Report on the holobiont as promising selection target to improve resilience and product quality

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Executive Summary

The deliverable D3.7 aims to support the scientific community to promote an adapted vision of plant breeding for organic agriculture that has been called "organic plant breeding". This vision advocates a holistic approach and comes back to the foundation and the four principles of organic agriculture, i.e., ecology, fairness, health, and care. After a short introduction (**Chapter 1**) and description of LIVESEED experiments (**Chapter 2**) this document is constituted a literature review of the holobiont (**Chapter 3**), the consequences in terms on organic plant breeding and seed production (**Chapter 4**), the results of various LIVESEED experiments (**Chapter 5**) and a synthesis on new perspectives based on the integration of these sources of information (**Chapter 6**), as well as detailed results of LIVESEED experiments in Annex 1 to 4.

Results from several tasks in WP2 and WP3 of the LIVESEED project which worked on the exploration of the microbiome were at the basis of deliverable D3.7: (i) about organic seed health strategy (task 2.3.1 on carrot seed health and task 2.3.2 on wheat with common bunt management), (ii) plant adaptation in contrasted environments (respectively task 3.2.3 with tomato and task 3.2.1 for maize), (iii) plant breeding for microbiome mediated disease resistance (task 3.3.2 with pea and maize), and (iv) an integrative approach in which robustness of seeds of peas and carrots were evaluated with both AOX-SHAM-inhibition and calorespirometry (task 3.3.1).

Chapter 3 is a literature review about the potential of the holobiont concept for organic seed production and organic plant breeding. Several aspects are covered: i) the change in the holobiont during the plant domestication process for comestible produces, ii) the complexity of microbial transmission by seeds, and then, iii) the important role of the holobiont in plant adaptation and co-evolution for improved resilience in the agroecosystems. Below are the main conclusions provided per subsection:

i) The change in the holobiont during the plant domestication process for comestible produces

Looking back at the domestication process and development of agriculture, especially during the last century of industrial agriculture, the genetic diversity of the associated microbiome might have been reduced and shifted, through the selection of traits which are mainly focused on harvest index and yield increases to cover humans' caloric demand. The consequences of modern agriculture and food systems are observed on the disorders of ecosystems and human health (malnutrition, obesity). This paper highlights how plant breeding can be more aligned with crop resilience and human health, even if compromising in terms of maximum yield, in order to favour a balance in microbiome and conserving some aspects of the robustness of wild plants.

ii) About seeds and the complexity of microbial transmission

In modern plant breeding crop health has mainly been thought of in terms of management of genes for disease resistance (e.g., through pyramidising monogenic resistance genes) neglecting that the plant microbiome might play an important role in adaptation. Seed microbiome studies are stressing the importance of the seed as a true vector of adaptation and health and invite to better take into consideration the seed production stage as relevant means to improve efficiency and resilience of organic systems. Organic seeds carrying a diversified living microbiome, full of adapted microorganisms -instead of sterile seed free from pathogens-, are a means to enhance the development of organic agriculture and sustainable cropping systems in general. Thus, sterilization of seed by heat or steam treatment might even have detrimental effects on seedling resilience, as it might destroy the beneficial microorganisms.

iii) About plant adaptation and evolution for resilience

The recent understanding of the living beings as holobiont reinforces the necessity to re-think plant breeding for organic systems which calls for health, resilience, diversity, enhancing adaptation and co-

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evolution of crops to their environment. The organic sector has rapidly understood the interest of the holobiont concept to better integrate the plants in their environment and practices for good adaption. The dynamics of coevolution, mainly in the context of climate instability, is perfectly included in the on-farm plant breeding activities but needs the conception of a new form of seed marketing in the formal plant breeding sector which, over time, has dissociated the environments of crop production from plant breeding and seed production.

Chapter 4 aimed to offer several new perspectives for organic seed production and organic plant breeding starting with the relationships between soil and seed, followed by a detailed description of various notions of heredity connecting genetic, epigenetic and holobiont knowledge that can contribute to the further development of organic plant breeding and seed production.

