4_4	4.05	2.03	2.02
BR7_1	4.51	2.90	2.89
BR7_2	4.42	2.71	2.70
BR7_3	4.09	2.19	2.18
BR7_4	3.32	1.88	1.87
BR8_1	4.36	2.78	2.77
BR8_2	4.85	2.98	2.96
BR8_3	4.38	2.32	2.31
BR8_4	4.75	2.54	2.54

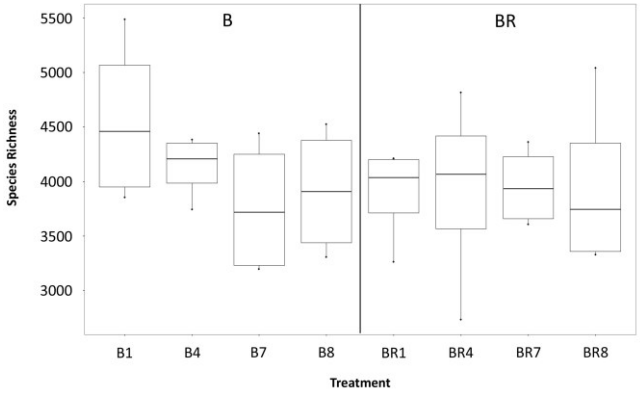

Figure S9. Alpha diversity of rhizosphere bacterial community collected at different cycles during induction of disease suppression determined with Illumina 16S amplicon sequencing. The alpha diversity was calculated with the Fisher's index. B: soil cycled with sugar beet only (remained conducive); BR: soil cycled with sugar beet and *R. solani* (became suppressive). The number following the treatment represents the cycle number. No statistical differences were observed between the samples, when analyzed with analysis of variance and LSD post hoc analysis.

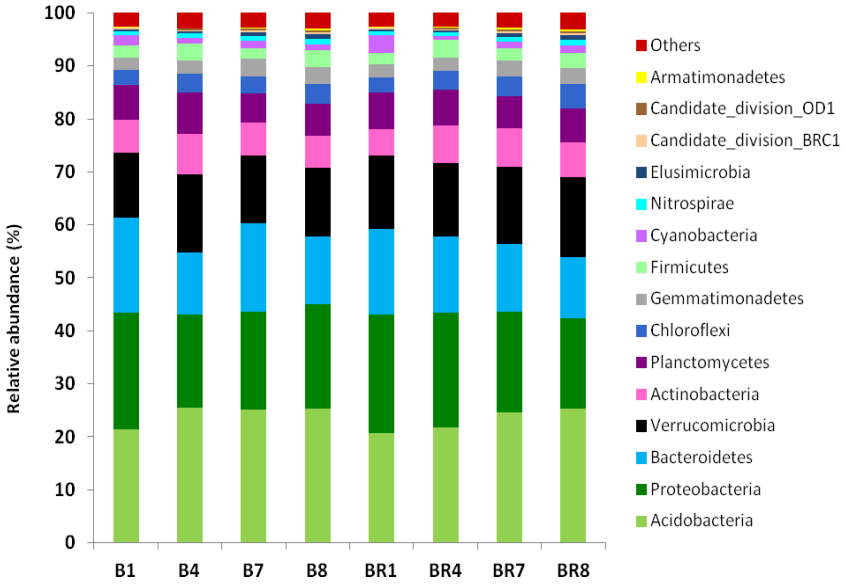

Figure S10. Relative abundance of the 15 most abundant rhizobacterial phyla collected at different cycles during induction of disease suppression determined with Illumina 16S amplicon sequencing. B: soil cycled with sugar beet in absence of *R. solani* remained conducive; BR: soil cycled with sugar beet in presence of *R. solani* became suppressive. The number following the treatment letter indicates the cycle number

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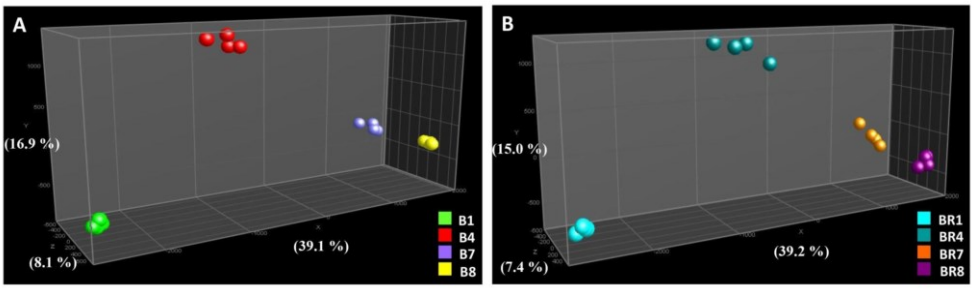

Figure S11. PCoA of the beta diversity of 16S amplicon data of the rhizosphere bacterial community collected at different cycles during induction of disease suppression. Principal component analysis (PCA) was calculated using the Bray-Curtis' coefficient. A) soil cycled with sugar beet in the absence of *R. solani* (remained conducive; B), and B) soil cycled with sugar beet in the presence of *R. solani* (became suppressive; BR). The number following the treatment represents the cycle number. At the axes, the percentage of variation explained is indicated.

Table S3. Statistical differences between cycles and soil treatment calculated by Permanova pairwise comparisons of the beta diversity of the 16S rRNA amplicon and the metatranscriptome data. An asterisk indicates a significant difference ($p<0.05$) between the samples.

Comparison		16S amplicon p-value	Metatranscriptome p-value
B1	BR1	0.425	0.035 *
B4	BR4	0.116	0.220
B7	BR7	0.442	0.016 *
B8	BR8	0.599	0.029 *
B1	B4	0.035 *	0.037 *
B4	B7	0.028 *	0.062
B7	B8	0.378	0.071
BR1	BR4	0.029 *	0.027 *
BR4	BR7	0.032 *	0.081
BR7	BR8	0.618	0.352

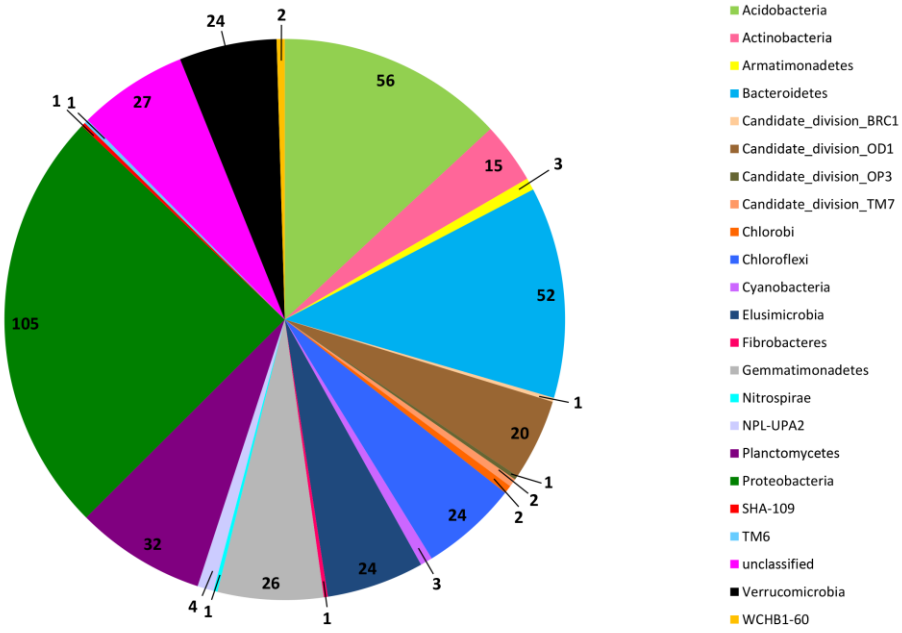

Figure S12. Bacterial phyla of OTUs that occur in higher abundance in suppressive soil after suppressiveness induction (approach B). The 16S amplicon sequences of BR7 and BR8 (suppressive) were pooled and compared to either BR1 or BR4 (conducive). OTUs that are in higher abundance in BR7+BR8 for both comparisons are shown here. Numbers within the pie indicate the number of OTUs per phylum.

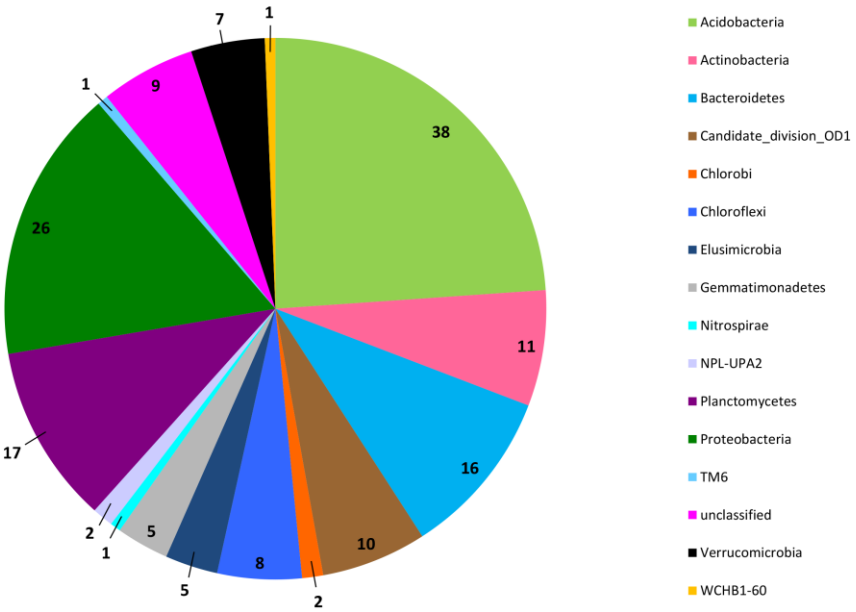

Figure S13. Bacterial phyla of OTUs that occur in higher abundance and are uniquely associated with suppressiveness (approach B). The 16S amplicon sequences of BR7 and BR8 (suppressive) were pooled and compared to either BR1 or BR4 (conducive). The same approach was used for treatment B, in which sequences of B7 and B8 (conducive) were pooled and compared to either B1 or B4 (conducive). Only those OTUs that were exclusively found in higher abundance in BR7+BR8 and not in B7+B8 compared to previous cycles are shown here. Numbers within the pie indicate the number of OTUs per phylum.

Table S4. Summary of the number of OTUs within the bacterial families found in higher abundance and uniquelyassociated with suppressiveness (approach B).

OTUs exclussive BR7-BR8		
Phylum	**Family**	**Num OTUs**
Acidobacteria	RB41	3
	unclassified	31
	Unknown_Family	3
Actinobacteria	Actinospicaceae	1
	Micromonosporaceae	2
	Nocardioidaceae	3
	Pseudonocardiaceae	1
	Streptomycetaceae	1
	unclassified	3
Bacteroidetes	Chitinophagaceae	5
	Cytophagaceae	4
	env.OPS_17	3
	unclassified	4
Candidate_division_OD1	unclassified	10
Chlorobi	SJA-28	2
Chloroflexi	unclassified	8
Elusimicrobia	unclassified	5
Gemmatimonadetes	Gemmatimonadaceae	3
	unclassified	2
Nitrospirae	Nitrospiraceae	1
NPL-UPA2	unclassified	2

OTUs exclussive BR7-BR8		
Phylum	**Family**	**Num OTUs**
Planctomycetes	Phycisphaeraceae	1
	Planctomycetaceae	5
	unclassified	11
Proteobacteria	Bdellovibrionaceae	2
	Caulobacteraceae	1
	Coxiellaceae	3
	Ellin6055	1
	Hyphomicrobiaceae	2
	mitochondria	1
	Nannocystaceae	1
	Phyllobacteriaceae	1
	Polyangiaceae	2
	Rhizobiales_Incertae_Sedis	1
	Rhodocyclaceae	1
	Sphingomonadaceae	2
	unclassified	7
	Xanthomonadaceae	1
TM6	unclassified	1
unclassified	unclassified	9
Verrucomicrobia	Opitutaceae	4
	unclassified	3
WCHB1-60	unclassified	1

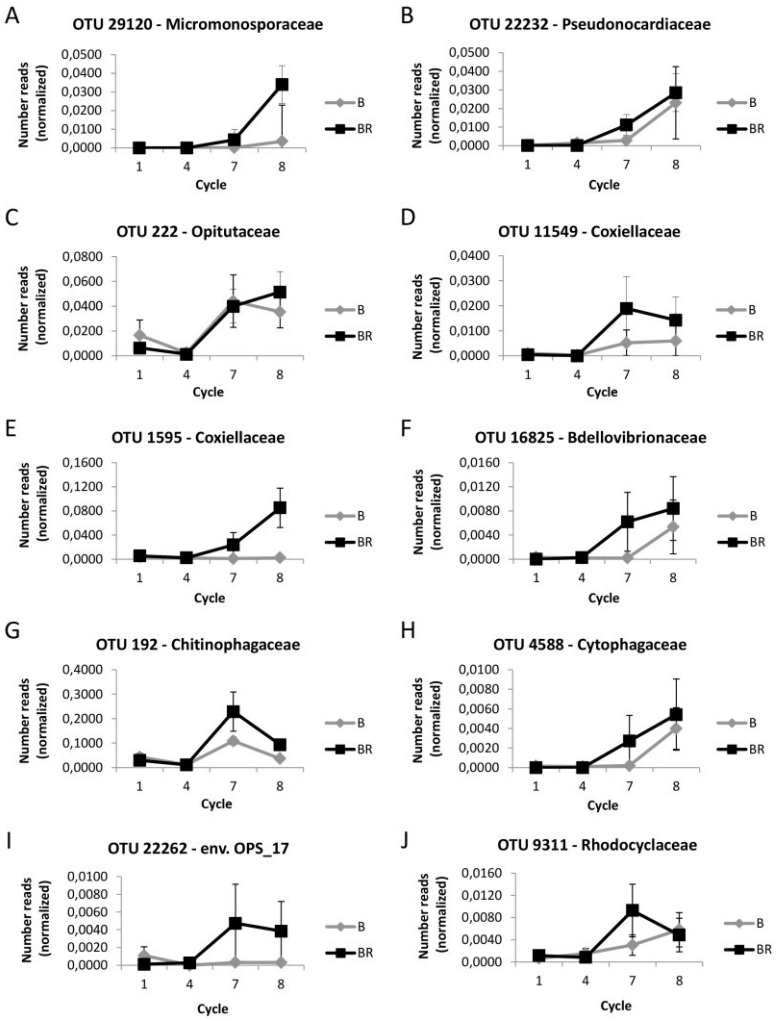

Figure S14. Dynamics of top 10 most abundant OTUs classified at family level found in higher abundance and uniquely associated with suppressiveness (approach B). The 16S amplicon sequences of BR7 and BR8 (suppressive) were pooled and compared to either BR1 or BR4 (conducive) and a similar approach was used for the data of soil sugar beet cycled in absence of *R. solani* (B) (conducive). The dynamics of the top 10 most abundant OTUs that were exclusively found in higher abundance in BR7+BR8 and not in B7+B8 compared to previous cycles are shown here (A-J). **A)** OTU 29120, belonging to the *Micromonosporaceae* (unclassified at genus level); **B)** OTU 22232, belonging to the *Pseudonocardiaceae* (genus *Actinophytocola*); **C)** OTU 222, belonging to the *Opitutaceae* (genus *Opitutus*); **D)** 11549, belonging to the *Coxiellaceae* (genus *Aquicella*); **E)** 1595, belonging to the *Coxiellaceae* (genus *Aquicella*); **F)** OTU 16825, belonging to the *Bdellovibrionaceae* (genus *Bdellovibrio*); **G)** OTU 192, belonging to the *Chitinophagaceae* (genus *Chitinophaga*); **H)** OTU 4588, belonging to the *Cytophagaceae* (unclassified at genus level); **I)** OTU 22262, belonging to the *env. OPS_17* (unclassified at genus level); **J)** OTU 9311, belonging to the *Rhodocyclaceae* (genus *Methyloversatilis*).

Table S5. Overview of the number of reads obtained from the metagenomic RNA sequencing and after the processing and filtration steps.

Sample	Raw reads	Trimmed	Retained reads (%)	Remaining rRNA (%)	Remaining eukaryotic mRNA (%)	ORFs (%)	Predicted protein features (%)	Assigned to functional categories (%)
B1_1	21455413	18477864	86,12	15.1	40.2	73.2	19.6	71.7
B1_2	8961902	7423219	82,83	5.7	19.3	79.7	19.6	77.8
B1_3	10036215	8233274	82,04	4.5	16.0	79.8	20.5	76.8
B1_4	9644475	7891552	81,82	6.0	17.2	80.4	18.0	77.1
B4_1	8154080	6558798	80,44	3.4	23.4	75.1	19.9	74.4
B4_2	8460560	6978686	82,48	5.4	23.3	78.3	18.1	74.7
B4_3	7797177	6215280	79,71	5.4	25.9	78.0	17.5	74.3
B4_4	15534657	13308492	85,67	2.4	32.7	74.2	18.1	73.0
B7_1	9661217	8149194	84,35	5.7	29.0	79.1	18.4	72.8
B7_2	8709548	7398214	84,94	5.4	14.4	80.5	21.3	77.0
B7_3	9170105	7777816	84,82	4.5	25.7	80.6	19.4	73.4
B7_4	14099207	12008504	85,17	2.8	28.7	75.5	19.8	74.5
B8_1	9601634	7900001	82,28	2.7	13.7	77.8	19.1	74.0
B8_2	10038912	8213071	81,81	3.8	13.7	80.1	18.3	77.5
B8_3	6130766	4956092	80,84	5.6	13.4	80.7	17.7	75.6
B8_4	7280396	6175372	84,82	5.3	15.8	75.1	19.1	67.4
BR1_1	16618729	14575260	87,70	16.3	24.6	62.7	11.8	51.6
BR1_2	11166550	9570318	85,71	2.9	32.2	80.3	20.3	74.9
BR1_3	10222683	8752699	85,62	2.9	29.9	80.4	20.5	75.8
BR1_4	9035274	7615475	84,29	4.8	26.4	79.1	18.4	75.3
BR4_1	5859217	5073755	86,59	5.4	27.5	78.6	19.4	75.1
BR4_2	6353724	5365076	84,44	4.8	24.2	77.7	21.0	77.1
BR4_3	19703626	17045026	86,51	4.0	21.8	81.4	22.3	75.9
BR4_4	23072530	20161793	87,38	4.6	38.9	78.1	19.8	72.6
BR7_1	3176811	2512858	79,10	9.1	21.6	78.6	19.3	75.5
BR7_2	11341414	9749404	85,96	5.3	23.7	80.2	24.1	78.0
BR7_3	30799084	29183678	94,76	3.6	14.2	89.4	21.4	75.7
BR7_4	10903604	9336854	85,63	2.8	23.4	77.0	21.2	75.9
BR8_1	6780112	5610167	82,74	9.6	12.9	77.8	19.1	74.0
BR8_2	24368973	23481702	96,36	1.7	1.5	91.7	22.9	76.7
BR8_3	8468720	7098928	83,83	5.6	15.8	80.7	17.7	75.6
BR8_4	10394742	9037234	86,94	8.1	21.7	75.1	19.1	67.4

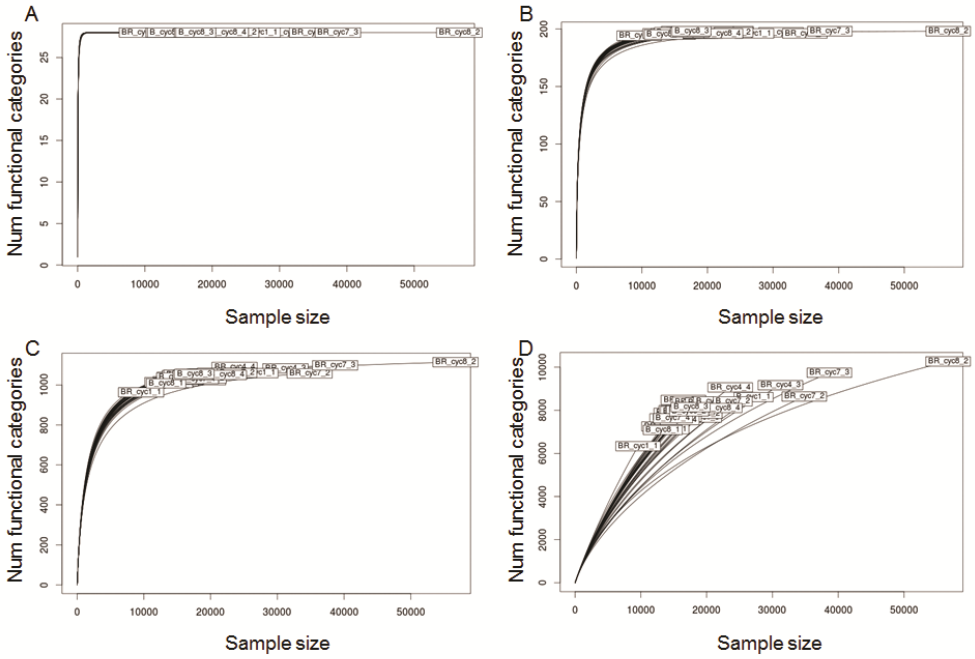

Figure S15. Rarefaction curves representing the depth in coverage of the RNA sequencing at different functional levels based on the SEED Subsystems annotation. A) Level 1; B) Level 2; C) Level 3; D) Level 4 (Functions).

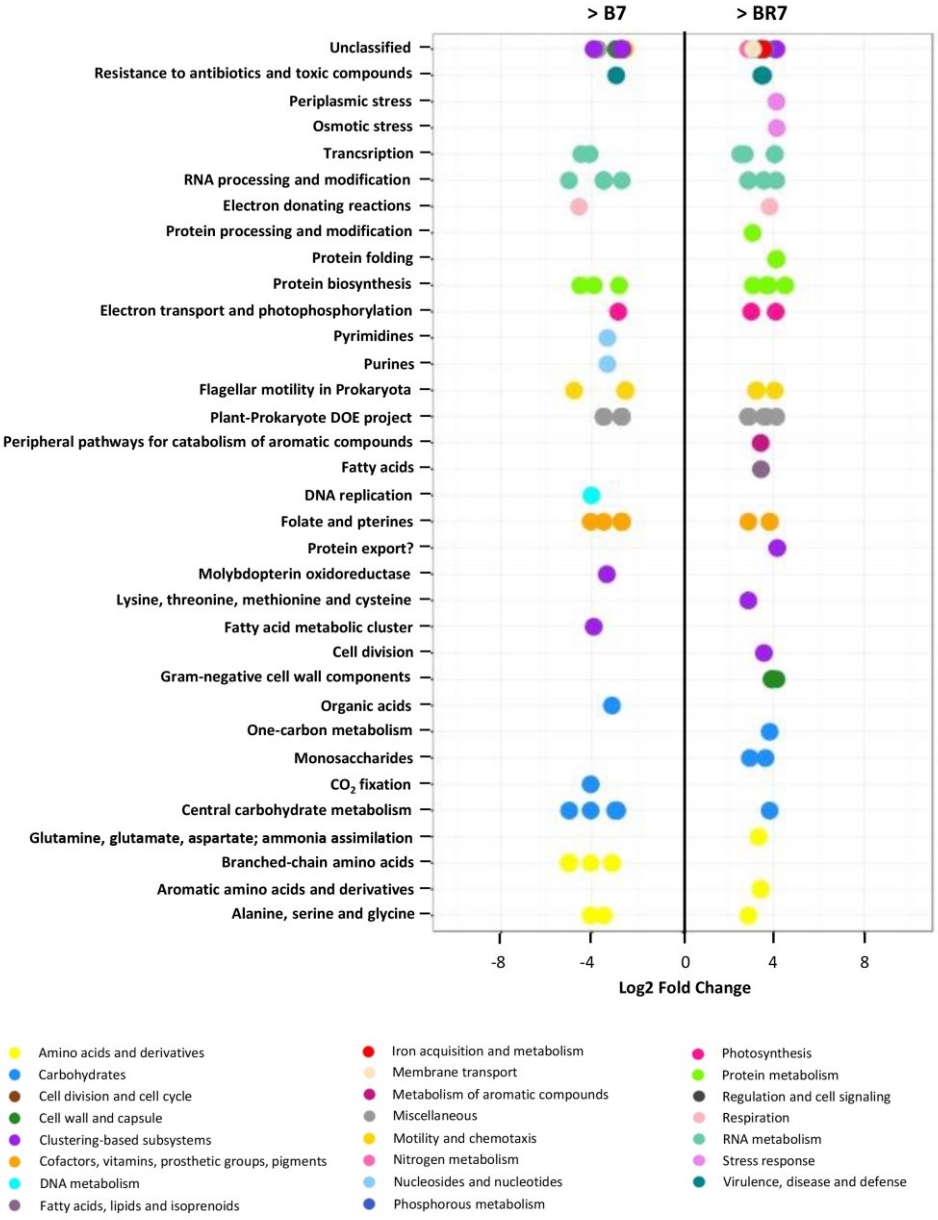

Figure S16. Differential abundance of transcripts between conducive and suppressive soil at cycle 7 (approach A). Dots represent the transcripts differentially expressed (calculated as log2 fold change ≥ 1 and p-vale < 0.05 calculated with Wald test) and are colored based on the functional category to which they belong. >B7: Transcripts found in higher abundance in treatment B (conducive); >BR7: transcripts found in higher abundance in treatment BR (suppressive).

