Keystone microbial taxa regulate the invasion of a fungal pathogen in agro-ecosystems

P. Trivedi [a, b, *], M. Delgado-Baquerizo [b, c], C. Trivedi [b], K. Hamonts [b], I.C. Anderson [b], B.K. Singh [b, d]

[a] Department of Bioagricultural Sciences and Pest Management, Colorado State University, Fort Collins, CO, United States
[b] Hawkesbury Institute for the Environment, Western Sydney University, Penrith South, New South Wales, Australia
[c] Cooperative Institute for Research in Environmental Sciences, University of Colorado, Boulder, CO, United States
[d] Global Centre for Land Based Innovation, Western Sydney University, Penrith South, New South Wales, Australia

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ABSTRACT

Uncovering potential soil drivers of soils pathogen suppression represent an essential step in order to develop alternative and sustainable management strategies for disease control and increased soil health. In this study, we tested the potential role of keystone microbial taxa and chemical/physical properties in the suppression (referred to as soil suppressiveness) of the soil-borne model pathogen *Fusarium oxysporum* using soil samples from various crop producing agro-ecosystems in Australia. Using random forest, we identified bacteria belonging to the phyla *Actinobacteria*, *Firmicutes* and *Acidobacteria* as the major microbial predictors for soil suppressiveness at a continental scale. Structural equation modeling approach revealed strong relationship between the relative abundance of phylum *Actinobacteria* and soil functions carried out by soil microbial communities (soil functioning) with pathogen inhibition. Overall our study provided a mechanistic framework showing how microbial communities, soil functionality, and abiotic properties being antagonistic to soil pathogens are linked and interactively shape the suppressive potential of soils at continental scale. This information, upon further validation can be incorporated in risk management tools for developing novel concepts such as "*Know before you Sow*" leading to increased farm productivity and profitability.

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Soil-borne plant pathogens, in particular fungi, are a serious threat to agricultural production worldwide and can cause losses up-to 20—35% in some production systems (Weller et al., 2002; Strange and Scott, 2005; Raaijmakers et al., 2009). However, farmers are being challenged to use less pesticides and fumigants and to apply more sustainable cropping practices (Weller et al., 2002). The intrinsic ability of soils to suppress pathogens is an important yet underutilized resource that can be exploited for controlling soil-borne pathogens (Schroth and Hancock, 1982; Mendes et al., 2011; Cha et al., 2016; Latz et al., 2016). Soil suppressiveness is interactively shaped by various factors such as abiotic properties, management practices, and the structure and activities of indigenous microbial communities (including their direct antagonistic effect on the pathogen or indirect effect mediated through maintenance of several key ecosystem functions) (Garbeva et al., 2004; Berg and Smalla, 2009; Philippot et al., 2013; van Elsas et al., 2012; Santhanam et al., 2015; Latz et al., 2016; Raaijmakers and Mazzola, 2016). A mechanistic framework for predicting the suppressive (or stimulatory) potential of soils is required to provide management options and measures to reduce the impact of soil-borne pathogens. However, the existing knowledge gap on the major drivers of soil suppressiveness both at local and regional scales limits our ability to develop such management tools (Dignam et al., 2016). Despite extensive research on the molecular mechanisms involved in disease suppression by bacteria (Haas and Défago, 2005; Berg and Smalla, 2009; Mendes et al., 2011; van Elsas et al., 2012; Chapelle et al., 2016), there is still a lack of knowledge on the identity of keystone microbial species that regulate the rates of pathogen invasion in soils (Philippot et al., 2013; Latz et al., 2016). This information is crucial for sustaining both plant health and soil fertility and could provide a potential target for novel agricultural management strategies.

Here, we aim to: (a) identify the most significant bacterial groups involved in soil suppressiveness; and (b) disentangle pathways to identify drivers of soil suppressiveness at a continental scale. We hypothesized that: (a) specific members of the keystone bacterial taxa will have a direct impact on suppressiveness (measured in terms of pathogen inhibition); and that (b) abiotic factors and microbial diversity will affect soil suppressiveness indirectly via regulation of the ability of soils to support soil functioning.