Chapter 5 summarised what was learnt from LIVESEED research:

i) Exploration of new biological domains with the method of calorespirometry

Respiratory heat rate (*Rq*) was significantly lower for the pea cvs G85, S91 and G78 and higher in the cvs EFB-2, S134, Respect-1 and EFB-1, but there was no correlation to root rot disease (**Annex 2A**). However, pea samples with higher values of Rq and RCO2 presented a high germination rate (**Annex 2B**). It was quite interesting that the seed samples of the susceptible cv. Respect-1 exhibited lower expression on the three AOX genes, was the cultivar exhibiting higher germination rates and higher values on the calorespirometric parameters. For breeding, it is important that the heritability of such AOX related traits as predictors for plant adaptation capacity are studied before recommendations are made to plant breeders and seed producers.

ii) Plant breeding for disease resistance

The main focus was to improve plant health by elucidating plant microbe interactions using a case study on pea and root rot caused by a complex of soil borne pathogens. Significant genotype effect as well as significant soil x genotype interaction on microbial community composition was detected. Results of the phenotypic root rot assessment in the field trials in Switzerland, France and Latvia could verify the resistance level of the selected pea lines selected with the screening tool. The data on microbiome data of pea roots from field trials revealed that disease pressure and sampling time are main drivers of microbiome community diversity. Diseased roots still harbour a complex fungal community with certain taxa being replaced. Year, location, and pea genotype showed differences in taxa richness and diversity (**Annex 3**).

iii) Understanding the microbiome evolution in on-farm plant breeding and diversified agronomical management

On-farm selection of tomato in France directly in an agroforestry context over two years has enlightened effects on microbiome structuration due to the strong environmental context but weak effect of variety on both bacterial and fungal communities of both varieties (**Annex 1**). The farming system was confirmed for its major impact on the soil rhizosphere microbiota for maize, and several fungal and bacterial taxa were found to be farming system-specific whatever the cultivars. Arbuscular mycorrhizae (phyla *Glomeromycota*) were among the most important functional groups in the fungal microbiota and *Achromobacter, Burkholderia, Erwinia, Lysinibacillus, Paenibacillus, Pseudomonas,* and *Stenotrophomonas* in the bacterial microbiota (Ares et al. 2021). Further research is ongoing (**Annex 4**).

iv) Managing seed production for seed health and resilient systems

We have particularly illustrated and confirmed that seed vigour and microbial activity on seeds and seedlings are inter-related for carrots: high seed vigour not only provides more tolerance to abiotic stresses as drought and cold but can also make seedlings more tolerant to pathogens. The findings have indicated that seed health and seed quality are intimately intertwined. A novel more holistic

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seed health strategy was developed and discussed with stakeholders. Practical recommendations that arise from this organic seed health and quality system integrate the role of diversity and the seed microbiome in seed quality aspects.

v) Sharing actors' knowledge for holobiont management

Several workshops were centred on the consideration of the microbiome for organic plant breeding and seed production to create awareness about the importance of the holobiont, develop common concepts and exchange knowledge.

Finally, in **Chapter 6** a synthesis with a scheme which aims to describe the different approaches of organic plant breeding and seed production (e.g., on-farm, small scale breeding, large companies) is proposed. A conceptual scheme is presented showing how hereditary information may be passed on at several levels (genetic, epigenetic and microbiome) and how this affects processes of adaptation and co-evolution and consequently also the resilience of agroecosystems. It is concluded that the recent discoveries and new understandings of the co-evolution between plants and their microbiome and the resulting dynamic interactions underline the importance of a holistic concept of organic plant breeding and seed production, also important for the further development of organic and conventional farming. The holobiont concept calls for a better balance and collaboration between different scientific approaches to improve the resilience of our food systems.

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1. Introduction

In 1943, Howard published the book An Agricultural Testament[1], in which he described the central concept of organic farming in which soil fertility was centred on building soil humus with an emphasis on a 'living bridge' between soil life, such as mycorrhizae and bacteria, and how this chain of life from the soil supported the health of crops, livestock, and mankind. Rudolf Steiner, another pioneer from the first decade of the 20th century also claimed the huge importance of microorganisms in the soil: "the soil surrounding a growing plant's roots is a living entity with a vegetative life of its own, a kind of extension of plant growth into the Earth[2]."

Nowadays, we are rediscovering and describing in detail the diversity and the role of microorganisms in the soil. This knowledge is fostering the conception of an authentic organic agriculture where all living beings are being considered through their complementary and synergetic roles. Conventional/industrial agriculture had been based on a very different vision based on Justus von Liebig (1803-1873), and his chemical concept of plant biology, who advocated that plants 'find new nutritive material only in inorganic substances'.