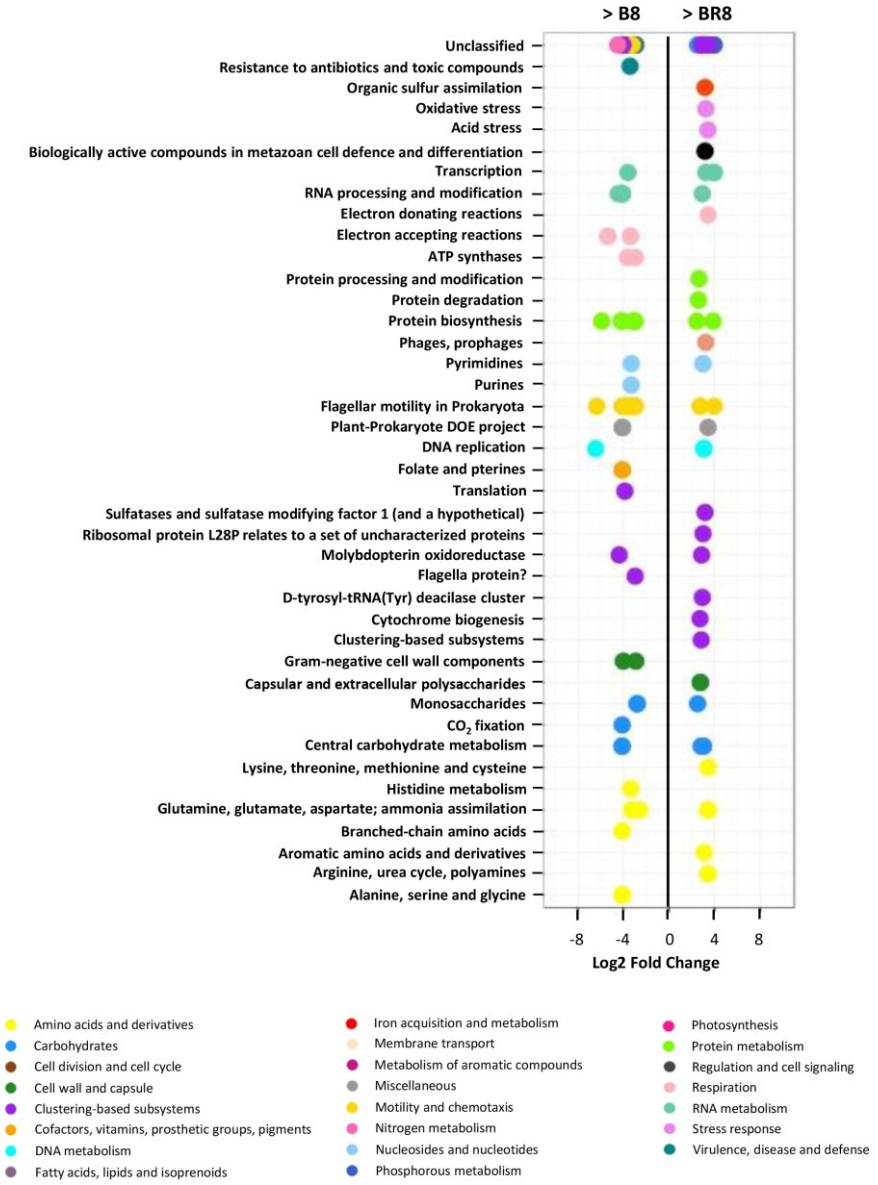

Figure S17. Differential abundance of transcripts between conducive and suppressive soil at cycle 8 (approach A). Dots represent the transcripts differentially expressed (calculated as log2 fold change ≥ 1 and p-vale < 0.05 calculated with Wald test) and are colored based on the functional category to which they belong. >B8: Transcripts found in higher abundance in treatment B (conducive); >BR8: transcripts found in higher abundance in treatment BR (suppressive).

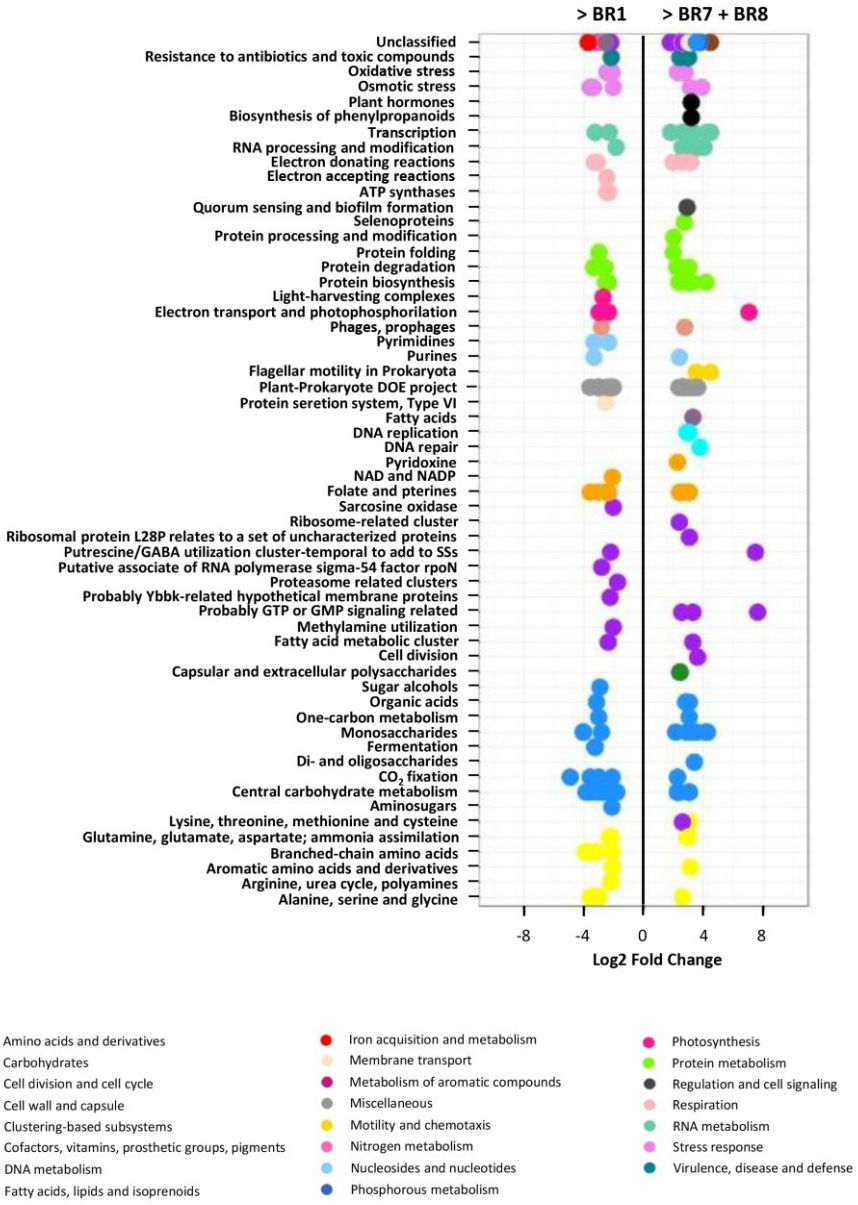

Figure S18. Differential abundance of transcripts between conducive and suppressive soil during suppressiveness induction by comparing cycles within BR (conducive BR1 and suppressive BR7+BR8) (approach B). Dots represent the transcripts differentially expressed (calculated as log2 fold change ≥ 1 and p-vale < 0.05 calculated with Wald test) and are colored based on the functional category to which they belong. >BR1: Transcripts found in higher abundance in BR1 (conducive); >BR7 + BR8: transcripts found in higher abundance in BR7 + BR8 (suppressive).

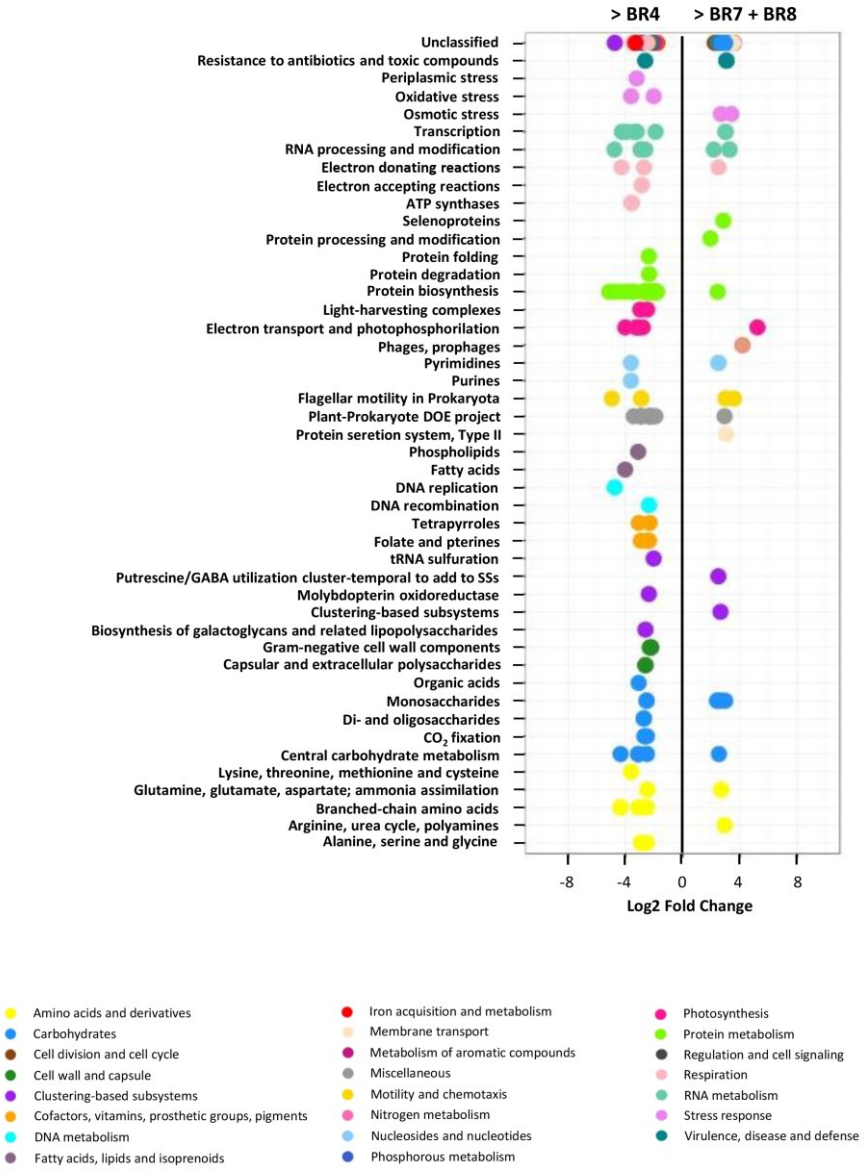

Figure S19. Differential abundance of transcripts between conducive and suppressive soil during suppressiveness induction by comparing cycles within BR (conducive BR4 and suppressive BR7+BR8) (approach B). Dots represent the transcripts differentially expressed (calculated as log2 fold change ≥ 1 and p-vale < 0.05 calculated with Wald test) and are colored based on the functional category to which they belong. >BR4: Transcripts found in higher abundance in BR4 (conducive); >BR7 + BR8: transcripts found in higher abundance in BR7 + BR8 (suppressive).

Table S6. Summary of the transcripts found in higher abundance in suppressive soil with approaches A and B. A letter Y indicates the comparisons for which transcripts upregulated in suppressive soil were found.

Level 1	Level 2	Level 3	Function	Approach A		Approach B	
				> BR7	> BR8	> BR7+BR8 vs BR1	> BR7+BR8 vs BR4
Amino Acids and Derivatives	Alanine, serine, and glycine	Alanine biosynthesis	Cysteine desulfurase CsdA-CsdE (EC 2.8.1.7), main protein CsdA	Y		Y	
	Arginine; urea cycle, polyamines	Arginine and Ornithine Degradation	Arginine decarboxylase, catabolic (EC 4.1.1.19)		Y		
			Succinylornithine transaminase (EC 2.6.1.81)				Y
		Polyamine Metabolism	Arginine decarboxylase, catabolic (EC 4.1.1.19)		Y		
	Aromatic amino acids and derivatives	Aromatic amino acid degradation	Aromatic amino acid transport protein AroP		Y	Y	
		Chorismate Synthesis	3-dehydroquinate dehydratase II (EC 4.2.1.10)	Y			
		Common Pathway For Synthesis of Aromatic Compounds (DAHP synthase to chorismate)	3-dehydroquinate dehydratase II (EC 4.2.1.10)	Y			
	Glutamine, glutamate, aspartate, asparagine; ammonia assimilation	Aspartate aminotransferase	Aspartate aminotransferase (EC 2.6.1.1)		Y		
		Glutamine, Glutamate, Aspartate and Asparagine Biosynthesis	Aspartate aminotransferase (EC 2.6.1.1)		Y		
			Aspartate racemase (EC 5.1.1.13)			Y	
			glutamine synthetase family protein	Y		Y	Y
	Lysine, threonine, methionine, and cysteine	Lysine biosynthesis AAA pathway 2	N-acetyl-gamma-aminoadipyl-phosphate reductase (EC 1.2.1.-)			Y	
		Threonine and Homoserine Biosynthesis	Aspartate aminotransferase (EC 2.6.1.1)		Y		

Level 1	Level 2	Level 3	Function	Approach A		Approach B	
				> BR7	> BR8	> BR7+BR8 vs BR1	> BR7+BR8 vs BR4
Carbohydrates	-	beta-glucuronide_utilization	Beta-glucuronidase (EC 3.2.1.31)		Y	Y	
		Sugar_utilization_in_Thermotogales	Pectate lyase precursor (EC 4.2.2.2)				Y
			Ribose ABC transport system, ATP-binding protein RbsA (TC 3.A.1.2.1)	Y		Y	Y
			Xylan oligosaccharide ABC transporter, permease component 2			Y	
	Central carbohydrate metabolism	Dehydrogenase_complexes	2-oxoglutarate dehydrogenase E1 component (EC 1.2.4.2)		Y		
		Entner-Doudoroff_Pathway	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)			Y	
		Ethylmalonyl-CoA_pathway_of_C2_assimilation	Mesaconyl-CoA hydratase		Y		Y
		Glycolysis_and_Gluconeogenesis	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)			Y	
		Glyoxylate_bypass	Aconitase hydratase (EC 4.2.1.3)			Y	
		Pentose_phosphate_pathway	Ribose-phosphate pyrophosphokinase (EC 2.7.6.1)			Y	
		TCA_Cycle	2-oxoglutarate dehydrogenase E1 component (EC 1.2.4.2)		Y		
			Aconitase_hydratase (EC 4.2.1.3)			Y	
			Succinate dehydrogenase iron-sulfur protein (EC 1.3.99.1)	Y			
	CO2 fixation	Calvin-Benson_cycle	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)			Y	
	Di- and oligosaccharides	Beta-Glucoside_Metabolism	Endo-beta-1,3-1,4 glucanase (Licheninase) (EC 3.2.1.73)			Y	

Level 1	Level 2	Level 3	Function	Approach A		Approach B	
				> BR7	> BR8	> BR7+BR8 vs BR1	> BR7+BR8 vs BR4
Carbohydrates	Monosaccharides	D-Galacturonate_and_D-Glucuronate_Utilization	Beta-glucuronidase (EC 3.2.1.31)		Y	Y	
			Pectate lyase precursor (EC 4.2.2.2)				Y
			Xylan oligosaccharide ABC transporter, permease component 2			Y	
		D-ribose_utilization	Ribose ABC transport system, ATP-binding protein RbsA (TC 3.A.1.2.1)	Y		Y	Y
		Fructose_utilization	Fructose ABC transporter, substrate-binding component FrcB			Y	
		L-rhamnose_utilization	Predicted L-rhamnose ABC transporter, substrate-binding component	Y		Y	Y
			Transcriptional regulator of rhamnose utilization, DeoR family			Y	Y
	One-carbon Metabolism	Serine-glyoxylate_cycle	Aconitase hydratase (EC 4.2.1.3)			Y	
			Succinate dehydrogenase iron-sulfur protein (EC 1.3.99.1)	Y			
	Organic acids	Methylcitrate_cycle	2-methylisocitrate dehydratase (EC 4.2.1.99)			Y	
		Propionate-CoA_to_Succinate_Module	2-methylisocitrate dehydratase (EC 4.2.1.99)			Y	
			Aconitate hydratase (EC 4.2.1.3)			Y	
Cell Division an Cell Cycle	-	Bacterial_Cytoskeleton	Cell division protein ZipA	Y			
		Cyanobacterial_Circadian_Clock	Circadian clock protein KaiC			Y	Y
		Macromolecular_synthesis_operon	RNA polymerase sigma factor RpoD	Y	Y	Y	Y
			SSU ribosomal protein S21p			Y	

Level 1	Level 2	Level 3	Function	Approach A		Approach B	
				> BR7	> BR8	> BR7+BR8 vs BR1	> BR7+BR8 vs BR4
Cell Wall and Capsule	-	Peptidoglycan_Biosynthesis	UDP-N-acetylmuramoylalanyl-D-glutamate--2,6-diaminopimelate ligase (EC 6.3.2.13)	Y			
		Peptidoglycan_biosynthesis--gjo	UDP-N-acetylmuramoylalanyl-D-glutamate--2,6-diaminopimelate ligase (EC 6.3.2.13)	Y			
		Recycling_of_Peptidoglycan_Amino_Acids	AmpG permease	Y			
	Capsular and extracellular polysacchrides	dTDP-rhamnose_synthesis	dTDP-glucose 4,6-dehydratase (EC 4.2.1.46)		Y	Y	
		Rhamnose_containing_glycans	dTDP-glucose 4,6-dehydratase (EC 4.2.1.46)		Y	Y	
	Gram-Negative cell wall components	KDO2-Lipid_A_biosynthesis	Lipid A export ATP-binding/permease protein MsbA (EC 3.6.3.25)	Y			
		Lipopolysaccharide_assembly	Survival protein SurA precursor (Peptidyl-prolyl cis-trans isomerase SurA) (EC 5.2.1.8)	Y			
		Lipopolysaccharide-related_cluster_in_ Alphaproteobacteria	Lipid A export ATP-binding/permease protein MsbA (EC 3.6.3.25)	Y			
Clustering-based Subsystems	-	CBSS-176280.1.peg.1561	Ribonuclease BN (EC 3.1.-.-)		Y		
		CBSS-196620.1.peg.2477	Ferrous iron transport protein B		Y		
		CBSS-211586.1.peg.3133	RNA polymerase sigma factor RpoS			Y	
		CBSS-296591.1.peg.2330	dTDP-glucose 4,6-dehydratase (EC 4.2.1.46)		Y	Y	
		CBSS-314269.3.peg.1840	Carbon monoxide dehydrogenase large chain (EC 1.2.99.2) parolog without usual motifs		Y	Y	

Level 1	Level 2	Level 3	Function	Approach A		Approach B	
				> BR7	> BR8	> BR7+BR8 vs BR1	> BR7+BR8 vs BR4
Clustering-based Subsystems	-	CBSS-316273.3.peg.448	TldD family protein, Actinobacterial subgroup			Y	
		CBSS-350688.3.peg.1509	DNA polymerase III alpha subunit (EC 2.7.7.7)		Y		
			FIG000325: clustered with transcription termination protein NusA			Y	
			Transcription termination protein NusA			Y	
		Cell_division-ribosomal_stress_proteins_cluster	SSU ribosomal protein S1p			Y	
		Cluster_with_second_YidC_in_Bacilli	Inner membrane protein translocase component YidC, short form OxaI-like			Y	
		Conserved_gene_cluster_associated_with_Met-tRNA_formyltransferase	Serine/threonine protein kinase PrkC, regulator of stationary phase		Y		
		EC49-61	Survival protein SurA precursor (Peptidyl-prolyl cis-trans isomerase SurA) (EC 5.2.1.8)	Y			
		Glutaredoxin_3_containing_cluster	FIG136845: Rhodanese-related sulfurtransferase	Y			
		LMPTP_YfkJ_cluster	Ribonuclease BN (EC 3.1.-.-)		Y		
		NusA-TFII_Cluster	FIG000325: clustered with transcription termination protein NusA			Y	
			Transcription termination protein NusA			Y	
		RNA_modification_cluster	Inner membrane protein translocase component YidC, short form OxaI-like			Y	
		Spore_Coat	Spore coat protein A		Y	Y	

Level 1	Level 2	Level 3	Function	Approach A		Approach B	
				> BR7	> BR8	> BR7+BR8 vs BR1	> BR7+BR8 vs BR4
Clustering-based Subsystems	Cell Division	CBSS-393130.3.peg.794	Thiamine biosynthesis protein ThiI	Y		Y	
	Clustering-based subsystems	CBSS-292415.3.peg.2341	Major facilitator superfamily (MFS) transporter		Y		Y
	Cytochrome biogenesis	CBSS-196164.1.peg.461	Cytochrome c-type biogenesis protein CcsA/ResC		Y		
	D-tyrosyl-tRNA(Tyr) deacylase (EC 3.1.-.-) cluster	CBSS-342610.3.peg.283	Ribonuclease BN (EC 3.1.-.-)		Y		
	Fatty acid metabolic cluster	COG1399	Ribonuclease E (EC 3.1.26.12)			Y	
	Lysine, threonine, methionine, and cysteine	CBSS-84588.1.peg.1247	Cysteine desulfurase CsdA-CsdE (EC 2.8.1.7), main protein CsdA	Y		Y	
	Molybdopterin oxidoreductase	CBSS-269799.3.peg.2220	Molybdopterin oxidoreductase (EC 1.2.7.-)		Y		
	Probably GTP or GMP signaling related	CBSS-176299.4.peg.1292	Holo-[acyl-carrier protein] synthase (EC 2.7.8.7)			Y	
			hypothetical protein NAS141_09886			Y	
			Mll7752 protein			Y	
	Protein export?	CBSS-393121.3.peg.2760	NLP/P60 family protein	Y			
	Putrescine/GABA utilization cluster-temporal,to add to SSs	GABA_and_putrescine_metabolism_from_cluters	GABA aminotransferase (EC 2.6.1.19)			Y	Y

Level 1	Level 2	Level 3	Function	Approach A		Approach B	
				> BR7	> BR8	> BR7+BR8 vs BR1	> BR7+BR8 vs BR4
Clustering-based Subsystems	Ribosomal Protein L28P relates to a set of uncharacterized proteins	A_Gram-positive_cluster_that_relates_ribosomal_protein_L28P_to_a_set_of_uncharacterized_proteins	LSU ribosomal protein L28p			Y	
			Serine/threonine protein kinase PrkC, regulator of stationary phase		Y		
	Ribosome-related cluster	A_Gammaproteobacteria_Cluster_Relating_to_Translation	Ribose-phosphate pyrophosphokinase (EC 2.7.6.1)			Y	
	Sulfatases and sulfatase modifying factor 1 (and a hypothetical)	Sulfatases_and_sulfatase_modifying_factor_1	Arylsulfatase (EC 3.1.6.1)		Y		
Cofactors, Vitamins, Prosthetic Groups, Pigments	Folate and pterines	5-FCL-like_protein	Succinate dehydrogenase iron-sulfur protein (EC 1.3.99.1)	Y			
		YgfZ	Aconitate hydratase (EC 4.2.1.3)			Y	
			Cysteine desulfurase CsdA-CsdE (EC 2.8.1.7), main protein CsdA	Y		Y	
			Ribose-phosphate pyrophosphokinase (EC 2.7.6.1)			Y	
			Succinate dehydrogenase iron-sulfur protein (EC 1.3.99.1)	Y			
		Ygfz-Iron	TctA citrate transporter			Y	
	Pyridoxine	Pyridoxin_(Vitamin_B6)_Biosynthesis	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)			Y	

Level 1	Level 2	Level 3	Function	Approach A		Approach B	
				> BR7	> BR8	> BR7+BR8 vs BR1	> BR7+BR8 vs BR4
DNA Metabolism	-	Restriction-Modification_System	Type I restriction-modification system, restriction subunit R (EC 3.1.21.3)				Y
		Type_I_Restriction-Modification	Type I restriction-modification system, restriction subunit R (EC 3.1.21.3)				Y
	DNA repair	DNA_repair,_bacterial_DinG_and_relatives	ATP-dependent helicase DinG/Rad3			Y	
	DNA replication	DNA-replication	DNA polymerase III alpha subunit (EC 2.7.7.7)		Y		
		DNA_topoisomerases,_Type_I,_ATP-independent	DNA topoisomerase III, Burkholderia type (EC 5.99.1.2)		Y		
			DNA topoisomerase V			Y	
		DNA_topoisomerases,_Type_II,_ATP-dependent	DNA topoisomerase VI subunit A (EC 5.99.1.3)			Y	
			DNA topoisomerase VI subunit B (EC 5.99.1.3)			Y	
		DNA-replication	DNA polymerase III alpha subunit (EC 2.7.7.7)		Y		
Fatty Acids, Lipids, and Isoprenoids	Fatty acids	Fatty_Acid_Biosynthesis_FASII	Holo-[acyl-carrier protein] synthase (EC 2.7.8.7)			Y	
		Fatty_acid_degradation_regulons	Long-chain fatty acid transport protein	Y			
Iron acquisition and metabolism	-	Campylobacter_Iron_Metabolism	Ferric siderophore transport system, periplasmic binding protein TonB			Y	Y
			Ferrous iron transport protein B		Y		
		Heme, hemin_uptake_and_utilization_systems_in_GramNegatives	Ferric siderophore transport system, periplasmic binding protein TonB			Y	Y
			Paraquat-inducible protein B			Y	

Level 1	Level 2	Level 3	Function	Approach A		Approach B	
				> BR7	> BR8	> BR7+BR8 vs BR1	> BR7+BR8 vs BR4
Iron acquisition and metabolism	-	Hemin_transport_system	Ferric siderophore transport system, periplasmic binding protein TonB			Y	Y
		Iron_acquisition_in_Vibrio	Ferrous iron transport protein B		Y		
		Transport_of_Iron	Ferrous iron transport protein B		Y		
			Iron-regulated protein A precursor	Y			
Membrane Transport	-	Citrate_Utilization_System_(CitAB,_CitH,_and_tctABC)	TctA citrate transporter			Y	
		Ton_and_Tol_transport_systems	Biopolymer transport ExbD protein			Y	
			Ferric siderophore transport system, periplasmic binding protein TonB			Y	Y
			Outer membrane lipoprotein omp16 precursor		Y		
			TPR repeat containing exported protein	Y			
	Protein secretion system, Type II	General_Secretion_Pathway	General secretion pathway protein E				Y
Metabolism of Aromatic Compounds	-	Benzoate_transport_and_degradation_cluster	Benzoyl-CoA oxygenase component B		Y	Y	
	Peripheral pathways for catabolism of aromatic compounds	Quinate_degradation	3-dehydroquinate dehydratase II (EC 4.2.1.10)	Y			