To test our hypotheses, we collected soil samples from major crop producing agro-ecosystems in Australia (n = 24; Supplementary Fig. 1). We characterized the structure (diversity) and composition (relative abundance) of the soil bacterial community using MiSeq and qPCR analysis (abundance only). We analyzed soil bacteria as they constitute the most dominant microbial communities in agricultural ecosystems (van Der Heijden et al., 2008) and have a very strong direct and indirect effect on pathogen establishment (Wei et al., 2015; Latz et al., 2016). Soil properties (including total C, pH) and functions (soil enzymatic activities) were determined by methods described in Trivedi et al. (2016). We calculated a soil functioning index by using the activity of seven enzymes involved in nutrient turnover as described by Delgado-Baquerizo et al. (2016). We then set up microcosm experiments and introduced *Fusarium oxysporum* as a model pathogen in different soil samples. We chose *F. oxysporum* as this a complex of host-specific forma specialis causing root-rots and vascular wilts in many crops around the world (Lockwood, 1986). In our study, we used *F. oxysporum* f. sp. *cucumerinum* J.H Owen that is responsible for vascular wilt in cucumber plants (*Cucumis* sp.; Owen 1955). Pathogen abundance over time was determined by pathogen specific qPCR analysis (Scarlett et al., 2013) and percent inhibition of the pathogen in different soils was calculated. In the microcosms, we noticed a sharp decrease in the pathogen numbers for most of the soil samples within 14 days. However, for most soil samples, the numbers stabilized after this time period as there were no significant differences in the pathogen populations between 14 and 21 days and therefore percent reduction in the pathogen populations was determined by using the data obtained on 21st day after initial inoculation (de Boer et al., 2003). We further used statistical modeling approaches viz. Random Forest (RF) and Structure Equation Modeling (SEqM, for *a priory* modeling see Supplementary Fig. 2) to assess the major drivers of soil suppressiveness across a continental scale. Details of the experimental set up and analysis are provided in Supplementary Material and Methods section.

Overall our sampling sites varied considerably in their soil properties (Supplementary Fig. 3a) and soil bacterial community structure and composition (Supplementary Fig. 3b) and thus provide an excellent framework to test our hypothesis. Due to the high taxonomic diversity in the soil microbial communities there can be large numbers of potential predictors for soil suppressiveness. We therefore conducted random forest (RF) analysis based on the relative abundance of major phyla (or classes for *Proteobacteria*) to identify the most important microbial groups involved in soil suppressiveness. Our RF models indicated that bacteria belonging to the phyla *Actinobacteria, Firmicutes and Acidobacteria* were the major predictors for soil suppressiveness in crop producing agro-ecosystems of Australia (Fig. 1a). Our analysis further showed that bacteria belonging to phyla *Actinobacteria* ($R^2 = 91.26$; $P < 0.001$) and *Firmicutes* ($R^2 = 45.01$; $P < 0.001$) were positively correlated with pathogen inhibition. Many culture based studies have shown that different members of these bacterial groups can directly antagonize pathogens through various mechanisms including production of antibiotics; volatiles; and siderophores (Chaurasia et al., 2005; Cha et al., 2016). Recent metagenomic based studies

have also provided strong linkages between the relative abundance of these groups and disease suppression (Mendes et al., 2011; Cha et al., 2016). These studies were however based on observations at local scales that limit our ability to make generalizations for predicting and manipulating the disease suppressive potential of soils. In this context our continental scale results represent an important step forward in evaluating the importance of soil taxa for soil suppressiveness.