Since the late 1990's, advances in molecular technologies allowed the DNA sequencing of microbial communities. This enabled the identification of the genetic background of all the microbes – bacteria, fungi, protozoa and viruses – that live associated within a plant (endophyte) or attached to seed, leaf or roots of a plant in a particular environment and make up its microbiome, e.g., the diverse community of microorganisms living in and on it. Then, for plants, the "holobiont" concept emerged and was described as "a unit of biological organization composed of a host and its microbiota" (Margulis, 1993; Bordenstein et al., 2016). It is associated with its hologenome which is the complete genetic content of the plant host genome, its organelles' genomes, and its microbiome which together constitute the genetic conception of the living beings.

In the same way as all agronomical developments which support the industrial agriculture since the 19th century have been inspired by the chemical vision of life, plant breeding for this type of agriculture has been strongly based on the science of genetics and has progressed thanks to developments in molecular biology. Nowadays, the scientific community aims to promote an adapted vision of plant breeding for organic agriculture that has been called "organic plant breeding". This vision advocates a holistic approach and comes back to the foundation and the principles of organic agriculture thanks to the holobiont concept of living beings.

2. LIVESEED experiments

The following tasks in WP2 and WP3 of the LIVESEED project worked on the exploration of the microbiome:

- organic seed health strategy (task 2.3.1 with carrot and task 2.3.3 on wheat with common bunt management by WR in the Netherlands, ITAB, France and FiBL-CH),
- plant adaptation in contrasted environments such as agroforestry or organic/conventional comparison coupled with plant breeding strategies (respectively task 3.2.3 with tomato by INRAE, France and task 3.2.1 for maize by IPC in Portugal),
- plant breeding for microbiome mediated disease resistance (task 3.3.2 with pea and maize, FiBL-CH),

[1] https://thenaturalfarmer.org/article/a-brief-history-of-organics-in-the-us/

[2] https://hawthornevalley.org/blog/2019/07/11/living-soils-for-our-earth-our-community-and-ourselves-part-1/

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- and an integrative approach in which robustness of seeds/plants from peas and carrots were evaluated with both AOX-SHAM-inhibition and calorespirometry (task 3.3.1 by UEV, Portugal).

2.1. Objectives and research questions

In LIVESEED, several experiments aimed to support the further development of the holobiont concept as a plant structure hypothesis for substantiating organic plant breeding (WP3) and seed production (WP2). Several LIVESEED approaches, such as phenotypic evaluation and microbiome analysis, were combined in order to better understand the opportunity and consequences of plant breeding and seed production on plant adaptation to their environment and their interaction with the soil life, with questions related to the following topics:

- seed health, vitality and conservation according to microbiome composition
- plant adaptation to growing conditions and plant health through microbiome evolution.

The following research questions were addressed:

➢ *About seed health*:

(1) Can a more diverse microbiome give the seed more tolerance to soil borne pathogens or pests and do organically produced seeds have a more diverse microbiome (task 2.3)?

(2) Can the tolerance be due to the physiology of the seed or is it due to the seed microbiome contributing to the higher vigour of seeds, and can the microbiome be deteriorated due to poor storage? What have we learnt from the effect of seed ageing on the microbiome (task 2.3.1)?

(3) What is the effect of organic versus conventional seed production conditions on the diversity in the seed microbiome (task 2.3.3)? Can we perform an integrative approach of seed vitality (vigour, AOX activity) and resilience assessments task 3.3.1)?

➢ *About plant adaptation to growing conditions and plant health through microbiome evolution*:

(1) Can we observe the microbiome evolution through generations in seeds and roots of tomato for adaptation to agroforestry systems compared to their shadeless control (task 3.2.3)?

(2) What microbiome adaptation can be observed in tomato in contrasted environments and according to different plant breeding strategies for maize (task 3.2.3)?

(3) Is disease resistance expression of pea lines related with a specific microbiome composition when tested in infected and healthy / disease-suppressive soils (task 3.3.2)?