Level 1	Level 2	Level 3	Function	Approach A		Approach B	
				> BR7	> BR8	> BR7+BR8 vs BR1	> BR7+BR8 vs BR4
Miscellaneous	Plant-Prokaryote DOE project	At1g14345	Inner membrane protein translocase component YidC, short form OxaI-like			Y	
		At2g23840	DNA-directed RNA polymerase alpha subunit (EC 2.7.7.6)			Y	
		At3g50560	2-hydroxychromene-2-carboxylate isomerase family protein, glutathione-dependent			Y	
		At5g37530	Aspartyl-tRNA synthetase (EC 6.1.1.12)	Y			
			Cysteine desulfurase CsdA-CsdE (EC 2.8.1.7), main protein CsdA	Y		Y	
		COG0523	LSU ribosomal protein L28p			Y	
			LSU ribosomal protein L33p			Y	
		COG2363	Cysteine desulfurase CsdA-CsdE (EC 2.8.1.7), main protein CsdA	Y		Y	
			Thiamine biosynthesis protein ThiI	Y		Y	
		DOE_COG2016	Thioredoxin			Y	
		DOE_COG3533	Hydrolase ass w/ COG3533Xanth			Y	
		Iron-sulfur_cluster_assembly	Chaperone protein HscB	Y			
			Cysteine desulfurase CsdA-CsdE (EC 2.8.1.7), main protein CsdA	Y		Y	
		PROSC	Aspartate aminotransferase (EC 2.6.1.1)		Y		
			Succinylornithine transaminase (EC 2.6.1.81)				Y

Level 1	Level 2	Level 3	Function	Approach A		Approach B	
				> BR7	> BR8	> BR7+BR8 vs BR1	> BR7+BR8 vs BR4
Motility and Chemotaxis	Flagellar motility in Prokaryota	Flagellar_motility	Flagellar basal-body rod modification protein FlgD	Y			
		Flagellum	Flagellar basal-body rod modification protein FlgD	Y			
			Flagellar transcriptional activator FlhD		Y	Y	Y
			RNA polymerase sigma factor RpoD	Y	Y	Y	Y
Nitrogen Metabolism	-	Allantoin_Utilization	Allantoicase (EC 3.5.3.4)	Y			
		Nitrate_and_nitrite_ammonification	Cytochrome c552 precursor (EC 1.7.2.2)			Y	
Nucleosides and Nucleotides	-	Hydantoin_metabolism	Dihydropyrimidinase (EC 3.5.2.2)		Y		
		Ribonucleotide_reduction	Ribonucleotide reductase of class II (coenzyme B12-dependent) (EC 1.17.4.1)			Y	Y
	Purines	De_Novo_Purine_Biosynthesis	Ribose-phosphate pyrophosphokinase (EC 2.7.6.1)			Y	
	Pyrimidines	Novel_non-oxidative_pathway_of_Uracil_catabolism	Beta-ureidopropionase, eukaryotic type (EC 3.5.1.6)				Y
			Dihydropyrimidinase (EC 3.5.2.2)		Y		
		Pyrimidine_utilization	Beta-ureidopropionase, eukaryotic type (EC 3.5.1.6)				Y
			Dihydropyrimidinase (EC 3.5.2.2)		Y		
Phages, Prophages, Transposable elements, Plasmids	Phages, Prophages	Phage_capsid_proteins	Phage major capsid protein				Y
		Phage_regulation_of_gene_expression	RNA polymerase sigma factor		Y	Y	
		r1t-like_streptococcal_phages	Phage major capsid protein				Y
Phosphorous Metabolism	-	Phosphate_metabolism	secreted alkaline phosphatase		Y	Y	

Level 1	Level 2	Level 3	Function	Approach A		Approach B	
				> BR7	> BR8	> BR7+BR8 vs BR1	> BR7+BR8 vs BR4
Photosynthesis	Electron transport and photophosphorylation	Photosystem_I	photosystem I subunit II (PsaD)	Y			
		Photosystem_II	photosystem II protein D1 (PsbA)	Y		Y	Y
Protein Metabolism	Protein biosynthesis	Ribosome_biogenesis_bacterial	Ribonuclease E (EC 3.1.26.12)			Y	
		Ribosome_LSU_bacteerial	LSU ribosomal protein L28p			Y	
			LSU ribosomal protein L33p			Y	
		Ribosome_SSU_bacterial	SSU ribosomal protein S1p			Y	
			SSU ribosomal protein S7p (S5e)	Y	Y	Y	Y
			SSU ribosomal protein S21p			Y	
		Ribosome_SSU_chloroplast	SSU ribosomal protein S5p (S2e), chloroplast	Y			
		Translation_elongation_factors_eukaryotic_and_archaeal	Translation elongation factor 1 beta subunit		Y		
		tRNA_aminoacylation,_Asp_and_Asn	Aspartyl-tRNA synthetase (EC 6.1.1.12)	Y			
			Aspartyl-tRNA(Asn) synthetase (EC 6.1.1.23)	Y			
		tRNA_aminoacylation,_Ser	Archaeal seryl-tRNA synthetase-related sequence			Y	

Level 1	Level 2	Level 3	Function	Approach A		Approach B	
				> BR7	> BR8	> BR7+BR8 vs BR1	> BR7+BR8 vs BR4
Protein Metabolism	Protein degradation	Aminopeptidases_(EC_3.4.11.-)	Membrane alanine aminopeptidase N (EC 3.4.11.2)		Y	Y	
		Proteasome_bacterial	ATP-dependent Clp protease ATP-binding subunit ClpX			Y	
		Proteolysis_in_bacteria,_ATP-dependent	ATP-dependent Clp protease ATP-binding subunit ClpX			Y	
		Putative_TldE-TldD_proteolytic_complex	TldD family protein, Actinobacterial subgroup			Y	
	Protein folding	GroEL_GroES	Heat shock protein 60 family co-chaperone GroES			Y	
		Peptidyl-prolyl_cis-trans_isomerase	Survival protein SurA precursor (Peptidyl-prolyl cis-trans isomerase SurA) (EC 5.2.1.8)	Y			
		Protein_chaperones	Chaperone protein HscB	Y			
	Protein processing and modification	Inteins	intein-containing	Y	Y	Y	Y
	Selenoproteins	Glycine_reductase,_sarcosine_reductase_and_betaine_reductase	Thioredoxin			Y	
		Selenocysteine_metabolism	selenocysteine-containing				Y
Regulation and Cell signaling	Quorum sensing and biofilm formation	Autoinducer_2_(AI-2)_transport_and_processing_(lsrACDBFGE_operon)	Autoinducer 2 (AI-2) ABC transport system, periplasmic AI-2 binding protein LsrB			Y	

Level 1	Level 2	Level 3	Function	Approach A		Approach B	
				> BR7	> BR8	> BR7+BR8 vs BR1	> BR7+BR8 vs BR4
Respiration	-	Biogenesis_of_c-type_cytochromes	Cytochrome c-type biogenesis protein CcsA/ResC		Y		
		Formate_hydrogenase	Formate dehydrogenase O alpha subunit (EC 1.2.1.2)			Y	Y
		Soluble_cytochromes_and_functionally_related_electron_carriers	Cytochrome c552			Y	
			Cytochrome c552 precursor (EC 1.7.2.2)			Y	
	Electron donating reactions	CO_Dehydrogenase	Carbon monoxide dehydrogenase large chain (EC 1.2.99.2) parolog without usual motifs		Y	Y	
		Formate_dehydrogenase	Formate dehydrogenase O alpha subunit (EC 1.2.1.2)			Y	Y
		Respiratory_dehydrogenases_1	Methanol dehydrogenase large subunit protein (EC 1.1.99.8)			Y	
		Succinate_dehydrogenase	Succinate dehydrogenase iron-sulfur protein (EC 1.3.99.1)	Y			
RNA Metabolism	RNA processing and modification	ATP-dependent_RNA_helicases,_bacterial	ATP-dependent RNA helicase Atu1833			Y	Y
			ATP-dependent RNA helicase NGO0650			Y	
			ATP-dependent RNA helicase RhlE			Y	
		mnm5U34_biosynthesis_bacteria	Cysteine desulfurase CsdA-CsdE (EC 2.8.1.7), main protein CsdA	Y		Y	
		RNA_processing_and_degradation,_bacterial	Ribonuclease E (EC 3.1.26.12)			Y	

Level 1	Level 2	Level 3	Function	Approach A		Approach B	
				> BR7	> BR8	> BR7+BR8 vs BR1	> BR7+BR8 vs BR4
RNA Metabolism	RNA processing and modification	tRNA_modification_Archaea	Cysteine desulfurase CsdA-CsdE (EC 2.8.1.7), main protein CsdA	Y		Y	
			Fe-S OXIDOREDUCTASE (1.8.-.-) Wyeosine biosynthesis				Y
			Thiamine biosynthesis protein ThiI	Y		Y	
		tRNA_modification_Bacteria	Chaperone protein HscB	Y			
			Thiamine biosynthesis protein ThiI	Y		Y	
		tRNA_processing	Ribonuclease BN (EC 3.1.-.-)		Y		
		Wyeosine-MimG_Biosynthesis	Fe-S OXIDOREDUCTASE (1.8.-.-) Wyeosine biosynthesis				Y
	Transcription	RNA_polymerase_bacterial	DNA-directed RNA polymerase alpha subunit (EC 2.7.7.6)			Y	
			DNA-directed RNA polymerase beta subunit (EC 2.7.7.6)			Y	
		Transcription_factors_bacterial	FIG000325: clustered with transcription termination protein NusA			Y	
			Transcription termination protein NusA			Y	
		Transcription_factors_cyanobacterial_RpoD-like_ sigma_factors	RNA polymerase sigma factor RpoD	Y	Y	Y	Y
		Transcription_initiation,_bacterial_sigma_factors	RNA polymerase principal sigma factor HrdA			Y	
			RNA polymerase principal sigma factor HrdD	Y	Y	Y	

Level 1	Level 2	Level 3	Function	Approach A		Approach B	
				> BR7	> BR8	> BR7+BR8 vs BR1	> BR7+BR8 vs BR4
RNA Metabolism	Transcription	Transcription_initiation,_bacterial_sigma_factors	RNA polymerase sigma factor RpoD	Y	Y	Y	Y
			RNA polymerase sigma factor RpoH-related protein RpoH2	Y		Y	
			RNA polymerase sigma factor RpoS			Y	
Secondary Metabolism	Biologically active compounds in metazoan cell defence and differentiation	Steroid_sulfates	Arylsulfatase (EC 3.1.6.1)		Y		
	Biosynthesis of phenylpropanoids	Phenylpropanoids_general_biosynthesis	UDP-glucose:sinapate glucosyltransferase (EC 2.4.1.120)			Y	
	Plant Hormones	Auxin_biosynthesis	Indole-3-acetate beta-glucosyltransferase			Y	
Stress Response	Acid stress	Acid_resistance_mechanisms	Arginine decarboxylase, catabolic (EC 4.1.1.19)		Y		
	Osmotic stress	Choline_and_Betaine_Uptake_and_Betaine_ Biosynthesis	HTH-type transcriptional regulator BetI	Y		Y	Y
			L-proline glycine betaine binding ABC transporter protein ProX (TC 3.A.1.12.1)			Y	Y
	Oxidative stress	Oxidative_stress	Paraquat-inducible protein B			Y	
		Redox-dependent_regulation_of_nucleus_processes	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)			Y	
		Regulation_of_Oxidative_Stress_Response	RNA polymerase sigma factor		Y	Y	

Level 1	Level 2	Level 3	Function	Approach A		Approach B	
				> BR7	> BR8	> BR7+BR8 vs BR1	> BR7+BR8 vs BR4
Stress Response	Periplasmic Stress	Periplasmic_Stress_Response	Survival protein SurA precursor (Peptidyl-prolyl cis-trans isomerase SurA) (EC 5.2.1.8)	Y			
Sulfur Metabolism	-	Galactosylceramide_and_Sulfatide_metabolism	Arylsulfatase (EC 3.1.6.1)		Y		
	Organic sulfur assimilation	Alkanesulfonate_assimilation	Arylsulfatase (EC 3.1.6.1)		Y		
Virulence, Disease and Defense	-	Bacterial_cyanide_production_and_tolerance_mechanisms	Formate dehydrogenase O alpha subunit (EC 1.2.1.2)			Y	Y
	Resistance to antibiotics and toxic compounds	Methicillin_resistance_in_Staphylococci	UDP-N-acetylmuramoylalanyl-D-glutamate--2,6-diaminopimelate ligase (EC 6.3.2.13)	Y			
		MexE-MexF-OprN_Multidrug_Efflux_System	Multidrug efflux transporter MexF			Y	
		Multidrug_efflux_pump_in_Campylobacter_jejuni_ (CmeABC_operon)	RND efflux system, outer membrane lipoprotein CmeC			Y	Y
		Multidrug_Resistance_Efflux_Pumps	RND efflux system, outer membrane lipoprotein CmeC			Y	Y
			Transcription repressor of multidrug efflux pump acrAB operon, TetR (AcrR) family	Y			

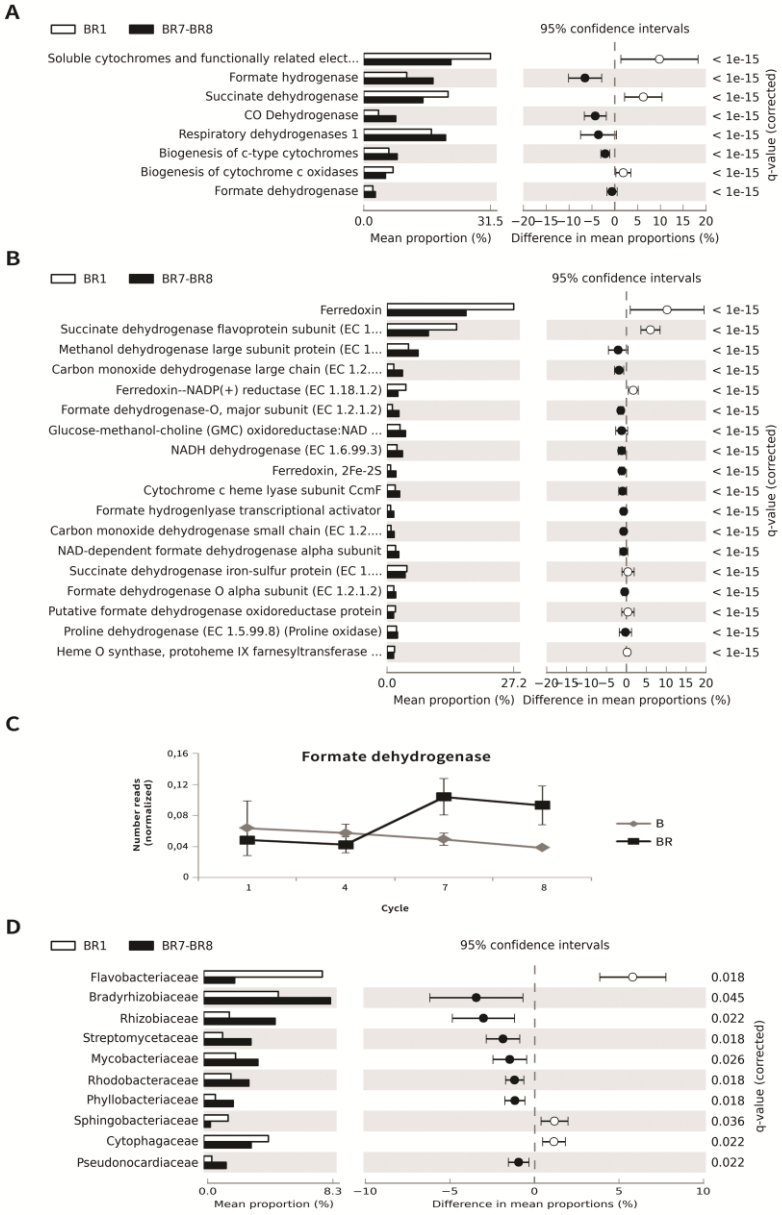

Figure S20. Abundance of transcripts involved in respiration, their dynamics, and assigned bacterial taxa in suppressive soil during suppressiveness induction (BR7+BR8 vs BR1; approach B). A) transcripts differentially expressed at level 3; B) transcripts differentially expressed at level 4; C) dynamics of the transcripts within the formate dehydrogenases during cycling; D) most active bacterial taxa associated with the transcripts of respiration that were more abundant in suppressive (black) and in conducive (white) soil.

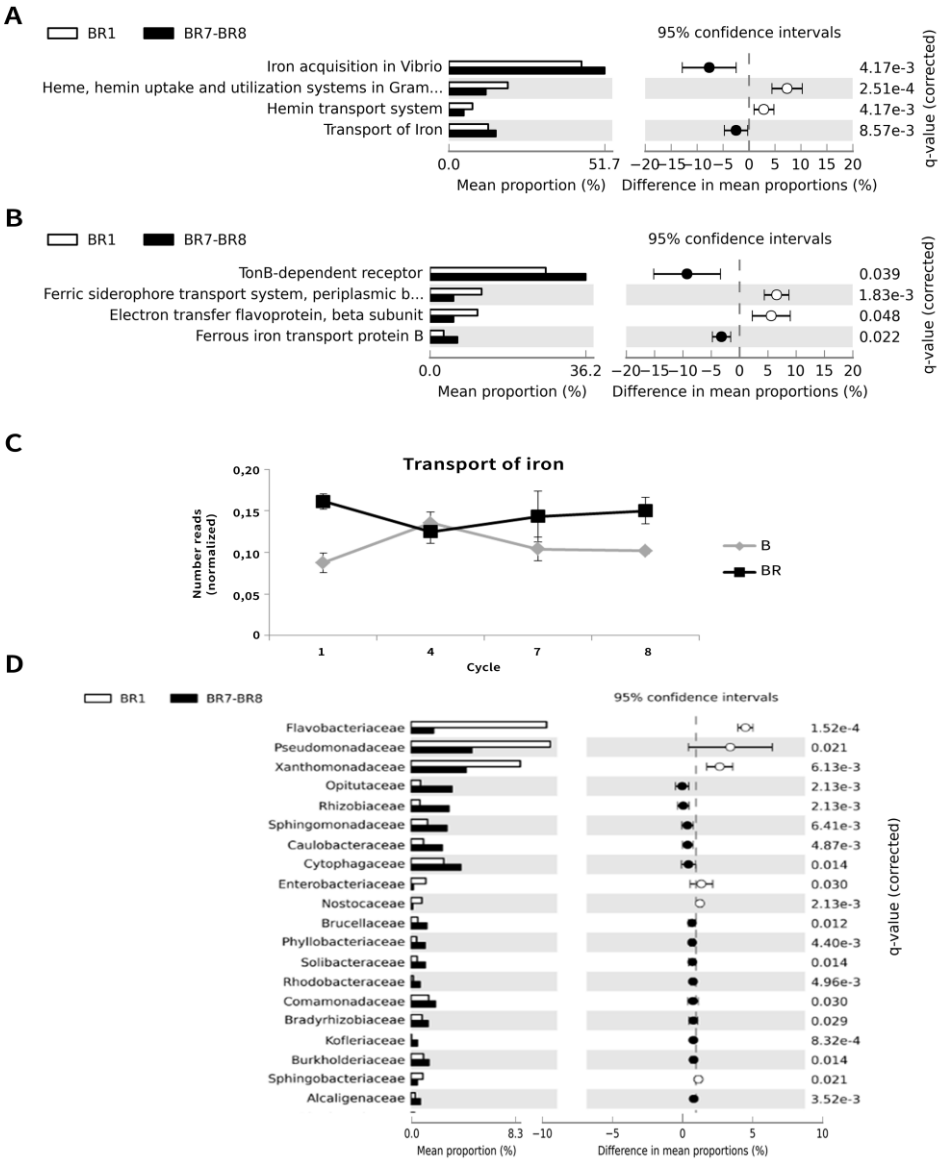

Figure S21. Abundance of transcripts involved in iron acquisition and metabolism, their dynamics, and assigned bacterial taxa in suppressive soil during suppressiveness induction (BR7+BR8 vs BR1; approach B) A) transcripts differentially expressed at level 3; B) transcripts differentially expressed at level 4; C) dynamics of the transcripts within the transport of iron; D) top 20 most active bacterial taxa associated with the transcripts of iron acquisition that were more abundant in suppressive (black) and in conducive (white) soil.

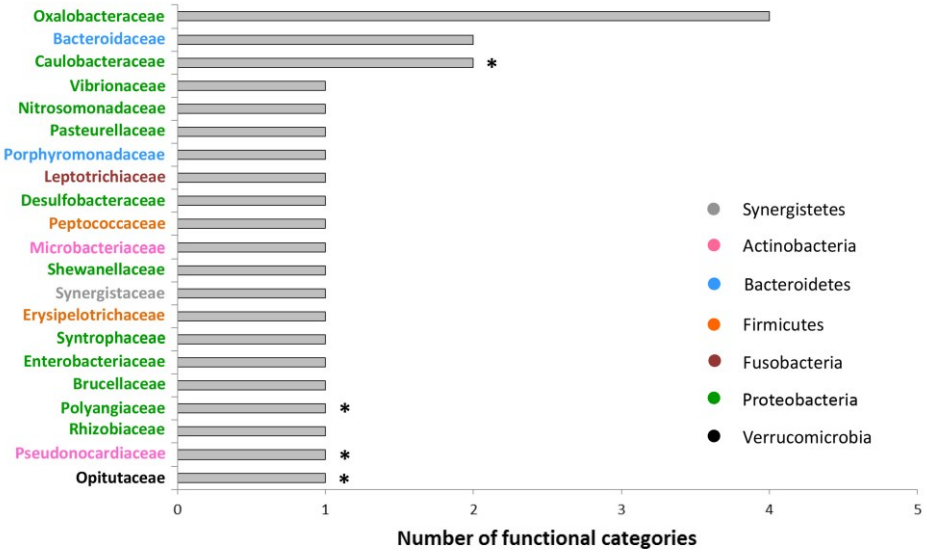

Figure S22. Summary of bacterial families activated in suppressive soil during suppressiveness induction based on metatranscriptome analysis. Bacterial taxa were assigned to all transcripts found in higher abundance in suppressive and in conducive soil for both approaches, and the total number of functions (level 3) were counted per bacterial family for the suppressive soil (BR7+BR8) compared with conducive soil (BR4). An asterisk indicates those families that are not only more active but also more abundant in suppressive soil, based on 16S amplicon data analyses (approaches A and B).

Table S7. Comparison of the bacterial families found more abundant (based on rRNA) and more active (based on mRNA) in the *R. solani* induced suppressive soil used in this study (Zwaagdijk) and another *Rhizoctonia* suppressive soil (Hoeven; Mendes et al (2011) and Chapelle et al 2015)).

Phyla	Family	rRNA-based (this study)	mRNA-based (this study)	rRNA-based (Mendes et al., 2011)	mRNA-based (Chapelle et al., 2015)
Acidobacteria	Acidothermaceae		Y		
	RB41	Y			
	Solibacteraceae		Y		
Actinobacteria	Actinospicaceae	Y		Y	
	Frankiaceae		Y		
	Geodermatophilaceae		Y		
	Glycomycetaceae		Y		
	Intrasporangiaceae		Y		
	Microbacteriaceae		Y		
	Micromonosporaceae	Y	Y	Y	
	Nocardiaceae		Y		
	Nocardioidaceae	Y	Y	Y	
	Nocardiopsaceae		Y		
	Mycobacteriaceae		Y		
	Pseudonocardiaceae	Y	Y	Y	
	Streptomycetaceae	Y	Y	Y	
Bacteroidetes	Bacteroidaceae		Y		
	Chitinophagaceae	Y			
	Cytophagaceae	Y			Y
	Cryomprphaceae	Y			
	env.OPS_17	Y			
	KD3-93	Y			
	Porphyromonadaceae		Y		
	Sphingobacteriaceae	Y			Y
Chlorobi	SJA-28	Y			
Chloroflexi	Chloroflexaceae		Y		
Firmicutes	Erysipelotrichaceae		Y		
	Peptococcaceae		Y		
Fusobacteria	Leptotrichiaceae		Y		
Gemmatimonadetes	Gemmatimonadaceae	Y		Y	

Phyla	Family	rRNA-based (this study)	mRNA-based (this study)	rRNA-based (Mendes et al., 2011)	mRNA-based (Chapelle et al., 2015)
Nitrospirae	Nitrospiraceae	Y		Y	
Planctomycetes	Phycisphaeraceae	Y		Y	
	Planctomycetaceae	Y	Y	Y	
Proteobacteria	Aurantimonadaceae		Y		
	Bradyrhizobiaceae		Y		
	Brucellaceae		Y		
	Caulobacteraceae	Y	Y	Y	
	Ellin6055	Y			
	Erythrobacteraceae		Y		
	Hyphomicrobiaceae	Y		Y	
	Hyphomonadaceae		Y		
	Parvularculaceae		Y		
	Phyllobacteriaceae	Y	Y	Y	
	Sphingomonadaceae	Y	Y	Y	Y
	Rhizobiaceae		Y		
	Rhizobiales_Incertae_Sedis	Y			
	Rhodobacteraceae		Y		
	Alcaligenaceae		Y		Y
	Burkholderiaceae	Y			Y
	Comamonadaceae		Y		Y
	Methylophilaceae		Y		
	Neisseriaceae		Y		
	Nitrosomonadaceae		Y		
	Oxalobacteraceae		Y		Y
	Rhodocyclaceae	Y	Y	Y	
	Bdellovibrionaceae	Y		Y	
	Cystobacteraceae				Y
	Desulfobacteraceae		Y		
	Kofleriaceae		Y		
	Nannocystaceae	Y		Y	
	Kofleriaceae		Y		
	Nannocystaceae	Y		Y	

Phyla	Family	rRNA-based (this study)	mRNA-based (this study)	rRNA-based (Mendes et al., 2011)	mRNA-based (Chapelle et al., 2015)
Proteobacteria	Polyangiaceae	Y	Y	Y	
	Syntrophaceae		Y		
	Alteromonadaceae		Y		
	Coxiellaceae	Y		Y	
	Enterobacteriaceae		Y		
	Francisellaceae		Y		
	Pasteurellaceae		Y		
	Shewanellaceae		Y		
	Vibrionaceae		Y		
	Xanthomonadaceae	Y		Y	
Synergistetes	Synergistaceae		Y		
Verrucomicrobia	Opitutaceae	Y	Y	Y	
	Subdivision 3				Y

A letter Y indicates the dataset for which the different bacterial families were detected more abundant or more active in a *Rhizoctonia* suppressive soil. From Chapelle et al (2015) the rhizosphere bacterial families responding to the inoculation with the soil-borne fungal pathogen *R. solani* based on mRNA analyses for the totality of functions was used. It should be noted that when investigating specific functions in more detail, other bacteria families might become significant (Chapelle et al, (2015) supplementary information), but these were not taken into account for this table.