Interestingly our RF models did not implicate bacteria belonging to *Gammaproteobacteria* and *Alphaproteobacteria* as significant drivers of soil suppressiveness in our soil samples (Fig. 1a). Various members of these groups have been studied in detail for their role in pathogen inhibition and development of disease suppression in soils in the past (Pandey et al., 2006; Weller, 2007; Trivedi et al., 2008, 2011). This apparent discrepancy may be due to two reasons: (a) bacteria belonging to *Gamma-* and *Alphaproteobacteria* are more abundant in the rhizosphere whereas we analyzed bulk soil samples as we were interested in determining the intrinsic potential of soils to suppress disease without the influence of plant systems; (b) these groups of bacteria might be involved in local soil suppressiveness and therefore were not a major predictor when a variety of soils collected across a continental scale were analyzed. In line with our results, after considering various criteria related to disease suppression, Cha et al. (2016) reported that *Actinobacteria* was the single phylum directly involved in disease suppression of vascular wilt. Recently, Latz et al. (2016) have also reported that the abundance of members of *Gammaproteobacteria* was not directly linked to soil suppressiveness, but their positive correlation with *Actinomyces* density make them appear significantly linked to disease suppression in a linear regression. Our analysis further revealed that bacteria belonging to phylum *Acidobacteria* were negatively correlated with the inhibition of pathogen in soils (Fig. 1d). To the best of our knowledge, members of *Acidobacteria* have not yet been reported for their role in disease suppression. In general, both fungi and *Acidobacteria* favors acid soils (Jones et al., 2009; Rousk et al., 2009) and therefore it is not unexpected that members of *Acidobacteria* are more numerous in soils where the pathogen is also abundant. Further, our RF analysis at lower taxonomic levels of *Actinobacteria* or *Firmicutes* showed that different families within these groups are important predictors of soil suppressiveness (Supplementary Fig. 4). This information can be used to increase the abundance of members of these groups through artificial inoculation or change in management practices that can lead to an increase in disease suppression potential.

We used structural equation modeling to build a system-level understanding of the main drivers of soil pathogen invasion accounting for direct and indirect effects of abiotic (soil C and pH); biotic [including (a) relative abundance based on MiSeq analysis of bacterial phyla that were important predictors for soil suppressiveness as determined by RF; (b) Shannon diversity calculated using MiSeq dataset; and (c) the abundance of total bacteria measured by qPCR analysis]; and soil functioning (i.e. enzyme activities) on soil suppressiveness (see Fig. 2). Our SEqM explained 94% of the variance found in the disease suppression ability of soil from Australian agro-ecosystems (Fig. 2a). We found that the relative abundance of *Actinobacteria* had the most significant and direct effect on pathogen inhibition. This effect was independent and was maintained even after considering the role of other biotic and abiotic components on pathogen inhibition.