Chapter 5

Role of oxalotrophic bacteria in suppression of damping-off disease caused by the fungal root pathogen *Rhizoctonia solani*

Ruth Gómez Expósito, Joeke Postma, Jos M. Raaijmakers and Irene de Bruijn

(To be submitted)

Abstract

Oxalic acid is produced by plants, animals and diverse microorganisms. For several plant pathogenic fungi, oxalic acid serves as a virulence factor by softening plant tissue and aiding in the activity of cell-wall degrading enzymes. Here, we investigated how widespread oxalotrophy is among soil bacteria and if degradation of oxalic acid by these bacterial genera is a strategy to control damping-off disease of sugar beet caused by the root pathogen *Rhizoctonia solani*. To this end, we enriched for and extracted soil bacterial communities that can use oxalate as a sole carbon source. Soil bioassays showed that application of the enriched oxalotrophic bacterial community to sugar beet seeds conferred significant suppression of *R. solani* damping-off disease in two out of three independent experiments. In contrast, a glucose-enriched soil bacterial community did not provide disease control in any of the three experiments. Subsequent isolation and characterization revealed that the oxalate-enriched bacterial community comprised of phylogenetically diverse bacterial families belonging to the phyla Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria. Bioassays with ten representatives of these oxalotrophic bacterial families showed that isolates classified as *Caulobacter* and *Nocardioides* species significantly suppressed *R. solani* damping-off disease. In conclusion, our results demonstrate that specific oxalotrophic soil bacteria constitute a novel and yet untapped resource for the control of economically important fungal plant pathogens.

Introduction

Oxalic acid ($C_2H_2O_4$) is an organic compound produced by animals, plants, fungi and bacteria. Production of oxalic acid has been described for several phytopathogenic fungi, including *Sclerotinia sclerotiorum (*Zhou and Boland, 1999; Cessna et al., 2000), *Sclerotium rolfsii* (Tang et al., 2011), *Botrytis cinerea* (Schoonbeek et al., 2007), *Aspergillus niger* (Ruijter et al., 1999) and *Rhizoctonia solani* (Nagarajkumar et al., 2005; Foley et al., 2016). The production of oxalic acid by pathogenic fungi is triggered under low nutrient conditions. Other major factors affecting oxalate production by fungi are carbon to nitrogen (C:N) ratio, pH and the type of C and N source. Among the most efficient carbon sources are carboxymethylcellulose, starch, D-glucose, D-galactose or D-xylose (Dutton and Evans, 1996). Fungi growing on media containing nitrates usually secrete higher amounts of oxalic acid than on media containing ammonium (Dutton and Evans, 1996). A pH higher than 3 has been reported to be essential for oxalic acid production (Chakrabarti and Samajpati, 1983; Ruijter et al., 1999; Xu et al., 2015).

For several fungal plant pathogens, such as *R. solani*, higher levels of oxalic acid production correlate with higher virulence (Nagarajkumar et al., 2005). The role of oxalic acid as a pathogenicity factor has been attributed to different mechanisms. First, oxalic acid may enhance infection of the host plant by lowering the pH of the colonized plant tissues, thereby creating favourable conditions for cell wall degrading enzymes such as polygalacturonases, pectinases, cellulases, hemicellulases or proteases (Cessna et al., 2000; Nagarajkumar et al., 2005; Schoonbeek et al., 2007; Li et al., 2014; Nakajima and Akutsu, 2014). Second, oxalic acid may act by sequestering Ca^{2+} ions in the plant cell wall, thereby weakening cell wall integrity and affecting Ca^{2+}-dependent defence responses (Cessna et al., 2000; Schoonbeek et al., 2007). Third, oxalic acid may modulate the oxidative burst, one of the first resistance responses of plant tissues upon infection. Several studies have indicated that oxalic acid acts by inhibiting the oxidative burst, reducing the ability of the host plant to defend against fungal attack (Cessna et al., 2000; Schoonbeek et al., 2007). However, research on *S. sclerotiorum* indicated that oxalic acid had dual opposing roles with an initial inhibition of the oxidative burst response and an enhanced oxidative burst at later stages of the infection (Williams et al., 2011).

Given the importance of oxalic acid as a virulence factor for numerous fungal plant pathogens, selection and application of microorganisms that can degrade oxalic acid has been proposed as a promising strategy for disease control (Yadav et al., 2012). For example, *Pseudomonas fluorescens* PfMDU2 reduced the severity of *R. solani* sheath blight on rice by 75%, most likely by detoxification of oxalic acid (Nagarajkumar et al., 2005). Recently, Bravo

et al. (2013) showed that oxalotrophic soil bacteria can migrate along fungal hyphae using them as "fungal highways" to seek for oxalate-rich compounds. Also Rudnick et al. (2015) proposed that oxalic acid may act as a signal molecule to guide mycophagous bacteria to the fungal hyphal tips for feeding. The ability of microorganisms to use oxalate, aerobically or anaerobically, as a carbon and energy source is referred to as oxalotrophy (Hervé et al., 2016). Oxalotrophic bacteria constitute a diverse taxonomic group, but a well-defined physiological group (Sahin, 2003). Hervé et al. (2016) examined the phylogenetic diversity of oxalotrophic bacteria across terrestrial and aquatic biomes and found that oxalate-degrading bacteria comprised three phyla, namely Actinobacteria, Firmicutes and Proteobacteria. The oxalotrophic bacterial genera reported to date include *Oxalobacter* (Abratt and Reid, 2010; Miller and Dearing, 2013; Giardina et al., 2014), *Oxalicibacterium* (Sahin and Aydin, 2006; Sahin et al., 2009), *Pseudomonas* (Sahin and Aydin, 2006; BravoMartin et al., 2013), *Streptomyces* (Sahin, 2004; Bravo et al., 2011), *Methylobacterium* (Sahin and Aydin, 2006; Bravo et al., 2011), and *Burkholderia* (Koch et al., 2014).

Oxalate can be degraded by three major pathways. In the first, oxalate is oxidized into CO_2 and hydrogen peroxide (H_2O_2) via oxalate oxidase, a pathway mainly utilized by plants (Svedružić et al., 2005; Lou et al., 2016), although oxalate oxidases have also been found in certain fungi (Escutia et al., 2005; Grąz et al., 2009; Yadav et al., 2012). Secondly, oxalate can be degraded to formate and CO_2 via oxalate decarboxylase, a pathway associated mainly with fungi (Svedružić et al., 2005) and bacteria (Tanner and Bornemann, 2000). Finally, oxalate can be degraded to formyl-CoA and CO_2 via oxalyl-CoA decarboxylase, a pathway associated mainly with bacteria (Svedružić et al., 2005). Furthermore, a variant of the third pathway was described for *Methylobacterium extorquens* (Schneider et al., 2012). Recently, Chapelle et al. (2015) proposed that in soils naturally suppressive to the root pathogen *R. solani*, oxalic acid produced by the pathogen activates specific plant responses and also enriches for specific rhizobacterial families with oxalotrophic traits that in turn suppress pathogen infection. More specifically, metatranscriptome analyses showed that members of the bacterial families *Oxalobacteraceae*, *Burkholderiaceae*, *Alcaligenaceae*, *Sphingomonadaceae*, *Cytophagaceae* and *Comamonadaceae* were activated on roots of sugar beet grown in the suppressive soil upon invasion by the fungal pathogen (Chapelle et al. 2015).

The overall aim of this study was to determine the density and phylogenetic diversity of oxalotrophic bacteria in a Dutch agricultural field soil used for sugar beet cultivation and to investigate their potential to suppress *Rhizoctonia* damping-off disease

of sugar beet. To this end, we enriched for oxalotrophic bacterial communities by incubation of the agricultural soil in a growth medium with oxalate as the sole carbon source. Subsequently, we isolated the oxalate-enriched bacterial community and tested their effect on *Rhizoctonia* damping-off disease in three independent soil bioassays. We then isolated and identified bacterial isolates from the oxalotrophy-enriched community and tested their efficacy, individually and in a synthetic community, to control *R. solani* damping-off disease.

Materials and methods

Soil collection and storage

The agricultural field soil was collected in June 2012 from a pear orchard located in Zwaagdijk, The Netherlands (52°41'53.549" N, 5°6'58.643" E) at a depth of 10-40 cm. The soil was air-dried, sieved (0.5 cm mesh) to remove plant and root material and stored at 8°C in darkness until use. The soil is conducive to *R. solani* damping-off disease of sugar beet. The soil physical properties were described previously in Gómez Expósito et al. (2015) and its chemical characteristics are described in Chapter 4.

Growth and storage conditions of the fungal pathogen

The fungal isolate *R. solani* AG2-2IIIB was provided by the Institute of Sugar Beet Research (IRS). It was kept in mineral oil at 10°C. Inoculum of the pathogen was prepared by growing *R. solani* on 1/5th potato dextrose agar (PDA, Oxoid) medium for one week at 25°C.

Enrichment and isolation of oxalotrophic bacteria

The naturally occurring oxalotrophic bacterial communities in the agricultural soil were enriched by transferring 2 g of soil in flasks containing 250 ml of Jin´s calcium oxalate (CaOx) medium (Jin et al., 2007) composed of 0.05 g/L K_2HPO_4, 0.05 g/L $MgSO_4$ * $7H_2O$, 4 g/L CaOx and 0.5 g/L yeast extract. As a control, the same amount of soil was added to the same medium with 4 g/L glucose. To avoid fungal growth, 100 µg/ml delvocid (DSM, The Netherlands) was added. Eight flasks per treatment were incubated at 25°C and 150 rpm for two weeks. Flasks were vortexed for 1 min and sonicated for 1 min to release bacterial cells from soil particles. The enrichments were filtered with miracloth (Calbiochem, US) and suspensions were centrifuged 5 min at 3,000 X *g* to remove most of soil particles. The supernatant was then centrifuged for 10 min at 9,000 X *g* to obtain most of the bacterial

cells. Cell pellets were resuspended in 10 ml of 10mM $MgSO_4$. For the recovery of pure bacterial cells, we used Nycodenz (a density gradient medium that allows the separation of organic material varying in weight and volume by separating the different type cells at different height upon centrifugation) as described in Chapelle et al. (2015) but using Nycodenz as gradient medium, prepared at 50% in 10 mM $MgSO_4$ (w/v). Five ml of the suspended bacterial cells were transferred to 15 ml tubes containing 7 ml of 50% Nycodenz and centrifuged for 45 min at 19,000 X *g* at 4°C. After centrifugation, bacterial cells (that appeared as a ring in the Nycodenz gradient) were harvested with a Pasteur pipette and washed two times with two volumes of 10 mM $MgSO_4$. Nycodenz was removed by centrifuging twice at 20,238 X *g* for 5 min. Bacterial cells were resuspended in 10 mM $MgSO_4$, their density was measured at OD_{600} and adjusted to 10^8 cells/ml using an OD_{600} equal to 1 equivalent to a density of 10^9 cells/ml (Fig. 1A).

Bacterial cell suspensions were mixed in a 1:1 ratio (v/v) with 80% (v/v) glycerol and stored at -80°C for further analyses (Fig. 1C). A second fraction of the bacterial suspension was centrifuged at 20,238 X *g* for 5 min and the bacterial cell pellet was stored at -80°C for DNA isolation. The remaining bacterial suspensions were used for the *in vivo* soil bioassay (Fig. 1B). The experiment was repeated two more times with some modifications. In brief, for the last two experiments, 10 g/L of CaOx or glucose were used, the media were incubated for 4 weeks and 10 replicates per medium were used. Harvesting of the cells was done as indicated above.

In vivo activity of oxalotrophy-enriched bacterial communities

The efficacy of the CaOx- and glucose-enriched bacterial communities to control *R. solani* damping-off disease was tested on sugar beet seedlings (Fig. 1B). Trays (19.5 x 6 x 3 cm) were filled with 250 g of agricultural soil with 20% (v/w) hydration. Fifteen sugar beet (*Beta vulgaris,* cultivar Rhino) seeds coated with thyram, hymexazol and poncho-beta were sown per tray and 100 µl of a suspension containing 10^8 cells/ml of the enriched bacterial community were inoculated onto each seed sown in the soil trays. Seeds treated with 10 mM $MgSO_4$ served as the control. Pots were placed in boxes with transparent lids in a growth chamber at 24°C with 70% humidity and a 16 h photoperiod and a light intensity of 150 micromol photons m^{-2} s^{-1}. Upon emergence of the sugar beet seedlings one week after sowing, *R. solani* was added to the soil by placing a fresh *R. solani* AG2-2IIIB agar plug (5 mm) with the mycelial side touching the upper root part of the first seedling in the row. *R. solani* disease symptoms were scored at regular intervals during 2-3 weeks by scoring the

number of plants suffering from damping-off disease. The bioassay was repeated three times, with 4 to 6 replicates per treatment.

Isolation and identification of oxalotrophic bacteria

Oxalotrophic bacteria were isolated by plating the bacterial community that was obtained in the first enrichment (Fig 1C and 2A). Fifty microliter of bacterial suspensions were serially diluted on Petri dishes containing 15 ml of R2A medium with an overlay of 5 ml of Jin´s calcium oxalate (CaOx) agar medium. Plates were incubated at 25°C for up to 1 month and checked once per week. The total number of colony forming units (CFU) after 1 month of growth was determined by plate counting. Colonies were re-plated on R2A+Jin´s CaOx medium for purification and, once pure, the isolates were checked for their ability to degrade CaOx on solid CaOx medium without the presence of R2A medium. Plates were incubated at 25°C for up to 6 weeks and plates were regularly monitored for halos around the bacterial colonies, which are indicative of CaOx degradation. Bacterial isolates were stored in 40% (v/v) glycerol at -80°C.

Hundred microliters of each glycerol stock containing the single bacterial isolates were transferred to 96-well plates and their 16S ribosomal RNA gene was sequenced at Baseclear (Leiden, The Netherlands) (Fig. 1C). Taxonomic assignment of each isolate was performed by comparing the obtained sequences to those deposited in the SILVA reference database (Quast et al., 2013). The isolates belonging to bacterial families found more active in a *R. solani* suppressive soil (Chapter 4) were selected for further analyses. For each of these families, phylogenetic trees were constructed by aligning the 16S sequences of the CaOx-degrading isolates and the type strains within each targeted family (retrieved from the Greengenes database (McDonald et al., 2012)) using Muscle (Edgar, 2004) and neighbor joining method in iTOL (http://itol.embl.de/) (Letunic and Bork, 2007).

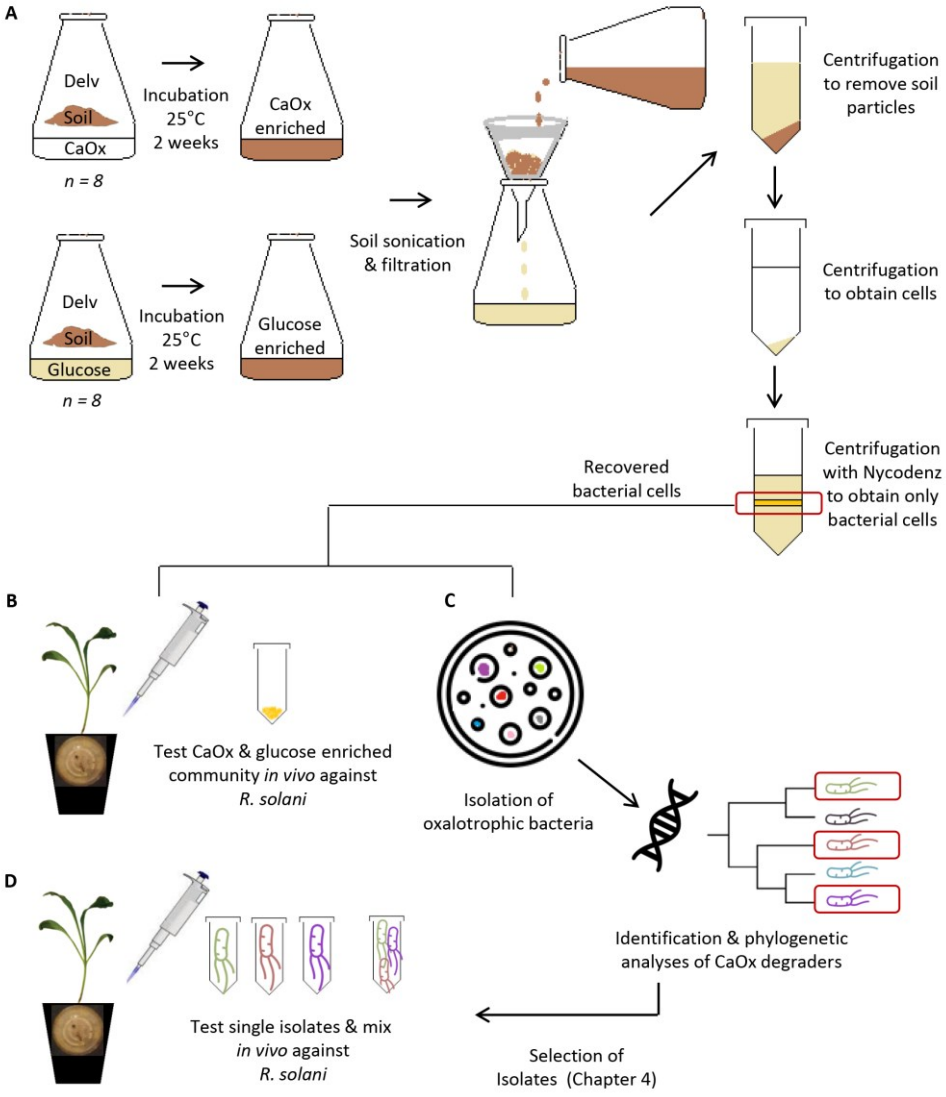

Figure 1. Schematic overview of the experimental design used to determine the role of oxalotrophic bacteria in *Rhizoctonia* disease suppression. A) Bacterial enrichments were performed by incubating 2 g of conducive soil in minimal medium containing delvocid (Delv) to eliminate fungal growth and either calcium oxalate (CaOx) or glucose as carbon source for 2 weeks at 25°C (*n=8*). Bacterial cells were harvested by sonication, filtration and several centrifugation steps, finalized by gradient centrifugation using Nycodenz; B) Bacterial cells from either the CaOx and glucose enrichments were applied to sugar beet seeds and challenged with *Rhizoctonia solani*; C) Cells from the CaOx enrichment were plated on CaOx medium and isolates collected and identified; D) Isolates within the bacterial families found more active in a *R. solani* suppressive soil (Chapter 4) were selected to test both their individual and their combined effect in suppressing *Rhizoctonia* disease.

In vivo activity of oxalotrophic bacterial isolates

Isolates belonging to families found more active in a *R. solani* suppressive soil (Chapter 4) were selected for the plant bioassays (Fig. 1D). In total, 10 isolates were selected and tested both individually and in a mixture with all 10 isolates. The selected isolates were grown on R2A plates at 25°C for five days to ensure enough biomass. Cells were harvested in 0.9% NaCl, washed 2 times with 0.9% NaCl at 7,000 X *g* for 10 min. The optical density of the bacterial suspensions was measured with a spectrophotometer at 600 nm and adjusted to 10^8 cells/ml. The bacterial mixture was prepared by mixing cell suspensions of all 10 isolates in an equal ratio (v/v). The bioassay was performed once and executed as described above.

Statistical analysis

Data were analysed in SPSS (version 23.0) by analysis of variance (ANOVA) followed by Dunnet post hoc tests.

Results

Enrichment and *in vivo* activity of oxalotrophic bacterial communities

We enriched for an oxalotrophic bacterial community by incubating soil samples in growth medium with calcium oxalate (CaOx) as the sole carbon source. The enriched bacterial communities were harvested by Nycodenz-gradient centrifugation and inoculated onto sugar beet seeds to test their efficacy to control *R. solani* damping-off disease. As a control, we included bacterial communities enriched in medium with glucose as the sole carbon source. The results of three independent enrichments and bioassays showed that the oxalate-enriched community significantly suppressed *R. solani* damping-off of sugar beet seedlings in two out of the three independent experiments (Fig. 2). Disease suppression by the oxalotrophic community was most prominent in the first bioassay. In contrast, the glucose-enriched community did not provide any disease control relative to the control treatment in any of the three experiments (Fig. 2).

Isolation and characterization of oxalotrophic bacteria

Bacterial isolates were obtained from the oxalate-enriched community used for bioassay A (Fig. 2A) by plating on medium containing CaOx as the sole carbon source. Plate counting

showed that 1 gram of dry soil, incubated in the medium with CaOx as the carbon source, contained 3.1 x 10^5 colony forming units (CFU) after 4 weeks of incubation. After dilution plating and subsequent colony purification, 537 isolates were obtained and identified by 16S-sequencing to belong to four different phyla: Actinobacteria (6%), Bacteroidetes (18%), Firmicutes (21.5%) and Proteobacteria (54.5%) (Table 1). The most abundant families within each phylum were *Micrococcaceae* and *Microbacteriaceae* (31.2% and 31.2% of the Actinobacteria, respectively), *Cytophagaceae* (94.9% of the Bacteroidetes), *Bacillaceae* (75.6% of the Firmicutes), and *Caulobacteraceae*, *Rhizobiaceae*, *Sphingomonadaceae*, *Xanthomonadaceae*, *Comamonadaceae* and *Oxalobacteraceae* (46.2%, 12.3%, 8.9%, 8.2%, 7.9% and 4.5% of the Proteobacteria, respectively) (Table 1, Fig. S1-S9).

Several isolates belonged to families previously found as more active in a *Rhizoctonia* suppressive soil, including *Micromonosporaceae*, *Nocardioidaceae*, *Caulobacteraceae*, *Rhizobiaceae*, *Bradyrhizobiaceae*, *Erythrobacteraceae*, *Sphingomonadaceae*, *Comamonadaceae* and *Rhodocyclaceae* (Chapter 4). Also, several of these families were found as more active in another *Rhizoctonia* suppressive soil, including *Microbacteriaceae, Cytophagaceae, Caulobacteraceae*, *Sphingomonadaceae*, *Xanthomonadaceae*, *Comamonadaceae* and *Oxalobacteraceae* (Chapelle et al., 2015). Phylogenetic analyses showed that most of our isolates clustered together. For example, within the *Caulobacteraceae*, 25.9% of the strains were closely related to *Caulobacter fusiformis*, and 32.6% were closely related to *Brevundimonas nasdae* (Fig. S1); all the isolates within the *Sphingomonadaceae* were closely related to *Sphingomonas soli* (Fig. S2); the majority of the isolates within the *Comamonadaceae* (60.8%) were closely related to *Caenimonas terrae* (Fig. S3); the majority of the isolates within the *Oxalobacteraceae* (76.9%) were closely related to *Oxalicibacterium solurbis* (Fig. S4); all isolates within the *Rhodocyclaceae* were closely related to *Denitratisoma oestradiolicum* (Fig. S5); the isolate within the *Erythrobacteraceae* was closely related to *Erythrobacter odishensis* (Fig. S6); within the *Rhizobiaceae*, 33.3% of the isolates were closely related to *Rhizobium selenitireducens* and 44.4% of the isolates were closely related to *Ensifer morelensis* (Fig. S7); the isolate within the *Bradyrhizobiaceae* was closely related to *Bradyrhizobium neotropicale* (Fig. S8); and all isolates within *Nocardioidaceae* were closely related to *Nocardioides daejeonensis* (Fig. S9).

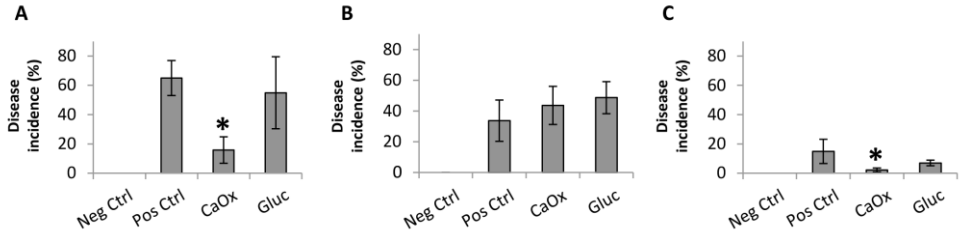

Figure 2. Effect of oxalotrophy-enriched bacteria on *Rhizoctonia* disease control on sugar beet seedlings. Conducive soil was inoculated in medium containing calcium oxalate (CaOx) or glucose (Gluc) as a sole carbon source and the enriched microbial community was inoculated at an initial density of 10^7 cells/sugar beet seed. The percentage of diseased sugar beet seedlings due to *R. solani* was determined after 14 (**A**), 21 (**B**), and 24 (**C**) days post inoculation (dpi) in three independent experiments. As controls, plants without *R. solani* (Neg ctrl) and plants without bacteria addition but with *R. solani* inoculation (Pos Ctrl) were included. An asterisk indicates a significant difference ($p < 0.05$) with the positive control treatment calculated by analysis of variance and Dunnet's post-hoc analyses.