Soil microbial groups can either directly impact pathogen inhibition by antagonizing the pathogens or indirectly by maintaining soil health via mediating processes involved in nutrient dynamics (Pandey et al., 2006; Lugtenberg and Kamilova, 2009; Trivedi et al., 2011). In accordance with our initial hypothesis, SEqM analysis revealed that soil functioning had a direct positive and significant
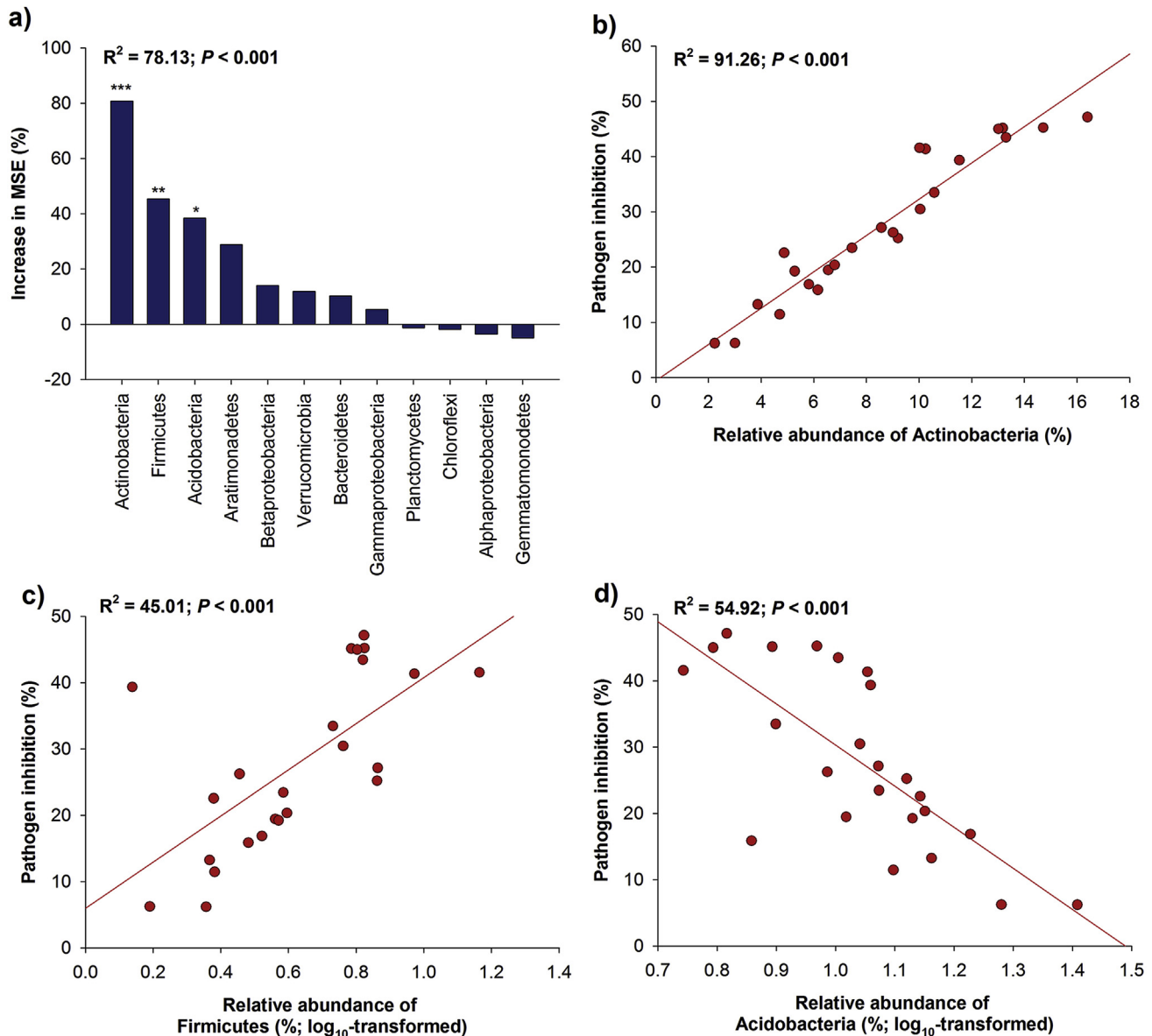
Fig. 1. Random forest analysis and correlations between the most important predictors for pathogen inhibition. 1a. Random Forest mean predictor importance (percentage of increase of mean square error) of bacterial relative abundance as drivers of pathogen inhibition. This accuracy importance was computed for each tree and averaged over the forest (5000 trees). Significance levels are as follows: *$P < 0.05$ and **$P < 0.01$. 1b-d. Relationships between the relative abundance of Actinobacteria (b); Firmicutes (c) and Acidobacteria (d, log transformed for normality) with pathogen inhibition (%).

impact on disease soil suppressiveness, indirectly driven by positive effects of bacterial diversity on soil functioning (Fig. 2). This is in line with a few recent studies that have provided empirical evidence that microbial diversity drives soil functioning in terrestrial ecosystems (Delgado-Baquerizo et al., 2016). Abiotic factors such as pH and C indirectly also impacted pathogen inhibition through their impact on soil functioning. Interestingly, microbial diversity as such had a slight negative impact on pathogen inhibition (Fig. 2). Recently, Wei et al. (2015) have reported that resource competition and community interactions are better predictors of invasion resistance towards a pathogen compared with resident community diversity. Our results suggest that specific functions such as pathogen inhibition are controlled by the relative abundance of a few groups of bacteria (such as *Actinobacteria* and *Firmicutes*) rather than the overall diversity (Garbeva et al., 2004; Latz et al., 2016). Similar morphology (albeit Actinomycetes have narrow mycelium

than fungi) and life cycle of Actinomycetes and Fungi (Doroghazi and Metcalf, 2013) can also result in niche overlap that can constrain growth of our model soil-borne pathogen (Wei et al., 2015).