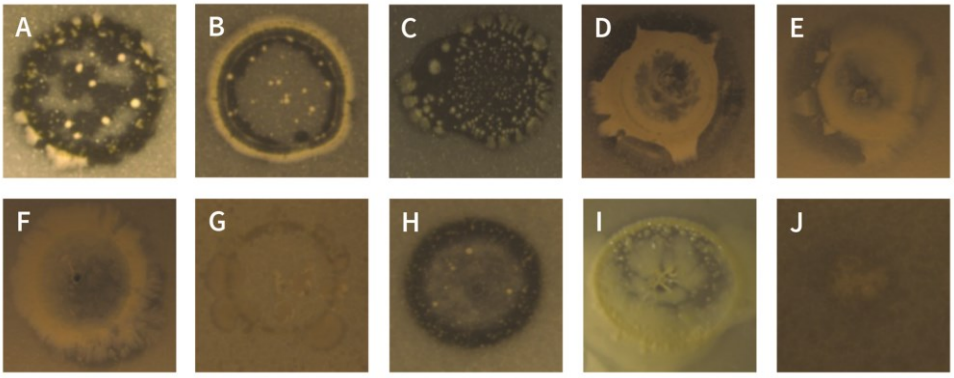

Figure 3. *In vitro* degradation of calcium oxalate by the selected bacterial isolates. Halos around the bacterial colonies represent degradation of calcium oxalate, after 6 weeks of incubation at 25˚C for **A)** *Caulobacter* sp. 43-37; **B)** *Sphingomonas* sp. 52-71; **C)** *Comamonadaceae* 41-92; **D)** *Oxalobacteraceae* 33-3; **E)** *Rhodocyclaceae* 42-88; **F)** *Brevundimonas* sp. 52-66; **G)** *Altererythobacter* sp. 63-11; **H)** *Rhizobium* sp. 42-41; **I)** *Bradyrhizobium* sp. 51-82; **J)** *Nocardioides* 53-15.

In vivo activity of oxalotrophic bacterial strains

Ten strains were selected that belonged to families previously found to be more active in the rhizosphere of sugar beet seedlings grown in a *R. solani* suppressive soil (Chapter 4). These families are *Caulobacteraceae*, *Sphingomonadaceae*, *Comamonadaceae*, *Oxalobacteraceae*, *Rhodocyclaceae*, *Erythrobacteraceae*, *Rhizobiaceae*, *Bradyrhizobiaceae*, and *Nocardioidaceae.* Their efficacy in suppressing *Rhizoctonia* damping-off *in vivo* was tested using the same setup as used in testing the oxalotrophic communities. For all ten strains, we first confirmed their oxalotrophic ability by growing on CaOx-amended medium as the sole carbon source (Fig. 3).

When applied to sugar beet seeds, 2 out of 10 strains (43-37 and 53-15) showed a significant reduction of *Rhizoctonia* damping-off disease compared to the non-inoculated control. The two strains reduced disease incidence by 40% and 45%, respectively, relative to the non-inoculated control (Fig. 4). When these two strains were part of a synthetic community of all ten strains, no significant disease suppression was observed (Fig. 4). They were classified as *Caulobacter* and *Nocardioides* species and were phylogenetically most closely related to *Caulobacter fusiformis* and *Nocardioides daejeonensis* (Fig. S1 and S9).

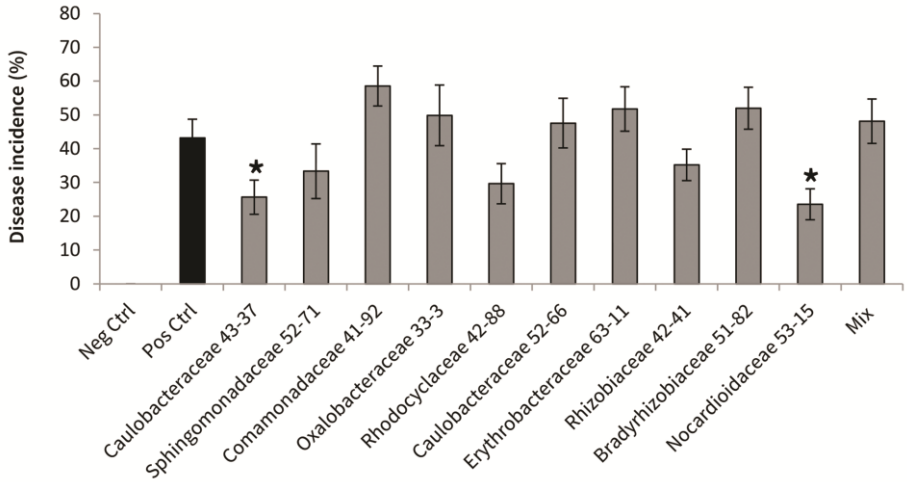

Figure 4. *In vivo Rhizoctonia* disease control on sugar beet seedlings by oxalotrophic isolates. From the oxalotrophy-enriched community single isolates were obtained and ten were selected for their ability to control *R. solani* damping-off when applied at an initial density of 10^7 cells/seed. The percentage of diseased sugar beet seedlings due to *R. solani* was determined after 21 days post inoculation (dpi). As controls, plants without *R. solani* (Neg Ctrl) and plants without bacteria addition but with *R. solani* inoculation (Pos Ctrl) were included. An asterisk indicates a significant difference ($p < 0.05$) with the control treatment calculated by analysis of variance and Dunnet's post-hoc analyses.

Table 1. Taxonomy of the oxalotrophic bacterial isolates obtained from the conducive agricultural soil after culturing with calcium oxalate as the sole carbon source.

Phylum	Class	Order	Family	Genus	# isolates
Actinobacteria	Actinobacteria	Micrococcales	Cellulomonadaceae	*Cellulomonas*	3
			Microbacteriaceae	*Agromyces*	1
				Microbacterium	9
			Micrococcaceae	*Arthrobacter*	10
			Promicromonosporaceae	*Cellulosimicrobium*	5
		Propionibacteriales	Nocardioidaceae	*Nocardioides*	4
Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	*Dyadobacter*	6
				Emticicia	86
				unclassified	1
	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	*Flavobacterium*	1
	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	*Chitinophaga*	3
				Parasegetibacter	1
Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	84
				Fictibacillus	3
			Paenibacillaceae	*Brevibacillus*	9
				Paenibacillus	12
			Planococcaceae	*Lysinibacillus*	1
				Paenisporosarcina	2
				Solibacillus	2
				unclassified	1
			Staphylococcaceae	*Staphylococcus*	1

Phylum	Class	Order	Family	Genus	# isolates
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	*Brevundimonas*	81
				Caulobacter	54
		Rhizobiales	Bradyrhizobiaceae	*Bradyrhizobium*	1
			Hyphomicrobiaceae	*Devosia*	5
			Rhizobiaceae	*Ensifer*	5
				Rhizobium	3
				Shinella	1
			Rhizobiales	unclassified	36
			Xanthobacteraceae	*Pseudolabrys*	1
		Rhodobacterales	Rhodobacteraceae	*Paracocccus*	1
		Rhodospirillales	Rhodospirillales	unclassified	4
		Sphingomonadales	Ellin6055	unclassified	1
			Erythrobacteraceae	*Altererythrobacter*	1
			Sphingomonadaceae	*Sphingomonas*	26
	Betaproteobacteria	Burkholderiales	Alcaligenaceae	unclassified	3
			Comamonadaceae	*Ramlibacter*	1
				Variovorax	2
				unclassified	20
			Oxalobacteraceae	*Herbaspirillum*	2
				unclassified	11
		Rhodocyclales	Rhodocyclaceae	uncultured	5
		unclassified	unclassified	unclassified	2
	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	2
		Xanthomonadales	Xanthomonadaceae	*Pseudoxanthomonas*	22
				Stenotrophomonas	1
				Thermomonas	1

Discussion

Microbial degradation or detoxification of oxalic acid produced as a virulence factor by plant pathogenic fungi has been proposed as an attractive strategy to control a range of plant diseases. This mechanism was also proposed to contribute to the natural suppressiveness of soils to damping-off disease caused by the soil-borne fungus *R. solani* (Chapelle et al., 2015). Here we showed that application of oxalotrophic soil bacterial communities to sugar beet seeds conferred significant suppression of *R. solani* damping-off disease in two out of three independent experiments, whereas glucose-enriched soil bacterial communities were not effective. Isolation and characterization revealed that the culturable oxalate-enriched bacterial community comprised phylogenetically diverse bacterial families belonging to the phyla Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria. Bioassays with ten representatives of these oxalotrophic bacterial families showed that *Caulobacter* and *Nocardioides* isolates significantly suppressed *R. solani* damping-off disease. These results confirm and extend previous studies that have shown that application of oxalotrophic bacteria reduced diseases caused by other oxalic acid producing pathogenic fungi, including *S. rolfsii* on bean (Dickman and Chet, 1998), grey mould disease caused by *B. cinerea* (Schoonbeek et al., 2007), and sheath blight of rice caused by *R. solani* AG1 (Nagarajkumar et al., 2005).

Oxalotrophy is a feature that is widely distributed among different bacterial taxa (Sahin, 2003). For example, Kost et al. (2013) showed that oxalotrophy is a trait of multiple plant-beneficial *Burkholderia* species (i.e. *B. phytofirmans*), but this trait seems to be absent in plant pathogenic (*B. glumae* and *B. plantarii*) and opportunistic human pathogenic (*B. cepacia*) *Burkholderia* species. They also showed that the *oxc* mutant of the plant beneficial endophyte *B. phytofirmans* PsJN is unable to grow on oxalate and had a reduced ability to colonize lupin and maize. Furthermore, oxalotrophic pathways in plant-beneficial *Burkholderia* Q208 were up-regulated during biofilm formation on the root surfaces of sugar cane (Paungfoo-Lonhienne et al., 2016).

The oxalotrophic bacterial taxa identified in our study belong to four different phyla, i.e. Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria. Hervé et al. (2016) recently reviewed the phylogenetic diversity of oxalotrophic bacteria in different natural habitats and concluded that oxalate-degrading bacteria were restricted to Actinobacteria, Firmicutes and Proteobacteria. Therefore, our study is the first, to our knowledge, where oxalotrophic Bacteroidetes were identified. The strains identified belong to the families *Cytophagaceae* (*Dyadobacter*, *Emticicia*), *Flavobacteriaceae* (*Flavobacterium*) and *Chitinophagaceae* (*Chitinophaga* and *Parasegetibacter*). Among the Actinobacteria, several

genera have already been reported as oxalotrophic, including *Microbacterium*, *Arthrobacter, Nocardioides* and *Cellulosimicrobium.* Among the Firmicutes, oxalotrophy has been described for *Bacillus* and *Paenibacillus* (Schoonbeek et al., 2007; Bravo et al., 2015; Maddaloni and Pascual, 2015). In our study we identified other oxalotrophic Firmicutes, including *Fictibacillus*, *Brevibacillus*, *Solibacillus* and *Lysinibacillus*. Among the Proteobacteria, *Rhizobium*, *Bradyrhizobium*, *Paracoccus*, *Variovorax*, *Pseudomonas* and *Stenotrophomonas* are well-known oxalotrophic genera (Sahin and Aydin, 2006; Anbazhagan et al., 2007; BravoCailleau et al., 2013; BravoMartin et al., 2013; Koch et al., 2014; Maddaloni and Pascual, 2015). Other oxalotrophic Proteobacterial genera identified in our study are *Brevundimonas*, *Caulobacter*, *Devosia*, *Ensifer*, *Sphingobium*, *Sphingomonas* and *Pseudoxanthomonas*.

When ten representatives of several of these different bacterial genera were tested for their *R. solani* suppressive ability, two strains (*Caulobacter* sp. 43-37 and *Nocardioides* sp. 53-15) significantly reduced *Rhizoctonia* damping-off. To our knowledge, neither *Caulobacter* nor *Nocardioides* have been previously reported as oxalotrophic bacteria and as antagonists of *R. solani*. In a *Rhizoctonia* suppressive soil *Caulobacteraceae* had a lower abundance than in a conducive soil (Mendes et al., 2011), but it was more active for three out of the four functions that were most upregulated in the *Rhizoctonia* suppressive soil (Chapelle et al., 2015). Regarding the role of *Nocardioidaceae* in suppressive soils, *Nocardiaceae* were more abundant in a soil suppressive to the bacterial pathogen *Streptomyces scabies* (Rosenzweig et al., 2012). However, in a *Rhizoctonia* suppressive soil, no enhanced abundance of *Nocardioidaceae* was observed (Mendes et al., 2011). Hence, the exact role of these two bacterial genera as well as the role of oxalotrophy in suppressiveness of soils to *R. solani* require further studies.

Because disease suppression is thought to be the result of the combined activity of multiple microbes rather than single species or strains (Lemanceau and Alabouvette, 1991; Raaijmakers et al., 1995; Weller et al., 2002; Mazzola, 2004), we also tested whether application of mixtures of different oxalotrophic bacterial species could enhance suppression of *R. solani*. Our results showed that application of the bacterial mixture did not enhance disease suppression when compared with the activity of the application of the single bacterial strains. Even though in some cases an enhancement of the production of secondary metabolites was observed in bacterial mixtures (de Boer et al., 2007; Garbeva and de Boer, 2009), also a loss of antimicrobial activity by microorganisms can occur due to

competitive interactions that may affect the activity or survival of key strains (Jousset et al., 2011). Since our mixture contained ten different oxalotrophic strains from nine different bacterial families, the interspecific competition may have adversely affected the biocontrol efficacy of individual strains, for example due to the degradation of bioactive secondary metabolites of one strain by other members of the consortium (Stockwell et al., 2010). In addition, the density of each of the microbial strains as well as their spatial distribution may not have been representative of their densities and niche distributions in the natural soil or rhizosphere habitat.

Collectively, the results presented in this study indicate that oxalotrophic bacteria are promising candidates for microbial control of *Rhizoctonia* damping-off disease and possibly other soil-borne diseases caused by oxalic acid-producing pathogens. Jayaraj et al. (2010) showed that spray application of oxalic acid on rice leaves reduced sheath blight due to *R. solani*. Since amendments of organic compounds have been associated with an enhanced disease suppressiveness of soils due to an increment in microbial activities (Bonanomi et al., 2010), application of oxalates directly to soil may serve to boost the oxalotrophic activity of the indigenous oxalotrophic microbial population, eliminating the practical and legislative difficulties of introducing microorganism into diverse environments.

Author contributions

All authors were involved in the design of the experiments. RGE performed the experiments, analyzed the data, created the figures and drafted the manuscript. All authors supervised the experimental design and contributed to the revision and writing of the manuscript. This research was funded by the Dutch Technology Foundation (STW).

Supplementary material

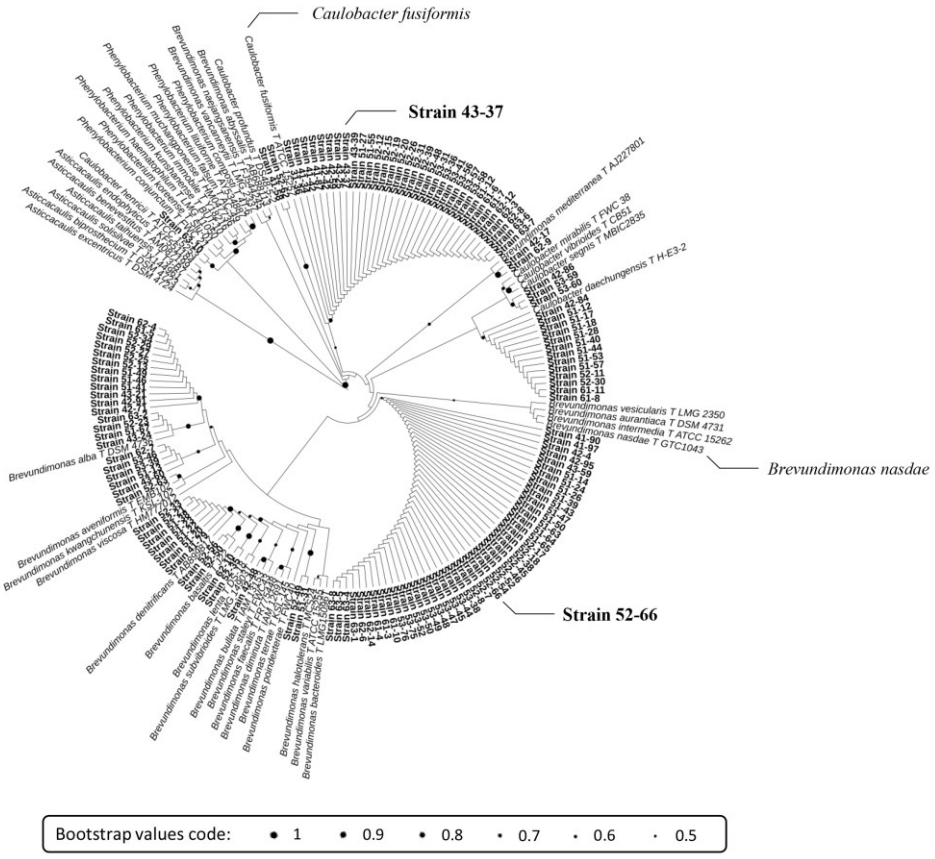

Figure S1. Phylogenetic tree of the 16S ribosomal gene (16S rRNA) of the type strains and strains obtained from the enrichment with CaOx belonging to the family *Caulobacteraceae*. The evolutionary relationship of the 16S rRNA sequences of the *Caulobacteraceae* isolates (in bold) and the type strains (in italics, downloaded from the Ribosomal Database Project (RDP)) was inferred by alignment with Muscle and neighbor-joining tree construction with 1,000 resampled data sets. The type strains are indicated with a 'T' followed by the strain name/ID number. The strains selected for further analyses (43-37 and 52-66) and their closest type strains (*Caulobacter fusiformis* and *Brevundimonas nasdae*) are highlighted.

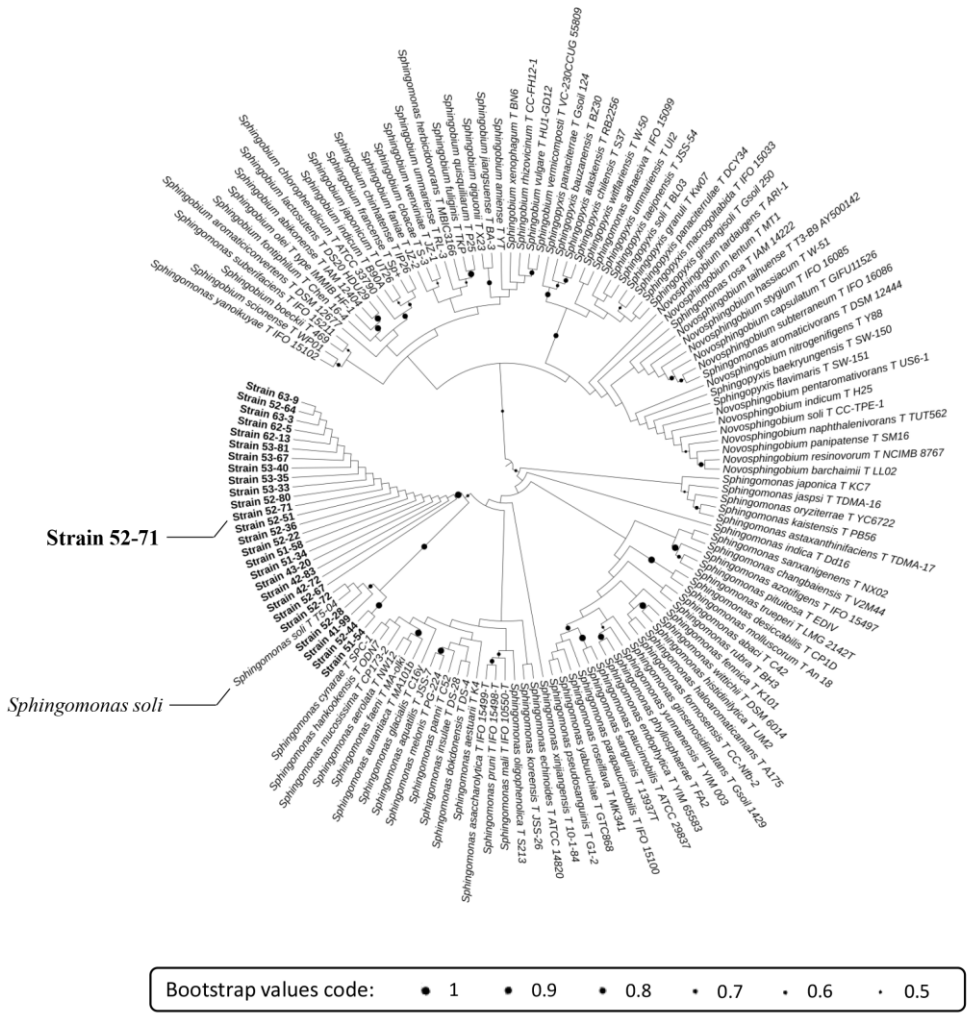

Figure S2. Phylogenetic tree of the 16S ribosomal gene (16S rRNA) of the type strains and strains obtained from the enrichment with CaOx belonging to the family *Sphingomonadaceae*. The evolutionary relationship of the 16S rRNA sequences of the *Sphingomonadaceae* isolates (in bold) and the type strains (in italics, downloaded from the Ribosomal Database Project (RDP)) was inferred by alignment with Muscle and neighbor-joining tree construction with 1,000 resampled data sets. The type strains are indicated with a 'T' followed by the strain name/ID number. The strain selected for further analyses (52-71) and its closest type strain (*Sphingomonas soli*) are highlighted.

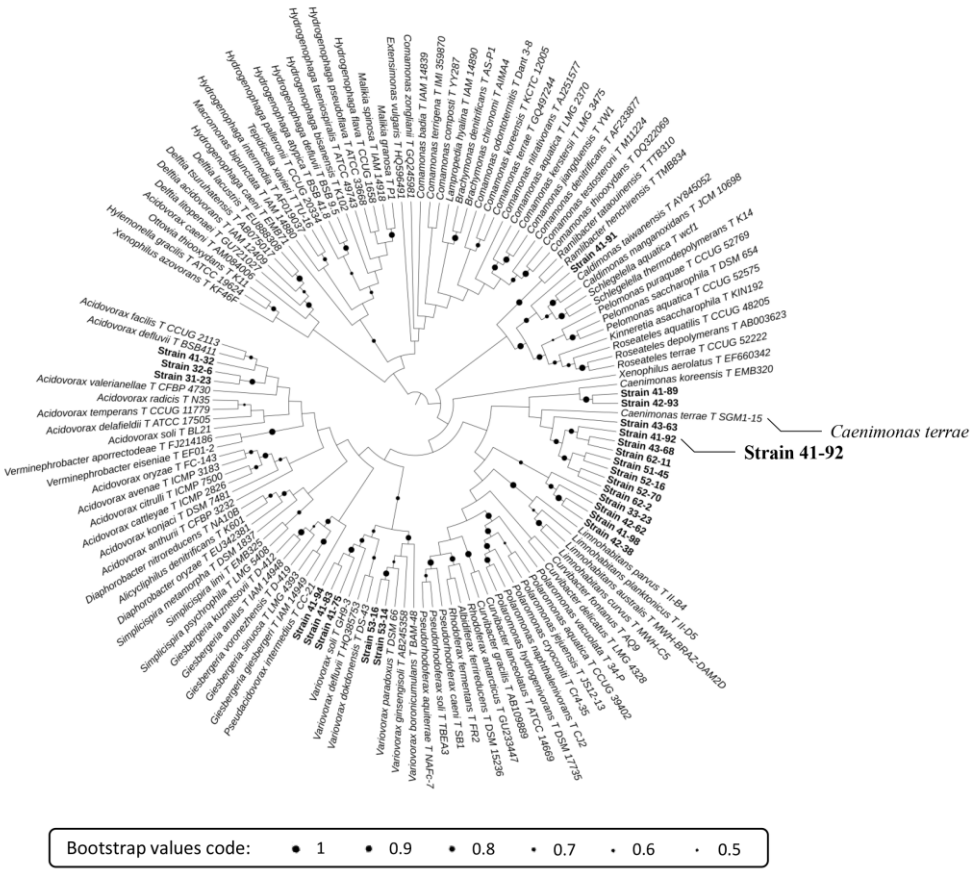

Figure S3. Phylogenetic tree of the 16S ribosomal gene (16S rRNA) of the type strains and strains obtained from the enrichment with CaOx belonging to the family *Comamonadaceae*. The evolutionary relationship of the 16S rRNA sequences of the *Comamonadaceae* isolates (in bold) and the type strains (in italics, downloaded from the Ribosomal Database Project (RDP)) was inferred by alignment with Muscle and neighbor-joining tree construction with 1,000 resampled data sets. The type strains are indicated with a 'T' followed by the strain name/ID number. The strain selected for further analyses (41-92) and its closest type strain (*Caenimonas terrae*) are highlighted.

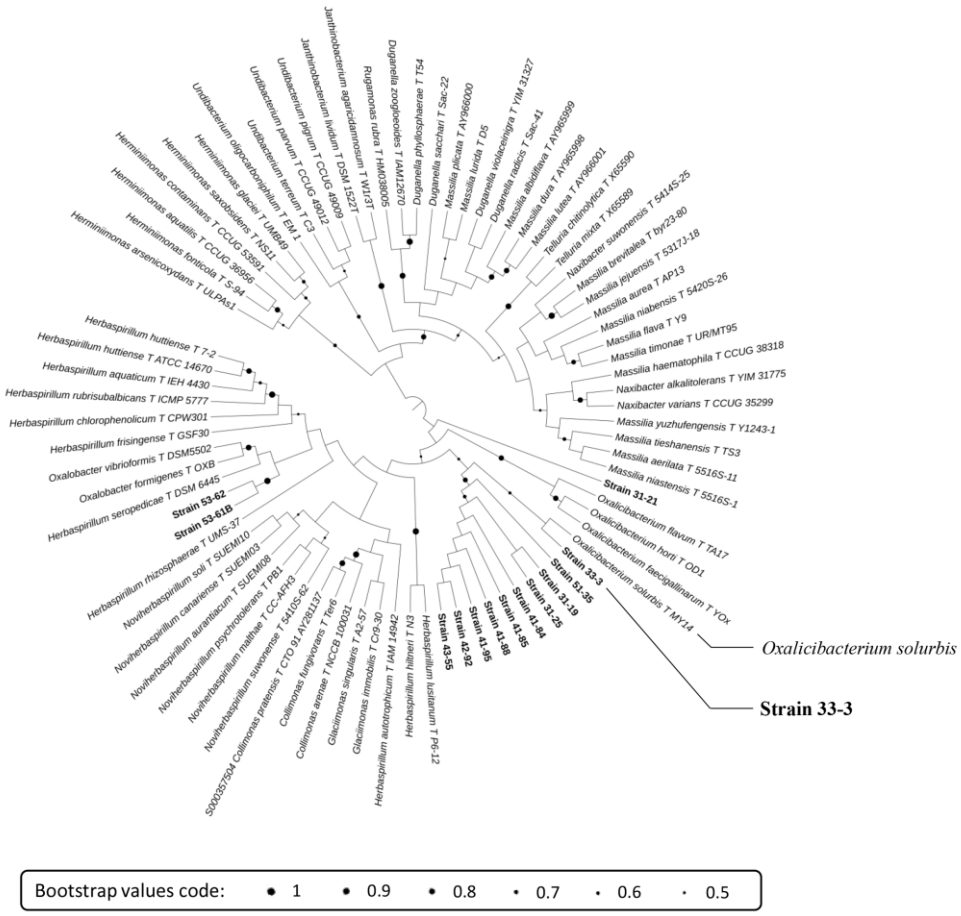

Figure S4. Phylogenetic tree of the 16S ribosomal gene (16S rRNA) of the type strains and strains obtained from the enrichment with CaOx belonging to the family *Oxalobacteraceae.* The evolutionary relationship of the 16S rRNA sequences of the *Oxalobacteraceae* isolates (in bold) and the type strains (in italics, downloaded from the Ribosomal Database Project (RDP)) was inferred by alignment with Muscle and neighbor-joining tree construction with 1,000 resampled data sets. The type strains are indicated with a 'T' followed by the strain name/ID number. The strain selected for further analyses (33-3) and its closest type strain (*Oxalicibacterium solurbis*) are highlighted.

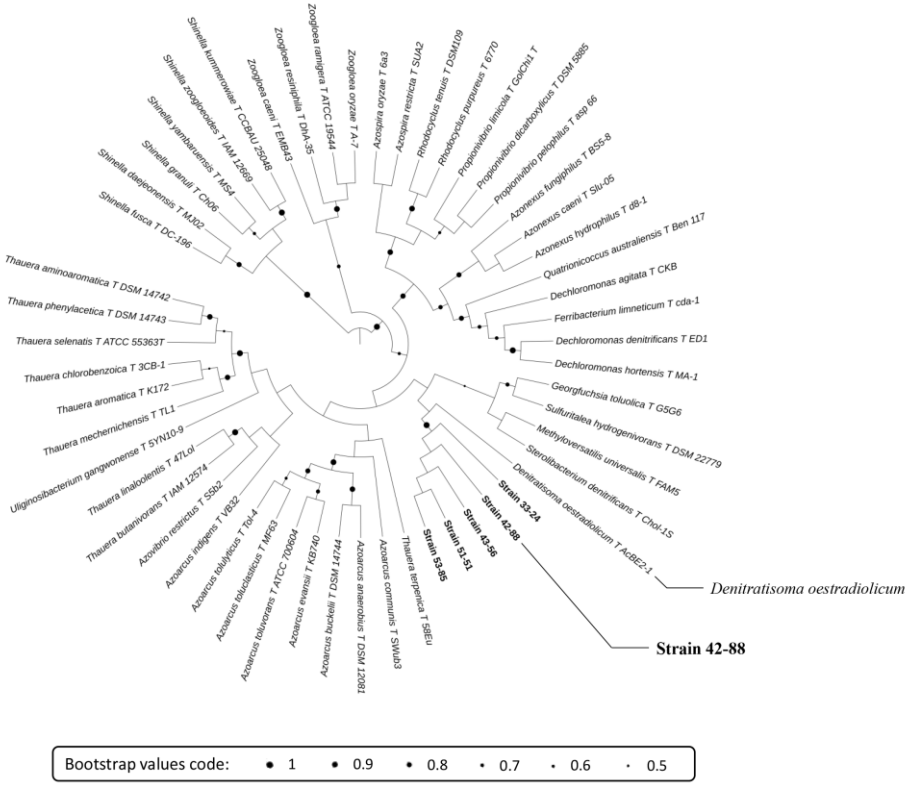

Figure S5. Phylogenetic tree of the 16S ribosomal gene (16S rRNA) of the type strains and strains obtained from the enrichment with CaOx belonging to the family *Rhodocyclaceae*. The evolutionary relationship of the 16S rRNA sequences of the *Rhodocyclaceae* isolates (in bold) and the type strains (in italics, downloaded from the Ribosomal Database Project (RDP)) was inferred by alignment with Muscle and neighbor-joining tree construction with 1,000 resampled data sets. The type strains are indicated with a 'T' followed by the strain name/ID number. The strain selected for further analyses (42-88) and its closest type strain (*Denitratisoma oestradiolicum*) are highlighted.

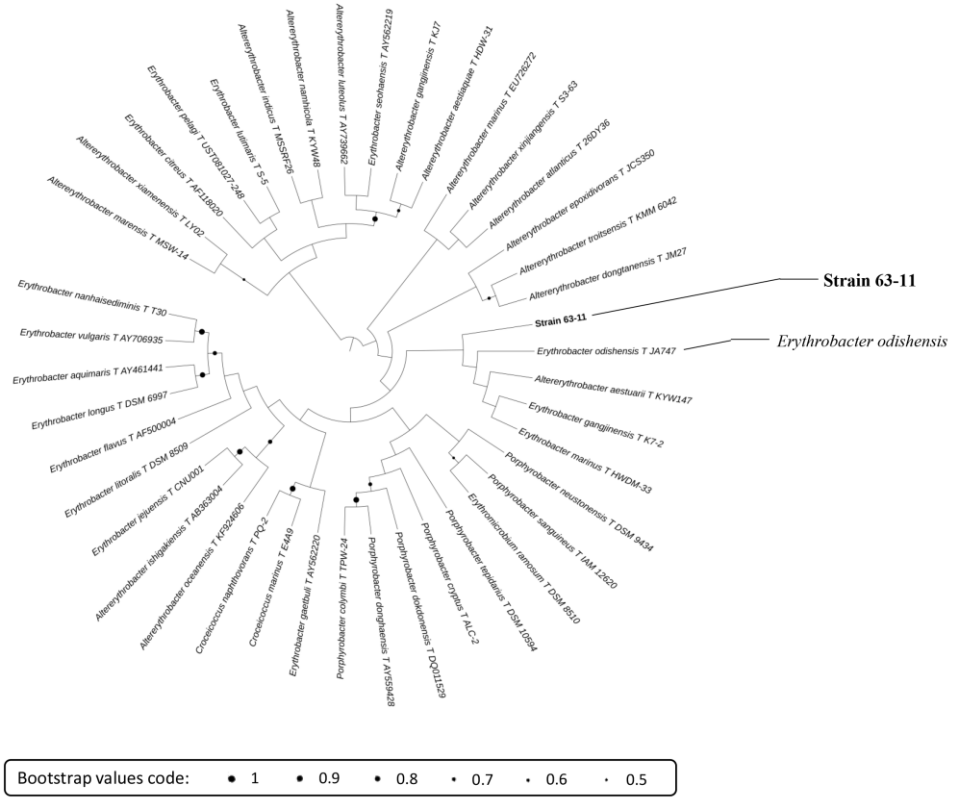

Figure S6. Phylogenetic tree of the 16S ribosomal gene (16S rRNA) of the type strains and strains obtained from the enrichment with CaOx belonging to the family *Erythrobacteraceae*. The evolutionary relationship of the 16S rRNA sequences of the *Erythrobacteraceae* isolates (in bold) and the type strains (in italics, downloaded from the Ribosomal Database Project (RDP)) was inferred by alignment with Muscle and neighbor-joining tree construction with 1,000 resampled data sets. The type strains are indicated with a 'T' followed by the strain name/ID number. The strain selected for further analyses (63-11) and its closest type strain (*Erythrobacter odishensis*) are highlighte

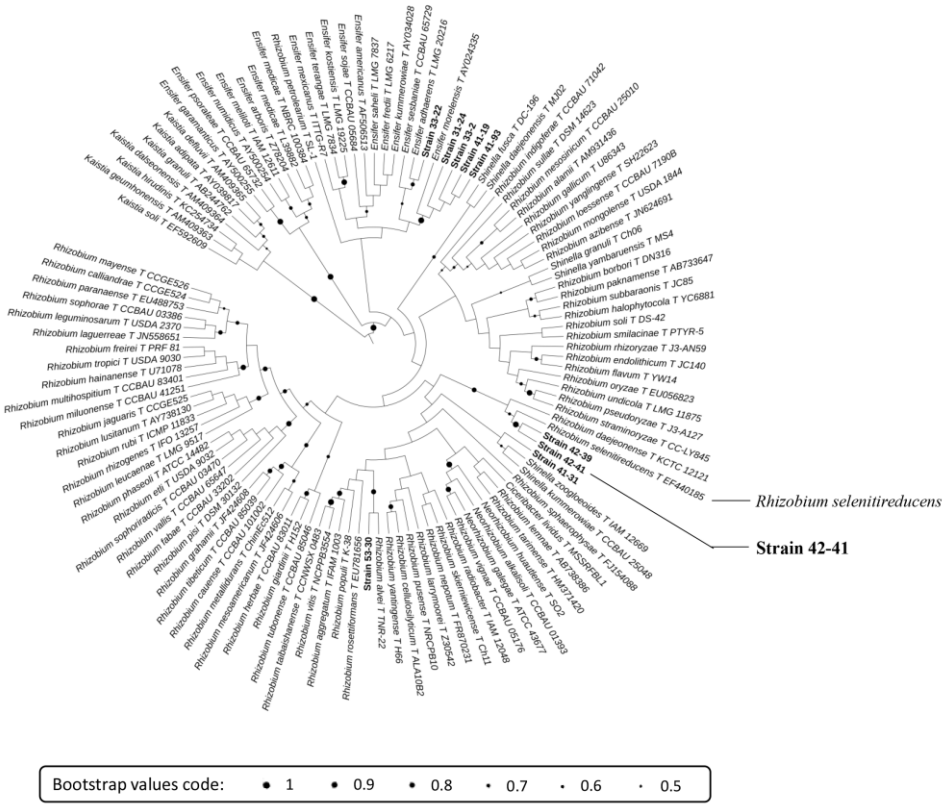

Figure S7. Phylogenetic tree of the 16S ribosomal gene (16S rRNA) of the type strains and strains obtained from the enrichment with CaOx belonging to the family *Rhizobiaceae*. The evolutionary relationship of the 16S rRNA sequences of the *Rhizobiaceae* isolates (in bold) and the type strains (in italics, downloaded from the Ribosomal Database Project (RDP)) was inferred by alignment with Muscle and neighbor-joining tree construction with 1,000 resampled data sets. The type strains are indicated with a 'T' followed by the strain name/ID number. The strain selected for further analyses (42-41) and its closest type strain (*Rhizobium selenitireducens*) are highlighted.

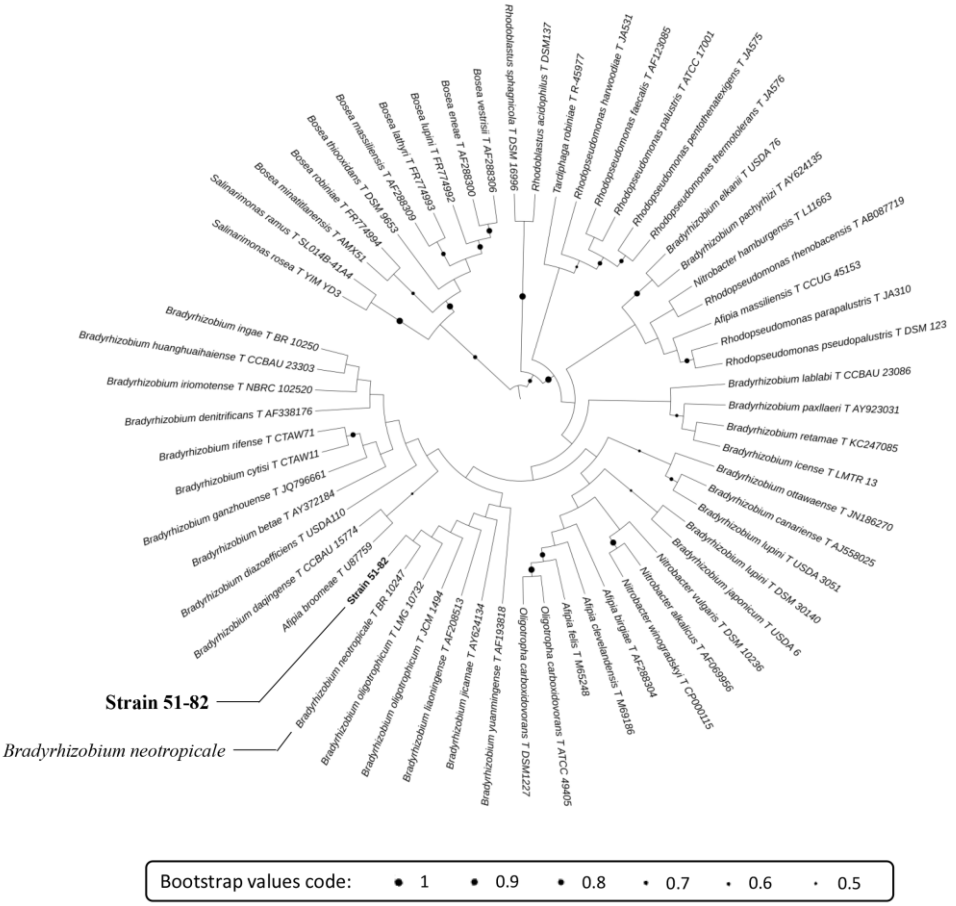

Figure S8. Phylogenetic tree of the 16S ribosomal gene (16S rRNA) of the type strains and strains obtained from the enrichment with CaOx belonging to the family *Bradyrhizobiaceae.* The evolutionary relationship of the 16S rRNA sequences of the *Bradyrhizobiaceae* isolates (in bold) and the type strains (in italics, downloaded from the Ribosomal Database Project (RDP)) was inferred by alignment with Muscle and neighbor-joining tree construction with 1,000 resampled data sets. The type strains are indicated with a 'T' followed by the strain name/ID number. The strain selected for further analyses (51-82) and its closest type strain (*Bradyrhizobium neotropicale*) are highlighted.

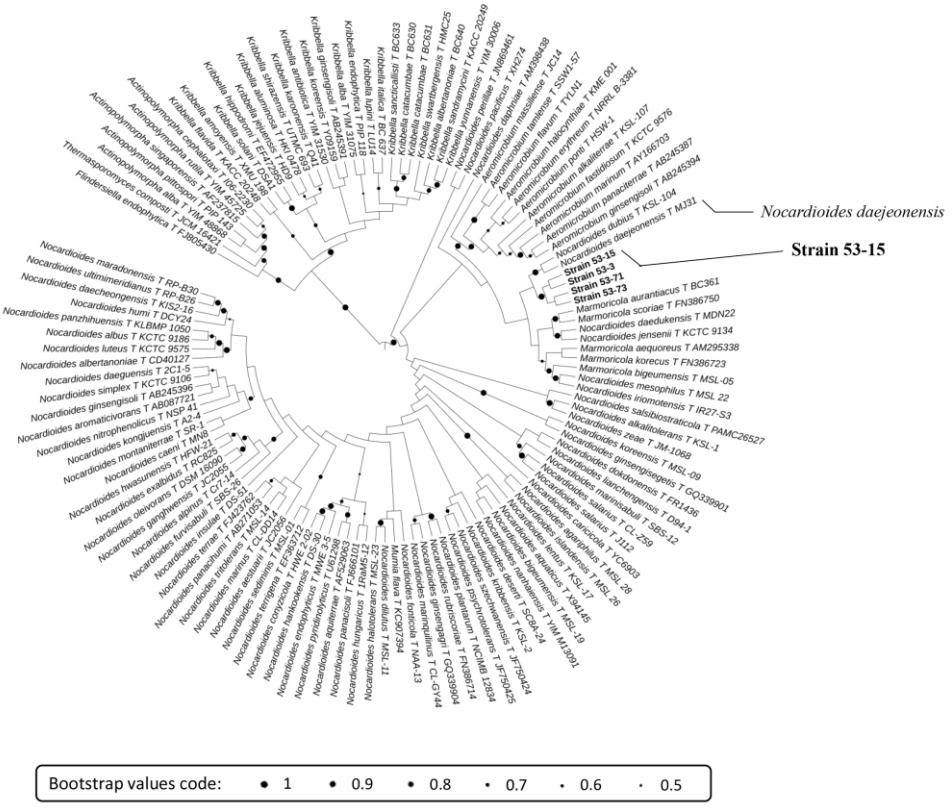

Figure S9. Phylogenetic tree of the 16S ribosomal gene (16S rRNA) of the type strains and strains obtained from the enrichment with CaOx belonging to the family *Nocardioidaceae.* The evolutionary relationship of the 16S rRNA sequences of the *Nocardioidaceae* isolates (in bold) and the type strains (in italics, downloaded from the Ribosomal Database Project (RDP)) was inferred by alignment with Muscle and neighbor-joining tree construction with 1,000 resampled data sets. The type strains are indicated with a 'T' followed by the strain name/ID number. The strain selected for further analyses (53-15) and its closest type strain (*Nocardoides daejeonensis*) are highlighted

Chapter 6

General discussion

Suppressive soils are soils in which soil-borne plant pathogens cause little or no disease due to specific microbial activities taking place in the soil and rhizosphere (Mazzola and Gu, 2002; Weller et al., 2002; Cha et al., 2016). Because of their microbiological origin, suppressive soils constitute an important source of microorganisms with specific antagonistic activities towards plant pathogens (Lemanceau et al., 2006; Rodríguez et al., 2015). Extensive work has been done to untangle which microbes contribute to disease suppression and through which mechanisms. Isolation and re-introduction of these microbes into natural environments may provide an effective alternative for plant disease control that is more ecologically sound than the application of broad-spectrum pesticides or soil fumigants. To date, re-introduction of several microbial genera isolated from disease suppressive soils has been shown to confer disease suppression. However, their suppressive capabilities are often restricted to assays conducted under controlled conditions and the majority of these microbes fail when applied in the field (Mazzola and Freilich, 2016). In this thesis, we investigated the role of different bacterial groups with antagonistic potential, both individually and in consortia, in suppressing the soil-borne fungal pathogen *Rhizoctonia solani* (Chapters 2, 3, 4 and 5) and in promoting growth of different plant species (Chapter 2).

For a better understanding of the microbiological basis of disease suppressive soils, we developed a small-scale reproducible bioassay to induce disease suppressiveness against *R. solani,* mimicking the dynamics of suppressiveness induction observed under field conditions (Chapter 4). This allowed us to analyze the successional changes in rhizobacterial community composition and functions associated with the soil immune response. Finally, the disease suppressive effects of bacterial groups with specific traits previously associated with disease suppressive soils (Chapter 4) and with traits found up-regulated in the induced suppressive soil (Chapter 5) were tested. In this chapter (Chapter 6), the major findings presented in this thesis are integrated and discussed in the context of other published studies. Figure 1 depicts the main research questions addressed in this thesis and summarizes the major findings.

Role of *Lysobacter* species in *Rhizoctonia* suppressive soils

Previous studies compared the microbial community composition of *Rhizoctonia* suppressive and non-suppressive (conducive) soils to seek for bioindicators of soil suppressiveness. Many different microorganisms have been suggested to play a role (Mendes et al., 2015; Chapelle et al., 2015; van der Voort et al., 2016), but none of these have been proven to be the sole causal factor in the disease suppression. One bacterial genus that has previously been associated with *Rhizoctonia* disease suppressive soils is *Lysobacter* (Postma et al., 2008; Postma et al., 2010). For different *Lysobacter* species and strains, isolation, often from suppressive soils, and effective suppression of *Rhizoctonia* disease in conducive soil has been demonstrated in the greenhouse (Giesler and Yuen, 1998; Puopolo et al., 2010). In the framework of this thesis, soil bioassays with 18 *Lysobacter* strains, mostly retrieved from *Rhizoctonia* suppressive soils, were performed but none of these showed a consistent reduction of damping-off disease of sugar beet caused by *R. solani* (Chapter 2). Phenotypic characterization of these 18 *Lysobacter* strains did reveal that most *Lysobacter* strains exhibited enzymatic activities involved in the degradation of the cell wall of pathogens as well as inhibition of hyphal growth or spore germination of a variety of plant pathogens, including *R. solani* (Chapter 2). Previous studies have indeed demonstrated that members of the genus *Lysobacter* harbour a substantial arsenal of genes encoding a variety of extracellular enzymes and secondary metabolites active against pathogens (Xie et al., 2012; de Bruijn et al., 2015). For example, production of extracellular enzymes such as proteases, endopeptidases, glucanases, lipases, chitinases, chitosanases or cellulases, has been demonstrated for *Lysobacter* species (Lapteva et al., 2012; Xu et al., 2015; Saraihom et al., 2016; Xu et al., 2016). Production of secondary metabolites by *Lysobacter* species includes antibacterial compounds such as lysobactin, WAP-8294A, tripopeptins, cephabacins, lactivicin and myxin, and antifungal compounds such as dihydromaltophilin and xanthobaccins (Xie et al., 2012). Furthermore, comparative genomics of five *Lysobacter* strains, four of which (*L. antibioticus* strain 76 (L08), *L. capsici* strain 55 (L14), *L. enzymogenes* C3 (L29), and *L. gummosus* strain 3.2.11 (L15)) were used in this thesis (Chapters 2 and 3), revealed multiple gene clusters involved in the biosynthesis of known and yet unidentified bioactive compounds (De Bruijn et al., 2015). These studies indicate that *Lysobacter* species have an enormous and untapped metabolic potential. Nevertheless, the four species did not show consistent suppression of *Rhizoctonia* damping-off disease when tested in soil bioassays. Moreover, analyses of their ability to colonize the roots indicated that these *Lysobacter* isolates are poor root colonizers on sugar beet and cauliflower (Chapter 2).

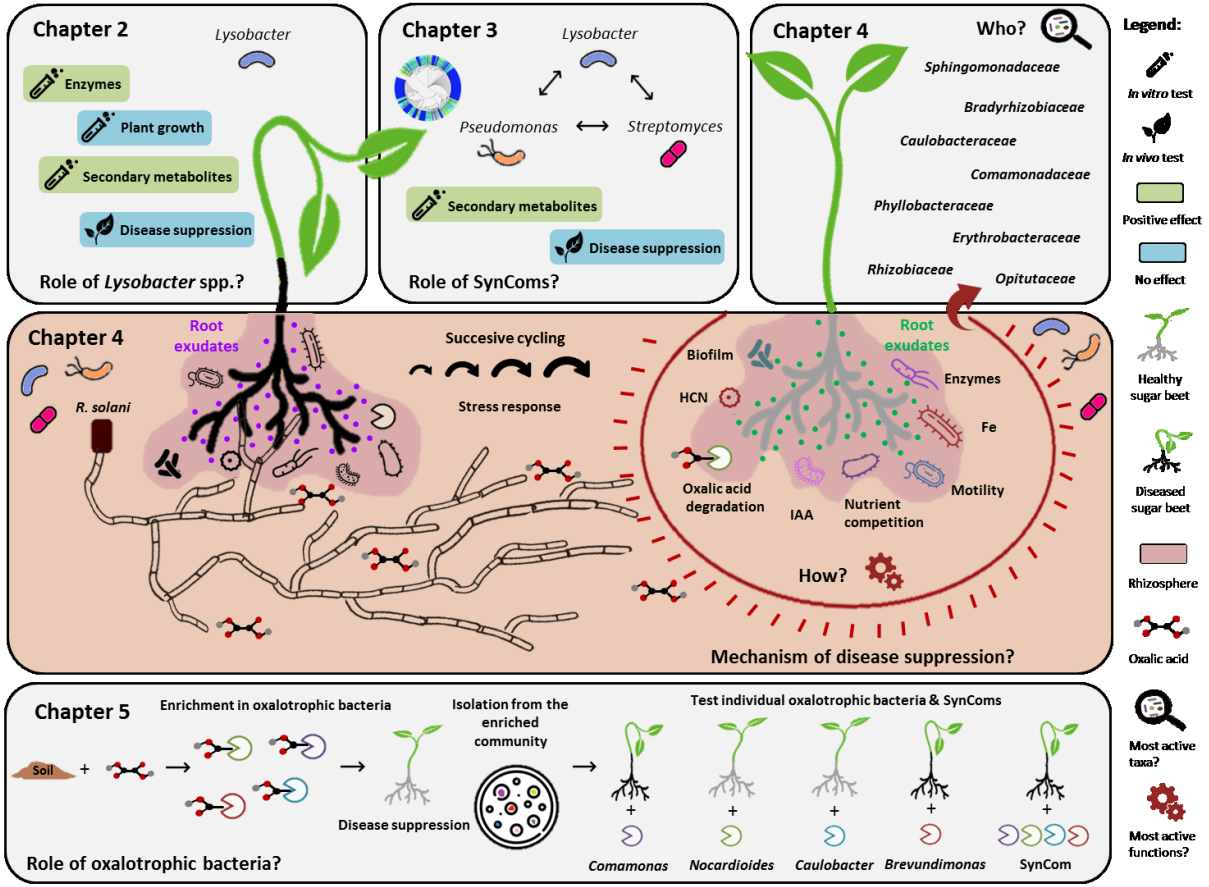

Figure 1. Unravelling the dynamics in bacterial communities and functions involved in the development of the soil immune response to suppress damping-off disease caused by *Rhizoctonia solani*. Schematic overview of the main research questions and most of the findings described in this thesis.