Overall our study suggests a framework showing how the linkages between microbial communities, soil functioning, and soil abiotic properties interactively shape the suppressive potential of soils (Garbeva et al., 2004; Berg and Smalla, 2009; Philippot et al., 2013). Our results suggest that microbial diversity (proxy for soil functioning) and the relative abundance of phylum *Actinobacteria* (proxy for disease suppression) have potential as biological indicators for the development of prediction models to enable the identification and characterization of the suppressive potential of soils. Both the MiSeq and qPCR (based on the gene copy numbers) revealed that more than 25% of pathogen inhibition was observed when the relative abundance of *Actinobacteria* was above 8%. This
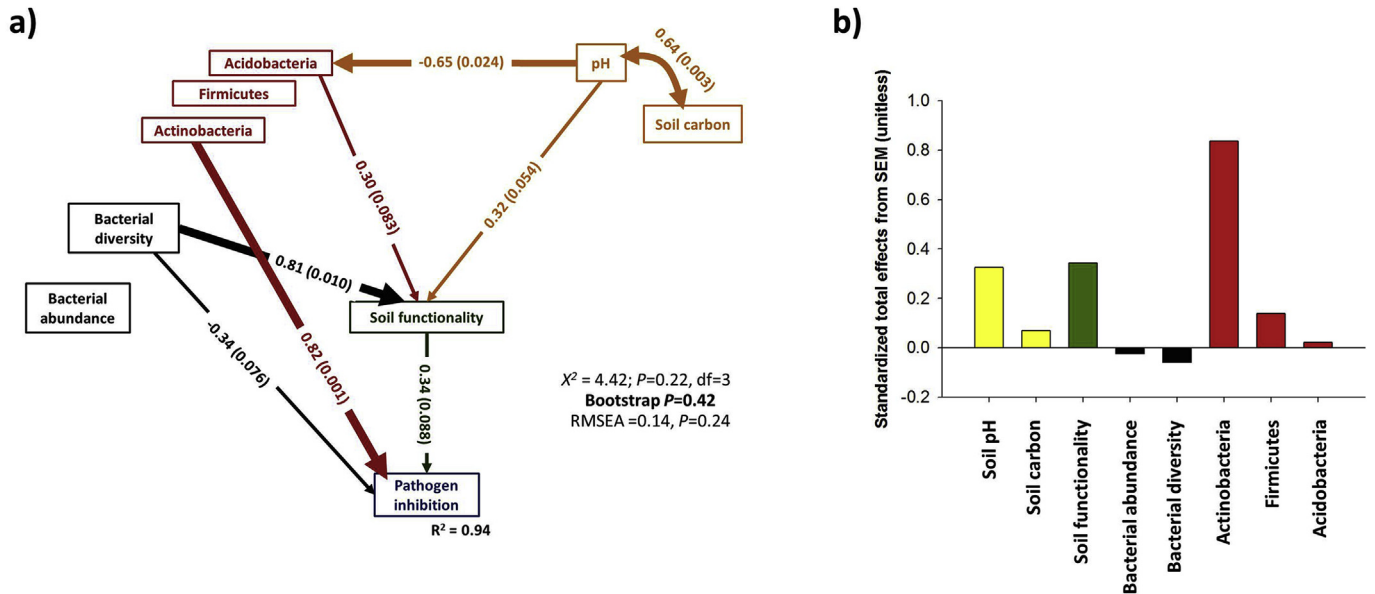
Fig. 2. Structural equation models based on the effects of soil properties (total C and pH), relative abundance of major predictors as determined by RF analysis, bacterial diversity, bacterial abundance, and Soil functioning on the pathogen inhibition. Numbers adjacent to the arrows are standardized path coefficients, analogous to partial regression weights and indicative of effect size of the relationship. Arrow width is proportional to the strength of path coefficients. R^2 indicates the proportion of variance explained. Model fitness details are provided adjacent to the path analysis. Results from covariate effects among microbial variables were not shown looking for visual simplicity. Panel b represent standardized total effects (direct plus indirect effects) derived from the structural equation models used.

similarity across continental scale suggests that it may be possible to develop a general approach to engineer disease-suppressive soil microbiomes (Raaijmakers and Mazzola, 2016). Integration of such semi-quantitative data and subsequent validation with other soil-borne pathogens, disease-incidences, yield and soil community characterization will lead to the development of risk management tools and competency standards for novel concepts such as "*Know before you Sow*" with the potential to deliver long-term solutions to soil-borne pathogen problems.

Data deposition

The raw sequence data have been deposited in the NCBI Sequence Read Archive (accession no. SUB2504549).

Authors contribution

P.T. designed the study. P.T., M.D.B., C.T., K.H., I.C.A., and B.K.S. analyzed the data. P.T. wrote the manuscript and all other authors contributed to revisions.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2017.03.013.

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