To investigate the role of *Lysobacter* in *Rhizoctonia* suppressive soils in more depth, we analyzed the changes in the indigenous *Lysobacter* populations in soil during the transition from a conducive to a suppressive state (Chapter 4). For quantifying the *Lysobacter* population, the 16 rRNA gene of the *Lysobacter* spp. was amplified by TaqMan using a specific probe as described in Postma et al. (2011). It appeared that the *Lysobacter* population tended to decrease with the successive growth cycles. Furthermore, the *Lysobacter* populations were very similar in a soil suppressive and a soil conducive to *R. solani* after 8 cycles of sugar beet growth (Fig. 2A). The dynamic changes of *Lysobacter* species during suppressiveness induction were also analyzed by counting the reads associated with *Lysobacter* in a metagenomic study based on 16S amplicon sequencing (Chapter 4). This confirmed the results obtained by TaqMan (Fig. 2B). When testing their potential to promote plant growth under *in vitro* conditions, no consistent plant growth promotion for any of the plant species tested was observed, neither *via* direct contact nor *via* volatile production (Chapter 2).

Role of bacterial consortia in *Rhizoctonia* suppressiveness

The control of soil-borne pathogens observed in disease suppressive soils has been proposed to be attributable to the combined activities of microbial consortia (Lemanceau et al., 2006). Microbial consortia or synthetic microbial communities have been suggested to provide an experimental means to unravel the conditions that are required to trigger interaction patterns like symbiosis or competition (Großkopf and Soyer, 2014). In the framework of this thesis, we created synthetic bacterial communities to investigate if the combined effect of different microorganisms with different modes of action will result in a stronger and more stable antagonistic activity against *R. solani* than their individual activities. *Pseudomonas* and *Streptomyces* have been consistently found in higher abundance in soils suppressive to *R. solani* (Postma et al., 2008; Postma et al., 2010; Mendes et al., 2011; Cha et al., 2016; Latz et al., 2016), and several isolates could suppress *R. solani* in greenhouse experiments (Adesina et al., 2009; Grosch et al., 2009; Patil et al., 2010; Wang et al., 2015). Thus, we combined *Lysobacter* with *Pseudomonas* and/or *Streptomyces* to determine if these consortia showed an enhanced or more consistent antifungal activity against *R. solani*. Although several of these bacterial combinations led to an increased antifungal activity *in vitro* compared with their individual activities, no consistent suppressive effect of damping-off disease of sugar beet was found for any of the synthetic communities in soil (Chapter 3). One reason for the lack of a consistent effect of our

synthetic communities against *R. solani* could be incompatibility of the modes of action, for example by production of enzymes by one strain that degrade secondary metabolites produced by the other (Stockwell et al., 2010; 2011).

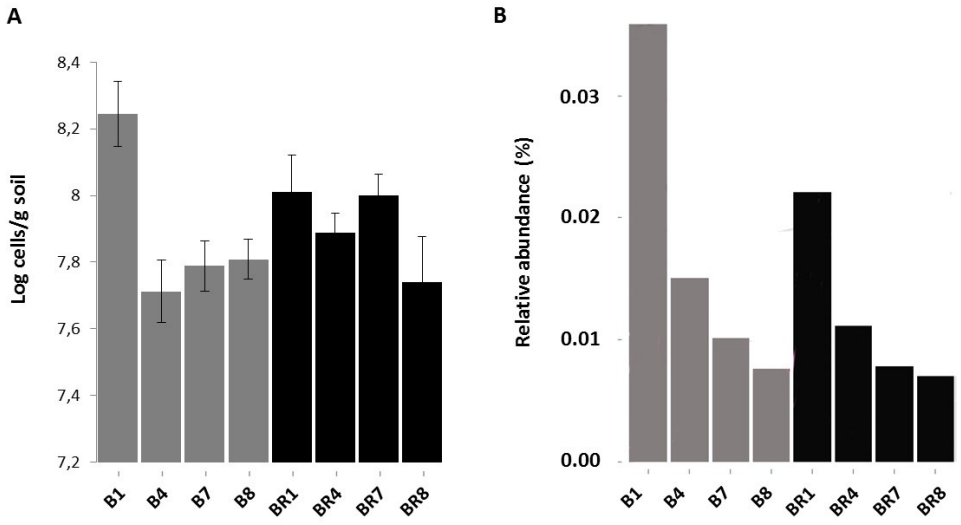

Figure 2. *Lysobacter* quantification in soils cycled with sugar beet, or with sugar beet and *R. solani*. In the latter (indicated by BR1 - BR8 in which the number refers to the cycle) there was induction of disease suppressiveness against *Rhizoctonia solani* whereas soils cycled with sugar beet only (B1 - B8) remained conducive. A) Number of *Lysobacter* cells per gram of rhizospheric soil (log cells/g soil) detected by TaqMan. B) Relative abundance (%) of *Lysobacter* reads detected by 16S amplicon sequencing.

Induction of the soil immune response towards *Rhizoctonia solani*: function over phylogeny

Suppressiveness induction by continuous mono-cropping has been observed in the field for several fungal pathogens, including *R. solani* (Postma et al., 2010; Mendes et al., 2011; Chapelle et al., 2015; van der Voort et al., 2016). This soil immune response towards *R. solani* could be mimicked under controlled conditions by successive growth of sugar beet seedlings in presence of the pathogen (Chapter 4), but not when the pathogen was absent. Presence of the pathogen has been suggested as a requirement for inducing suppressiveness, in a similar manner as the acquired immune response in animals necessitates exposure to the pathogen (Raaijmakers and Mazzola, 2016). Sayama et al.

(2001) demonstrated that inoculation of "living mycelium" was required to trigger disease suppression of *R. solani* on sugar beet. Collectively, these results suggest that virulence factors or other metabolites produced by *Rhizoctonia* or in interaction with its host plant may be sensed as signal molecules by the neighboring microbial community to undertake a response against its invasion.

The ability to induce soil suppressiveness allowed studying the successional changes in bacterial community composition and functions during the transition from a disease conducive to a suppressive state. Analysis of the dynamics in the community composition revealed that the successive growth of sugar beet had a significant effect in shaping the bacterial community composition. Plants secrete exudates into the rhizosphere and their chemical composition is, in part, specific to the plant species (Garbeva et al., 2008; Bouffaud et al., 2014; Ofek et al., 2014) and even to the genotype of a given plant species (Micallef et al., 2009; Hardoim et al., 2011; Weinert et al., 2011). Root exudates not only serve as a source of nutrients for soil microbes, but also as chemo attractant in microbial recruitment (Dakora and Phillips, 2002; Berendsen et al., 2012). Under environmental stress conditions (i.e. herbivore or pathogen attack), plants can modify the composition of these root exudates leading to the recruitment of different microbes or to the adjustment of their activities to relieve the stress (Rudrappa et al., 2008; Bever et al., 2012; Philippot et al., 2013; Gu et al., 2016). Combined with previous results obtained for another *Rhizoctonia* suppressive soil (Mendes et al., 2011), we expected distinct changes in the rhizobacterial community composition during the induction of disease suppressiveness. Our results, however, showed that the rhizobacterial community composition in suppressive and conducive soils were highly similar. Only a few operational taxonomic units (OTUs) belonging to specific families differed in abundance. This could mean that other soil (micro)organisms, rather than rhizobacteria, play a major role in suppressiveness to *R. solani.*

When looking into the transcriptome of the rhizobacterial community, changes were much more prominent between suppressive and conducive soil. This suggests that the transition from a conducive into a suppressive soil is mainly associated with changes in the activity of the resident bacterial community rather than with changes in the bacterial community composition. Among the transcripts found more active in suppressive soil were those involved in stress responses, most likely caused by the fungal invasion. Also transcripts involved in "classic" mechanisms previously associated with disease suppression, including niche exclusion, competition for iron and production of secondary metabolites (i.e. extracellular enzymes and HCN) were upregulated in suppressive soil.

Interestingly, the majority of these functions was restricted to a limited number of bacterial families, including *Sphingomonadaceae*, *Rhizobiaceae*, *Bradyrhizobiaceae*, *Caulobacteraceae*, *Phyllobacteraceae*, *Erythrobacteraceae*, *Comamonadaceae*, *Opitutaceae* or *Brucellaceae*.

In an attempt to identify specific bacterial families playing a key role in suppression of *R. solani*, we did a comparative analysis of bacterial families more abundant and more active in the *Rhizoctonia* suppressive soil generated in this study by successive cycling and in a natural *Rhizoctonia* suppressive soil studied previously (Mendes et al., 2011; Chapelle et al., 2015). *Sphingomonadaceae* appeared as the sole bacterial family that was both more abundant and more active in both suppressive soils. The bacterial families *Micromonosporaceae*, *Nocardioidaceae*, *Pseudonocardioidaceae*, *Streptomycetaceae*, *Planctomycetaceae*, *Caulobacteraceae*, *Phyllobacteriaceae*, *Rhodocyclaceae*, *Polyangiaceae* and *Opitutaceae* were found as more abundant in both suppressive soils and more active in this study. Important to mention is that several bacterial families detected more active for the majority of functions up-regulated in this study were not found as more active for the majority of functions up-regulated in the study by Chapelle et al. (2015). However, certain of these bacterial families were found more active only for specific functions suggested to be involved in disease suppression, and these included *Caulobacteraceae, Rhodocyclaceae*, *Phyllobacteraceae*, *Brucellaceae* and *Micromonosporaceae* (see Fig. S4 in Chapelle et al., 2015).

Overall, our results indicate that no single microorganisms but rather microbial consortia are associated with disease suppressiveness and that the composition of these microbial consortia is, in part, soil type specific. The induction of suppressiveness observed in the field and shown in this thesis in short-term bioassays represents an intriguing example of host-mediated microbiome engineering, where the protective microbiome is artificially selected over multiple generations (Mueller and Sachs, 2015), and, in this case, in the presence of the pathogen. Since certain functions appear to be important for suppressiveness, the suppressive microbiomes could also be engineered by stimulating these specific functions. For example, amendments of certain organic compounds, such as chitin, often enhance disease suppression (Hjort et al., 2014; Postma and Schilder, 2015). It has been suggested that chitin acts by stimulating the growth and chitinolytic activities of chitinolytic microbes that in turn degrade the cell wall of pathogenic fungi (Sneh, 1981; Singh et al., 1999; Hjort et al., 2014). In our study however, we did not observe suppression of *Rhizoctonia* damping-off on sugar beet by chitinolytic bacteria isolated from suppressive

soil (Chapter 4). Thus, chitinolytic activity per se seems does not seem to be a key mechanism involved in *Rhizoctonia* disease suppression.

Chapelle et al. (2015) proposed that oxalic acid secreted by *R. solani* creates a stress environment that elicits the responses of certain groups of bacteria with oxalotrophic abilities. Also our analyses revealed that the number of transcripts of genes encoding enzymes involved in the degradation of oxalate were higher in suppressive soil (Chapter 4). Enrichment of the indigenous soil oxalotrotrophic bacterial communities and subsequent re-inoculation into conducive soil led to significant disease suppression. Among the isolated oxalotrophic bacterial species, *Caulobacter* and *Nocardiodes* isolates reduced *Rhizoctonia* damping-off disease (Chapter 5). Thus, bacterial oxalotrophy appears to be a bacterial trait involved in suppressiveness to *R. solani*. Whether the amendment of soil with oxalate can trigger the soil immune response against *R. solani* is subject of ongoing experiments.

Specificity of disease suppressiveness

Plant species shape their own plant microbiome (Lareen et al., 2016) and may each harbor a different "protective microbiome". Here we tested if the protective microbiome of a soil suppressive to *R. solani* AG2-2IIIB on sugar beet (Chapter 4) is also protective against *Rhizoctonia* damping-off on other plant species (cauliflower and lettuce) and caused by other *R. solani* anastomosis groups. The results showed that the protective effect of the suppressive soil works for cauliflower but not for lettuce (Fig. 3). It should be noted that, two cycles of growth with cauliflower were necessary to observe a significant decrease in disease incidence relative to the control, suggesting that the suppressive microbiome may need a period of adaptation to the rhizosphere environment of cauliflower seedlings (Fig. 3B). Conversely, the suppressive community established for sugar beet needed two growth cycles to "disassemble" and cease conferring protection to lettuce (Fig. 3C).

Since the interaction of a particular plant species with the appropriate pathogen or strain of the pathogen appears to be essential to maintain the suppressive effect, we investigated whether or not suppressiveness could be induced for other *R. solani* anastomosis groups by successive growth cycles of their respective host plants. Results showed that suppressiveness could not be induced by successive growth cycles of lettuce or bean (Fig. 4). Maybe the induction period needs to be longer for these plant species or for the tested *R. solani* strains, but this was not further pursued here.

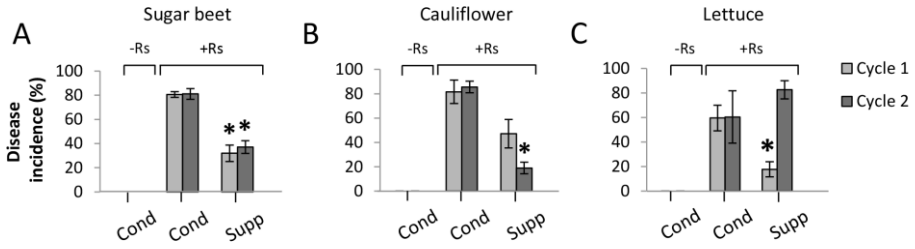

Figure 3. A *Rhizoctonia* disease suppressive soil from sugar beet suppresses damping-off on sugar beet and cauliflower but not lettuce. The percentage of seedlings suffering from damping-off disease, when grown in a soil suppressive to *R. solani* AG2-2IIIB was determined for two cycles of growth for A) sugar beet and *R. solani* AG2-2IIB; B) cauliflower and *R. solani* AG2-1/21; C) lettuce and *R. solani* AG1-1B. Error bars indicate standard error of the means (n=4). An asterisk indicates a significant difference ($p<0.05$) between the samples compared with the positive control (conducive soil + *R. solani* (Cond + Rs)) analyzed with analysis of variance and Dunnet's post hoc analysis.

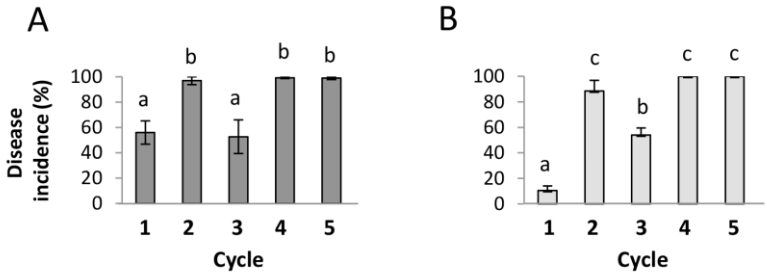

Figure 4. Attempted induction of soil suppressiveness in the presence of *R. solani* and lettuce or bean seedlings. Percentage of seedlings suffering from damping-off symptoms along five successive cycles of growth for A) lettuce in presence of *R. solani* AG1-1B and B) common bean in the presence of *R. solani* AG2-2. Error bars indicate standard error of the means (n=8). Bars with a different letter indicate a significant difference ($p<0.05$) between the samples analyzed with analysis of variance and Hochsberg post hoc analysis

Concluding remarks and future perspectives

Nowadays crop losses due to plant pests and diseases are a common problem worldwide. Improving productivity is crucial to reduce rural poverty and to increase food security worldwide (Flood, 2010; Cerda et al., 2017). Therefore, managing and preserving soil health is essential for sustainable agriculture and optimum ecosystem functioning (Larkin, 2015). The use of pesticides is a traditional control strategy, but the development of pathogen resistance, and an increasing public concern about the side effects on plant, animal and human health necessitate the development of alternative and sustainable control methods. The use of biological control agents (BCAs) (control of plant diseases caused by living organisms) has been suggested as an effective and more ecologically-friendly alternative. Suppressive soils constitute a valuable source of BCAs (Weller et al., 2002), but to date only a few microorganisms isolated from suppressive soils have been successful in disease control. In most cases, isolation of these microorganisms follows a "taxonomy-based" approach and their activities are typically tested in *in vitro* assays that do not mimic field conditions. Mazzola and Freilich (2016) reviewed the current limits in biological control, including an absence of studies analyzing the evolutionary processes leading to the assembly of an effective microbiome in suppressive soils. In the work presented in this thesis, we elucidate, for the first time, the successional changes in rhizobacterial community composition and functions during the soil immune response. I revealed that the early stages of disease suppressiveness towards *R. solani* are mainly due to changes in the expression of specific functions of the resident microbiome, rather than to changes in the community composition. Thus, "trait-based" approaches are preferred over taxonomy-based approaches in identifying the specific microorganisms and mechanisms underlying disease suppressiveness. To get a better insight in the mechanisms, a combination of different methodologies such as metataxonomic, metatranscriptomic, metaproteomic and/or metabolomic analyses are imperative. Since different microbes can perform the same function, knowing "how" a soil becomes suppressive may allow us to engineer the whole microbial community involved in traits that contribute to disease suppression. Furthermore, specific amendments may stimulate *in situ* the expression of a trait in different microbes involved in disease suppression. We showed that oxalic acid produced by *R. solani* may be a potential amendment that can alter the microbial community composition towards a disease suppressive community.

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Summary

Suppressive soils are soils in which soil-borne plant pathogens cause little or no disease due to specific microbial activities. Exploiting disease suppressive soils to control soil-borne plant diseases requires a deep understanding of the microbes and functions involved in disease suppression. To date, several microbial groups have been suggested as potential candidates involved in *Rhizoctonia* disease suppressive soils, but the mechanism of disease suppression is still poorly understood.

The research described in this thesis firstly addressed the role of *Lysobacter*, *Pseudomonas* and *Streptomyces* species in suppressing damping-off disease caused by *Rhizoctonia solani.* All these genera have previously been associated with *Rhizoctonia* suppressive soils and also have been shown to positively affect plant growth and health. Most of the *Lysobacter* strains included in this study showed a high metabolic potential to combat *R. solani* and other plant pathogens *via* the production of extracellular enzymatic activities and secondary metabolites active against different plant pathogens (chapter 2). When tested in soil bioassays, however, none of the *Lysobacter* strains consistently promoted plant growth or suppressed *R. solani*. Their lack of activity *in vivo* could be attributed to a poor ability to colonize the plant roots and/or a lack of expression of the genes encoding the bioactive enzymes and metabolites.

Application of microbial consortia with complementary or synergistic activities, also referred to as synthetic communities or 'SynComs', has been proposed to resolve inconsistent effects in disease suppression observed for single microbial strains. Therefore, the biocontrol activity of consortia of *Lysobacter, Pseudomonas* and/or *Streptomyces*, all isolated from *R. solani* disease suppressive soils, was evaluated (chapter 3). Using a combination of three *Lysobacter* strains as the baseline, no consistent control of *Rhizoctonia* damping-off disease on sugar beet seedlings was obtained in four independent experiments for any of the bacterial consortia tested.

To investigate the key microbes and mechanisms involved in *Rhizoctonia* suppressive soils, whole community analyses were adopted (chapter 4). A small-scale, reproducible bioassay was developed in which the induction of disease suppressiveness observed in the field was mimicked. This "soil immune response" could be induced under controlled conditions by successive growth of sugar beet seedlings in presence of *R. solani.* Successive growth of sugar beet in absence of *R. solani* did not result in suppressiveness induction, indicating that initial infections by the fungal pathogen are essential. By using different 'omics' approaches, the successional changes in rhizobacterial community composition and functions during the soil immune response were studied. Results indicated that the successive growth of sugar beet was the major driver shaping the

rhizobacterial community composition. Surprisingly, analysis of the rhizobacterial community composition revealed a high similarity between suppressive and conducive soils, with only a few operational taxonomic units (OTUs) found more abundant in suppressive soil. These OTUs belonged to the bacterial phyla Acidobacteria, Proteobacteria, Planctomyces, Bacteroidetes and Actinobacteria.

Metatranscriptome analyses revealed that the disease suppressive state correlated with an enhanced rhizobacterial activity rather than with shifts in rhizobacterial community composition. Interestingly, the up-regulated functions in the suppressive soil were restricted to a limited number of bacterial families, including *Rhizobiaceae*, *Bradyrhizobiaceae*, *Sphingomonadaceae, Caulobacteraceae, Comamonadaceae*, *Phyllobacteriaceae*, *Erythrobacteraceae* or *Opitutaceae*. Important to mention is that some of these bacterial families were found to be more active but not more abundant. Among the bacterial traits that were enhanced in the suppressive soil were those involved in primary metabolism, indicating a higher bacterial activity in suppressive than in conducive soil. In addition, an increase of transcripts involved in stress responses and in several "classic" mechanisms of disease suppression, including competition for nutrients and iron, as well as production of lytic enzymes and hydrogen cyanide was observed. Also genes encoding enzymes involved in oxalic acid degradation were more expressed in suppressive soil.

Oxalic acid is produced by *R. solani* and is considered as pathogenicity factor. We hypothesized that oxalic acid production triggers stress conditions in the plant and the rhizobacterial community, leading to the activation of specific bacterial functions, including degradation of oxalic acid. This would also explain why the induction of suppressiveness only occurred when the host plant was grown in presence of the pathogen. To further explore the potential role of oxalic acid degradation as one of the triggers of the soil immune response, the role of oxalotrophic bacteria in suppressing *R. solani* was investigated (chapter 5). The indigenous oxalotrophic bacteria were enriched using calcium oxalate as the sole carbon source and the resulting oxalotrophic community significantly suppressed damping-off disease caused by *R. solani*. Characterization of the enriched oxalotrophic community showed that several bacterial families were represented. Application of selected oxalotrophic bacterial genera to sugar beet seeds further revealed that strains classified as *Caulobacter* and *Nocardioides* significantly suppressed *Rhizoctonia* damping-off disease.

In conclusion, the research presented in this thesis elucidates, for the first time, the successional changes in rhizobacterial community composition and functions during the transition of a soil from a disease conducive to a disease suppressive state. The results

highlight that research on disease suppressive soils should combine taxonomic and functional analyses to unravel the complexity of the underlying mechanisms and to unravel the importance of function over phylogeny.

Samenvatting

In ziektewerende bodems komen bepaalde plantenziekten niet of nauwelijks voor vanwege de antimicrobiële activiteit van specifieke groepen bodemmicro-organismen. Om deze natuurlijke bescherming van planten beter te kunnen benutten in de landbouw voor de bestrijding van bodemgebonden plantenziekten, is fundamentele kennis nodig van de verantwoordelijke microben en de werkzame mechanismen. Voor gronden die ziektewerend zijn tegen de schimmel *Rhizoctonia solani*, de veroorzaker van wortelrot, zijn diverse microbiële groepen aangewezen als potentiële kandidaten maar de mechanismen die een rol spelen in deze ziektewerende gronden zijn nog grotendeels onbekend.

Het onderzoek in dit proefschrift beschrijft de rol van rhizosfeerbacteriën behorende tot de geslachten *Lysobacter*, *Pseudomonas* en *Streptomyces* in de ziekteonderdrukking van *R. solani*. Soorten uit deze drie geslachten zijn al eerder geassocieerd geweest met *Rhizoctonia*-ziektewerkende gronden en kunnen ook een plantengroeistimulerende werking hebben. De meeste *Lysobacter*-isolaten die onderzocht zijn in dit proefschrift onderdrukken de groei van *R. solani* en andere plantenpathogenen *in vitro* door de productie van extracellulaire enzymen en secundaire metabolieten (hoofdstuk 2). Echter, wanneer de activiteit van deze *Lysobacter* isolaten getest werd in een biotoets met planten, bleek dat geen van de isolaten in staat was de plantengroei te stimuleren of de wortelrot veroorzaakt door *R. solani* te onderdrukken. Dit gebrek aan *in vivo* activiteit zou te wijten kunnen zijn aan een beperkt vermogen van deze bacteriën om plantenwortels te koloniseren en/of aan een te lage expressie van genen die verantwoordelijk zijn voor de productie van de schimmelremmende metabolieten. Een andere mogelijke verklaring is dat deze isolaten niet afzonderlijk maar alleen in combinatie met elkaar of met andere bacteriesoorten met complementaire of synergistische activiteit actief zijn en pas dan deze ziekte kunnen onderdrukken. Om dit te onderzoeken zijn meerdere biotoetsen uitgevoerd waarin de activiteit van microbiële consortia, ook wel synthetische gemeenschappen of 'syncoms' genoemd, getest werd (hoofdstuk 3). Consortia gemaakt van *Lysobacter*, *Pseudomonas* en *Streptomyces*-isolaten afkomstig uit *Rhizoctonia* ziektewerende grond en met een combinatie van drie *Lysobacter*-isolaten als basis, vertoonden echter geen betere of meer consistente ziekteonderdrukking van *R. solani*.

Om de identiteit van de microben en de mechanismen van ziekteonderdrukking in de *Rhizoctonia*-ziektewerende gronden beter in kaart te brengen, werd de dynamiek van de bacteriële gemeenschap geanalyseerd alsook de functies die tot expressie kwamen tijdens de inductie van de ziektewerendheid (hoofdstuk 4). Ziektewerendheid van bodems tegen specifieke bodemschimmels zoals *R. solani* kan geïnduceerd worden in het veld door

continuteelt van een vatbaar gewas in aanwezigheid van de ziekteverwekkende schimmel. In deze studie kon ziektewerendheid tegen *R. solani* ook onder gecontroleerde omstandigheden in de kas worden geïnduceerd. Deze 'immuunrespons van de bodem' kon worden geactiveerd door kiemplanten van suikerbiet herhaaldelijk op te kweken in dezelfde bodem in aanwezigheid van *R. solani*. Door het toepassen van verschillende 'omica' technieken werden vervolgens de temporele veranderingen in de samenstelling van de bacteriële gemeenschap geanalyseerd alsook de functies die tot expressie kwamen tijdens de immuunrespons van de bodem. Tegen de verwachting in bleek dat de samenstelling van de bacteriële gemeenschap in de 'gezonde' bodem nauwelijks anders was dan die van de 'zieke' bodem; slechts enkele bacteriesoorten (operationele taxonomische eenheden; OTUs) waren in relatief hogere aantallen aanwezig in de gezonde bodem. Deze OTUs behoorden tot de bacteriële phyla Acidobacteriën, Proteobacteriën, Planctomyces, Bacteroidetes en Actinobacteriën. Metatranscriptomica analyse toonde aan dat de ziektewerendheid correleerde met een verhoogde activiteit van bepaalde bacteriële taxa. Deze verhoogde activiteit was zichtbaar in een beperkt aantal bacteriële families, o.a. Rhizobiaceae, Bradyrhizobiaceae, Sphingomonadaceae, Caulobacteraceae, Comamonadaceae, Phyllobacteriaceae, Erythrobacteraceae en Opitutaceae. De functies die geactiveerd werden in de ziektewerende grond waren gerelateerd aan primair metabolisme, wat een indicatie is voor een verhoogde activiteit van de rhizosfeerbacteriën. Ook genen betrokken bij stressreacties en enkele 'klassieke' mechanismen van ziekteonderdrukking, waaronder competitie om ijzer alsmede de productie van lytische enzymen en waterstofcyanide, bleken geïnduceerd te worden tijdens de immuunrespons van de bodem. Daarnaast kwamen ook genen die coderen voor enzymen voor de afbraak van oxaalzuur verhoogd tot expressie. Oxaalzuur wordt geproduceerd door *R. solani* en fungeert als een virulentiefactor. Op basis van deze resultaten was de hypothese van het vervolgonderzoek dat oxaalzuurproductie door *R. solani,* direct en/of indirect (via de plant), specifieke groepen rhizosfeerbacteriën en functies activeert die leiden tot de afbraak van oxaalzuur en onderdrukking van de ziekte veroorzaakt door *R. solani*. Om de mogelijke rol van de afbraak van oxaalzuur door rhizosfeerbacteriën als een belangrijk mechanisme van ziektewerendheid verder te onderzoeken, werden oxalotrofe bodembacteriën verrijkt in een medium met calciumoxalaat als enige koolstofbron (hoofdstuk 5). Deze verrijkte oxalotrofe bacteriegemeenschap werd vervolgens geïdentificeerd en individueel getest op hun vermogen om de ziekte veroorzaakt door *R. solani* te onderdrukken. Uit phylogenetische analyses bleek dat deze verrijkte oxalotrofe gemeenschap bestond uit

meerdere bacteriële families, waarvan alleen Caulobacter en Nocardioides isolaten in staat waren om de ziekte veroorzaakt door *R. solani* te onderdrukken.

Samenvattend laat het onderzoek beschreven in dit proefschrift zien dat de combinatie van taxonomische en functionele analyses essentieel is om de micro-organismen en complexe mechanismen die operationeel zijn in ziektewerende gronden te ontrafelen. Het onderzoek toonde tevens aan dat tijdens de transitie van een zieke naar een gezonde bodem, veranderingen in de expressie van specifieke microbiële functies in de rhizosfeer belangrijker zijn dan de veranderingen in taxonomische samenstelling van het microbioom van de rhizosfeer.

Resúmen

Los suelos supresivos son suelos en los que patógenos de plantas que se transmiten a través del suelo provocan escasa o ninguna enfermedad debido a actividades microbianas específicas. El aprovechamiento de suelos supresivos con el fín de controlar estas enfermedades en las plantas requiere de un profundo conocimiento tanto de los microbios, como de las funciones que llevan a cabo y que están implicadas en suprimir estas enfermedades. Hasta la fecha, varios grupos microbianos han sido sugeridos como posibles candidatos que podrían estar implicados en suprimir enfermedades causadas por el hongo patógeno *Rhizoctonia*. Sin embargo, los mecanismos a través de los cuales estos microbios actuarían inhibiendo estas enfermedades son todavía desconocidos.

La investigación descrita en esta tesis estuvo en primer lugar dirigida a determinar si diferentes especies de *Lysobacter*, *Pseudomonas* y *Streptomyces* juegan un papel importante en la supresión de la enfermedad provocada por *Rhizoctonia solani*. Estos géneros bacterianos han sido previamente asociados con suelos supressivos contra *Rhizoctonia*. Ciertas cepas de *Lysobacter*, *Pseudomonas* y *Streptomyces* han demostrado tener, además, efectos positivos en el crecimiento y en la salud de plantas. La mayoría de las cepas de *Lysobacter* incluídas en este estudio revelaron un alto potencial metabólico capaz de combatir tanto *R. solani* como otros patógenos de plantas, mediante la producción de enzimas extracelulares y metabolitos secundarios (capítulo 2). Sin embargo, cuando estas cepas de *Lysobacter* fueron probadas en ensayos de suelo, ninguna de ellas fue capaz ni de promover el crecimiento de plantas, ni de suprimir la infección provocada por *R. solani* de forma consistente. Esta falta de actividad en suelos podría ser atribuída a una escasa capacidad de colonización de las raíces por parte de *Lysobacter* y/o debido a una falta de expresión de los genes productores de enzimas y otros metabolitos en suelo.

La aplicación de consorcios microbianos con actividades complementarias o sinérgicas, también conocidas como ´SynComs´, han sido propuestos con el fín de minimizar la inconsistencia en los resultados observados tras la aplicación individual de cepas microbianas. Por ello, en este trabajo se evaluó el efecto de consorcios formados por *Lysobacter, Pseudomonas* y/o *Streptomyces* en la supresión de enfermedad provocada por *R. solani* (capítulo 3). Usando la combinación de tres cepas de *Lysobacter* como referente, no se observó una consistente supresión de *Rhizoctonia* en ninguno de los cuatro experimentos independientes realizados.

Para determinar cuáles son entonces los microbios clave (y los mecanismos a través de los cuales operan) involucrados en suprimir enfermedades causadas por *Rhizoctonia* en suelos supresivos, se procedió a analizar la totalidad de las comunidades bacterianas (capítulo 4). Mediante un ensayo a pequeña escala se reprodujo la inducción

de supresión contra *Rhizoctonia* en un suelo, de modo similar a como esta inducción ocurre de forma natural en suelos agrícolas. Esta 'respuesta inmune del suelo' pudo ser inducida en condiciones experimentales tras el cultivo continuado de plántulas de remolacha en presencia de *R. solani*. Cuando las plántulas de remolacha fueron cultivadas de forma continuada en ausencia de *R. solani,* no se pudo inducir supresión, lo que indica que infecciones iniciales por parte del hongo patógeno son esenciales. Mediante el uso de diferentes técnicas de análisis ómicos, se pudieron estudiar los cambios sucesionales ocurridos tanto en las comunidades bacterianas como en sus funciones durante el proceso de inducción de respuesta immune. Los resultados indicaron que el crecimiento continuado de plántulas de remolacha es el factor principal que selecciona las comunidades bacterianas. Sorprendentemente, los análisis revelaron una gran similitud entre las comunidades bacterianas en suelo conductivo y supresivo. Sólo unas pocas unidades taxonómicas operativas (UTOs), pertenecientes a los filos Acidobacteria, Proteobacteria, Planctomyces, Bacteroidetes y Actinobacteria, fueron encontrados en mayor abundancia en suelo supresivo.

El análisis del metatranscriptoma reveló que el suelo en estado supresivo está más relacionado con un aumento en ciertas actividades bacterianas que con cambios en la composición bacteriana en sí. Curiosamente, las funciones encontradas incrementadas en suelo supresivo fueron asociadas a un número limitado de familias bacterianas, incluyendo *Rhizobiaceae*, *Bradyrhizobiaceae*, *Sphingomonadaceae, Caulobacteraceae, Comamonadaceae*, *Phyllobacteriaceae*, *Erythrobacteraceae* u *Opitutaceae*. Es importante mencionar que algunas de estas familias bacterianas fueron encontradas más activas en suelo supresivo, pero no más abundantes. Entre las funciones encontradas incrementadas en suelo supresivo, se hallaron aquellas implicadas en el metabolismo primario, indicando que hay una mayor actividad bacteriana en suelo supresivo que en conductivo. También se evidenció un incremento de ciertas funciones involucradas en respuestas a situaciones de estrés y en varios mecanismos 'clásicos' de supresión de enfermedades (incluyendo competencia por nutrientes e hierro, producción de enzymas líticas y cianuro de hidrógeno), así como enzimas responsables de la degradación de ácido oxálico.

Rhizoctonia solani segrega ácido oxálico, el cual es considerado como un factor de patogenicidad. Por ello, planteamos la hipótesis de que la producción de ácido oxálico crearía condiciones de estrés en la planta y en las bacterias circundantes, lo que desencadenaría la activación de funciones bacterianas específicas, incluyendo la degradación de ácido oxálico. Esto explicaría también por qué el estado supresivo pudo ser inducido sólo cuando las plántulas fueron cultivadas en presencia del patógeno. Para

investigar en más detalle si la degradación del ácido oxálico es realmente el desencadenante de la 'respuesta inmune' del suelo, investigamos el papel de las bacterias capaces de degradar ácido oxálico (bacterias oxalotróficas) en la supresión de *R. solani* (capítulo 5). Las bacterias oxalotróficas autóctonas del suelo fueron enriquecidas usando oxalato de calcio como única fuente de carbono. La aplicación de la comunidad de bacterias oxalotróficas resultante tras el enriquecimiento en semillas de remolacha fue capaz de suprimir los síntomas de 'decaimiento de las plántulas' ('damping-off') provocados por *R. solani*. La caracterización de la comunidad de bacterias oxalotróficas obtenida reveló que varias familias estaban representadas. La aplicación individual de cepas pertenecientes a los géneros *Caulobacter* y *Nocardioides* suprimieron, de forma significativa, los síntomas de enfermedad causados por *R. solani*.

En conclusion, la investigación presentada en esta tesis muestra, por primera vez, los cambios sucesionales en la composición y funciones de bacterias del suelo durante la transición de un suelo en estado conductivo a un estado supresivo. Estos resultados subrayan la necesidad de combinar estudios taxonómicos y funcionales, con el fín de desentrañar la complejidad de los mecanismos que tienen lugar en suelos supresivos y la importancia del estudio de las funciones sobre estudios taxonómicos.

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When I started my PhD, I was performing most of my experiments in the Phytopathology lab while being located at the Biointeractions and Plant health unit (PRI). Although at the beginning this was a bit inconvenient (especially when I forgot something and had to walk all the way back through Radix from one place to the other to get it), it gave me the opportunity of getting to know many nice colleagues (and to do some extra exercise). Thanks to all PRI-colleagues for being so nice and always willing to help me. Special thanks to Mirjam (who helped me to perform the TaqMan assay), Marga (for her tips regarding the analyses of the TaqMan results), and Cor (for his advices on the design of TaqMan probes). Also special thanks to Patricia and Els N. Also thanks to all the Phyto-colleagues, for being so nice and helpful. Special thanks to all phyto-PhDs with whom I could enjoy borrels and lab-outings. Special thanks also to the 'Beggers' (Viviane, Chunxu, Yiying, Víctor, Juan, Menno, Judith, Xu, Emilie, Allison and Christin), for the nice discussions

Halfway of my PhD, I moved to NIOO, to the department of Microbial Ecology, where I continued and finished my PhD and where I got to know more nice people. Thanks to all ME-colleagues, for more discussions about work, coffee breaks and more borrels. Special thanks go to the members of the ´Cabrones Crew´. I enjoyed sharing dinners, drinks, parties and all the co-productions we made together! Chunxu, you always had the perfect speech for when I was highly stressed. Somehow, your wise words had a 'healing' effect (lasting at least for some hours!). And if that did not work, your contagious optimism and joy did! Vic, I enjoyed (some of) your jokes, and I very much appreciate the scientific-related advices you gave me. Juan, my desk-mate, every day you asked me how I was feeling and you brought some of the latin spirit that I miss so much to our office. Thanks to the rest my former and current groups members and specially to Kay, Desalegn, Lucas, Nurmi and Natalia for always being super nice to me and for their sweet and supportive words. Yiying, thanks for always worrying about my well-being, for all the funny conversations we had and for the thousand times you offered me your help to fix my figures in Photoshop.

Special thanks should of course go to ´as minhas meninas´. It was very nice to share coffee breaks, dinners, parties and excursions with you! Only with you girls, banal things such as peaches and beans become fun! Afnan, thanks for teaching me Arabic أفنان, صديقتي العزيزة اشكرك كثيرا على صبرك ووقتك في تعليمي اللغة العربية; Késia, I really enjoyed your cooking (specially your 'Bolo Salgado'…superb!); Ohana, from time to time it was fun to forget science and have chats about 'girly stuff'. I 'WISH' you girls all the best in the future.

More special thanks to my former and current office-mates (Vivi, Yiying, Juan, Paolo, Késia, Juan, Marcio, Ruth S., Julia, Manoeli, Leonardo, Johnny, Ronny, Anthony and Xiaojiao), for sharing PhD experiences, more coffee and sweets and for being comprenhensive with me on stressful moments. Thanks to the rest of the former and current PhD candidates with whom I could share my experiences. That made me realize that the roller coaster of emotions I felt during my PhD was something ´normal´. In addition to the ones I already named, special thanks go to Adam and Thiago. May the force be with you guys! Olaf, thanks for your help with the statistical analyses. Mattias, thanks for taking care of the raw NGS data and for your help with the bioinformatic problems. Manoeli, I highly appreciate your patience when I was learning ´R´. Thanks to all the technicians for their assistance in the laboratory and in the greenhouses. Special thanks to Roel for his help with the magnifiers and microscopes. Thanks to Reinier and Salva, for their contributions to the project. Thanks to Sang Yoon and to Adrian, for the never-ending laughs. Thanks to Almudena Medina, who believed in me and gave me the opportunity to first come to the Netherlands and encouraged me to apply for this position.

Lieber Fabian, vielen lieben Dank für dein bedingungslose Unterstützung, Geduld und Liebe. Ich bin mir bewusst, das ich in den letzten Monaten keine leichte Person war und dass der Druck das Schlimmste aus mir hervorgebracht hat. Allerdings warst du immer für mich da und versuchst mich zu retten. Vielen Dank auch für deinen tollen job mit dem Entwurf meines Thesis Cover.

Por último, pero no por ello menos importante, tengo que agradecer a mi familia y amigos en España, por el apoyo que me han dado durante estos largos cinco años y por hacerme creer que podía hacerlo. En especial a mis padres y a mi hermano, por preocuparse por mí y por darme siempre ánimos para poder terminar 'mi trabajo en el laboratorio'. Sonia, gracias por tu apoyo y por escuchar los inumerables e interminables audios contándote todas mis frustraciones. Gracias también a mis amigas de toda la vida (Marta, Cris, Rosa, Bea, Ali, Marta 2, Elena y 'Delegá'), pos los ánimos y por siempre pedirme que 'volviera ya, joé'.

Curriculum vitae
Publications

Ruth Gómez Expósito was born on 1st April 1983 in Jerez de la Frontera (Cádiz), Spain. In 2008 she completed her BSc degree in Biology at the University of Granada. After that, she continued her education by taking courses on physico-chemical analyses and in hematology. In 2010 she started her MSc studies in Microbiology at the University of Granada. During her MSc thesis at the group of Environmental Microbiology and Biodegradation (Superior Council of Scientific Research, CSIC), she studied the diversity of bacteria degrading petroleum compounds in aerobic and anaerobic conditions for bioremediation under the supervision of Dr Silvia Marqués Martín and Dr Alejandro Acosta González. In 2011 she obtained the personal grant Leonardo-Adeit for post-graduate training abroad and she moved to The Netherlands. At the Netherlands Institute of Ecology (NIOO-KNAW), she carried out an internship under the supervision of Dr Almudena Medina, where she studied the interaction between the mycophagous bacteria *Collimonas* and arbuscular mycorrhiza . In 2012 she started her PhD research under the supervision of Prof. Dr Jos Raaijmakers (Netherlands Institute of Ecology, NIOO-KNAW), Prof. Dr Francine Govers (Wageningen University), Dr ir Joeke Postma (Plant Research International) and Dr Irene de Bruijn (NIOO-KNAW). The findings of her PhD research are described in this thesis.

E-mail:ruth_gomez_exposito@hotmail.com

Gómez Expósito, R., de Bruijn, I., Jack, A., Postma, J., Raaijmakers, J.M. Successional changes in rhizosphere microbiome composition and functions during induction of the soil immune response to a fungal root pathogen (Chapter 4, to be submitted).

Gómez Expósito, R., Postma, J., Raaijmakers, J.M., de Bruijn, I. Role of oxalotrophic soil bacteria in suppression of damping-off disease caused by the fungal root pathogen *Rhizoctonia solani* (Chapter 5, to be submitted).

Gómez Expósito, R., de Bruijn, I., Postma, J., Raaijmakers, J.M. New perspectives on rhizosphere bacteria and disease suppressive soils (Chapter 1, submitted).

Gómez Expósito, R., Postma, J., Raaijmakers, J.M., de Bruijn, I. Evaluation of bacterial consortia to enhance plant disease control (Chapter 3, to be submitted).

Carrion, V.J., Perez-Jaramillo, J., Mendes, L.W., Mendes, R., Gómez Expósito, R., Medema, M., de Hollander, M., Raaijmakers, J.M. Mining the endophytic microbiome in natural disease suppressive soils (to be submitted).

Mendes, L.M., Carrion, V.J., de Hollander, M., Gómez Expósito, R., Mendes, R., Tsai, S.M., Raaijmakers. J.M. Differential response of common bean root microbiome to pathogen invasion (to be submitted).

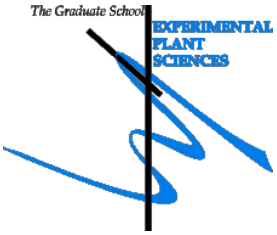
Gómez Expósito, R., Postma, J., Raaijmakers, J.M., and De Bruijn, I. (2015). Diversity and activity of *Lysobacter* species from disease suppressive soils. Front Microbiol 6 (1243).

de Bruijn, I., Cheng, X., de Jager, V., Gómez Expósito, R., Watrous, J., Patel, N, Postma, J., Dorrestein, P.C., Kobayashi, D., Raaijmakers, J.M. (2015). Comparative genomics and metabolic profiling of the genus *Lysobacter*. BMC Genomics 16 (991).

Education statement

Education Statement of the Graduate School

Experimental Plant Sciences



Issued to: Ruth Gómez Expósito
Date: 15-jun-17
Group: Laboratory of Phytopathology
University: Wageningen University & Research

1) Start-up phase	*date*
► **First presentation of your project**	
Title: Ecology and genomics of Lysobacter species: toward the development of new disease control strategies	Apr 05, 2013
► **Writing or rewriting a project proposal**	
Tittle: Ecology and genomics of Lysobacter species: toward the development of new disease control strategies	Sep-Dec 2012
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*3.0 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS Get2gether - Soest, NL	Jan 29-30, 2015
EPS Get2gether - Soest, NL	Jan 28-29, 2016
► **EPS theme symposia**	
EPS Theme 2 symposium "Interactions between plants and biotic agents", Utrecht, NL	Jan 24, 2013
EPS Theme 2 symposium "Interactions between plants and biotic agents", Amsterdam, NL	Feb 25, 2014
EPS Theme 3 symposium "Metabolism and adaptation", Wageningen, NL	Mar 11, 2014
► **NWO Lunteren days and other National Platforms**	
86th KNPV working group Soilborne Pathogens and Soil Microbiology	Mar 21, 2013
87th KNPV working group Soilborne Pathogens and Soil Microbiology	Oct 03, 2013
89th KNPV working group Soilborne Pathogens and Soil Microbiology	Oct 30, 2014
NVMM-KNVM Scientific Spring meeting - Arnhem, NL	Mar 22-23, 2016

▶ **Seminars (series), workshops and symposia**	
STW tech talks, annual congress 2012	Nov 10, 2012
Invited Seminars 2012 (Gabriele Berg, Jan Bergevoet, Birgit Piechulla, Gilles van Wezel)	2012
Invited Seminars 2013 (Andrew Sugden, David M. Weller, Pieter Dorrestein, Kathrin Riedel, Ross Mann)	2013
Invited Seminars 2014 (Robert Czajkowski, Eoin Brodie, Gilles van Wezel, Peter Dunfield, Cristina Barreto, Marco Kai, Laure Weiskopfm, Dirk Schaerlaekers)	2014
Invited Seminars 2015 (Michael Bonkowski, Kornelia Smalla, Harald Gross, Kevin Foster, Jeroen Jansen)	2015
Workshop on Microbial Volatiles - NIOO-KNAW, Wageningen, NL	Apr 14, 2014
Workshop in Environmental Metagenomics Symposium	Sep 19, 2014
Workshop on the MS-Tools (JSB)	Mar 06, 2014
Farewell Symposium Prof. J.A. van Veen - Perspectives in Microbial Ecology, Wageningen, NL	Jun 27, 2014
Farewell Symposium Prof. Pierre de Wit	Jun 05, 2014
Inaugural speech Prof. Jos Raaijmakers	Nov 13, 2015
Inaugural speech Prof. Liesje Mommer	Feb 25, 2016
▶ **Seminar plus**	
▶ **International symposia and congresses**	
12 Symposium on Bacterial Genetics and Ecology (Bageco 12)	Jun 09-13, 2013
Symposium The Edible Microbiome, Graz, Austria	Jun 14, 2013
2nd Thünen Symposium on Soil Metagenomics, Braunschweig, Germany.	Dec 11-13, 2013
International society for microbial ecology (ISME 15) (Seoul, South Korea)	Aug 24-29, 2014
10th International PGPR Workshop, Liège, Belgium.	Jun 16-19, 2015
▶ **Presentations**	
Poster: BAGECO meeting, Ljubjana, Slovenia	Jun 09-13, 2013
Talk: 87th KNPV working group Soilborne Pathogens and Soil Microbiology	Oct 03, 2013
Poster: Symposium on Soil Metagenomics, Braunschweig, Germany	Dec 11-13, 2013
Poster pitch: Spring school Host-Microbe Interactomics, Wageningen, NL	Jun 02-04, 2014
Poster: ISME 15, Seoul, South Korea	Aug 24-29, 2014
Talk: 10th International PGPR Workshop, Liège, Belgium	Jun 16-19, 2015
Poster: NIOO science days, Heeze, NL	Nov 11-12, 2015
Talk: NVMM-KNVM, Arnhem, NL	Mar 22-23, 2016
▶ **IAB interview**	
▶ **Excursions**	
Institute Sugar Beet Research (IRS), 3rd STW meeting, Bergen op Zoom, NL	May 21, 2013
Bejo Zaden B.V., 5th STW meeting, Warmenhuizen, NL	May 22, 2014
Micropia, Amsterdam,NL	Oct 30, 2014
EcoStyle, 9th STW meeting, Oosterwolde	Jun 22, 2016
Subtotal Scientific Exposure	*20.5 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Bioinformatics: a User's Approach	Mar 04-08, 2013
EPS Spring School Host-Microbe interactomics, Wageningen, NL	Jun 02-04, 2014
The Power of RNAseq, Wageningen, NL	Feb 10-12, 2016
► **Journal club**	
Literature discussion, Bacterial Ecology & Genomics Group of Phytopathology, WUR	2012-2014
Literature discussion, Microbial Ecology Department, NIOO-KNAW	2015-2016
► **Individual research training**	
Subtotal In-Depth Studies	*6.3 credits**

4) Personal development	*date*
► **Skill training courses**	
Mini-symposium "How to write a World-class Paper", Wageningen, NL	Oct 10, 2013
Postdoc Career Development Initiative (PCDI) Retreat - Life Sciences	Mar 25-27, 2015
Last Stretch of the PhD programme, Wageningen, NL	Sep 25, 2015
Working Outside Academia Workshop	Sep 29, 2016
Writting for impact workshop, Wageningen, NL	Jan 13, 2015
Dutch Course at KOKOTOPIA	May-Nov 2012
Arabic course at International Student Organization Wageningen (ISOW)	Oct 2015-Oct 2016
Literacy including EndNote introduction	Dec 06-07, 2016
► **Organisation of PhD students day, course or conference**	
2013 Organizing labouting	Apr-Jun 2013
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*4.9 credits**

TOTAL NUMBER OF CREDIT POINTS*	34,7

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

The research described in this thesis was performed in the Laboratory of Phytopathology of Wageningen University (WUR), the Business Unit of Biointeractions and Plant Health of Plant Research International (PRI), and the Department of Microbial Ecology at the Netherlands Institute of Ecology (NIOO-KNAW). This research was financially supported by the Technology Foundation (STW).

This is NIOO-thesis number 144.

Cover: representation of the suppressiveness induction process of a soil towards the fungal pathogen *Rhizoctonia solani*.
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