These research questions will be answered in three parts: Chapter 3 on literature review of the holobiont, chapter 4 on organic breeding and seed production, and chapter 5 on the results of various LIVESEED experiments. In chapter 6 these perspectives are integrated and synthesised. The LIVESEED experimental results combined with a recent literature survey will support recommendations for:

- organic plant breeding and seed production strategies
- strengthening the biological foundations of the principles of organic agriculture.

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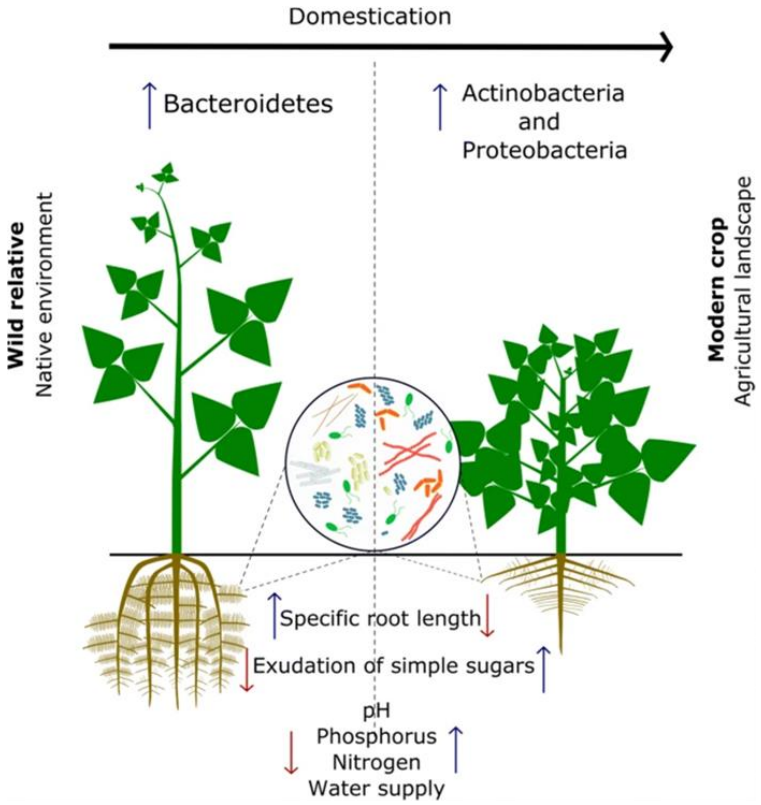
3. What we know from literature about the holobiont concept – Thoughts for organic seeds and organic plant breeding

3.1. About plant domestication for comestible produce

The development of new eco-evolutive knowledge makes it possible today to study interactions between plants and microorganisms. Every plant is colonised by microbial communities (fungi, bacteria, protists, archea, viruses, etc), either internal or external, differentiated among plants' organs (Compant et al., 2019). Environmental factors, particularly soil and climate (Fierer, 2017; Kong et al., 2019), as well as plant species and genotype (Peiffer et al., 2013; Bergna et al., 2018; Simonin et al., 2020; Xiong et al., 2021) and management practices (Hartmann et al., 2015) shape the microbiome associated with the different plant compartments, including seeds.



Pérez-Jaramillo et al, 2018

Figure 1. Impact of domestication on soil management, plant phenotype, plant physiology, and rhizobacterial diversity. In this hypothetical schematic representation, the root morphology of the wild relative of bean substantially differs from that of the modern counterpart. Readily available macronutrients and water associated with agricultural management led to shallower roots in the modern crop cultivars as compared to the roots of the wild relatives which are rooting deeper with conspicuous lateral roots. Domesticated crop plants presumably also exude more "simple" sugars than their wild relatives. The impact of the domestication process on rhizobacterial community composition is reflected in a decrease in Bacteroidetes abundance on modern crop plants, while the abundances of the Actinobacteria and Proteobacteria are increased (Pérez-Jaramillo et al. 2018).

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Domesticated crop species are the result of and reflect different types of evolutionary processes, arising as wild species become exposed to new very distinctive selection environments associated with human cultivation and use (Purugganan, 2019). Several studies on beans, sugar beet, barley, lettuce and *Arabidopsis,* reviewed in a meta-analysis by Pérez-Jaramillo et al. (2018), suggested that domestication led to compositional changes in the root microbiome. The most common domestication syndrome is related with the changes in the type and amounts of secondary metabolites, such as the loss of specific compounds that are toxic for humans or livestock or the reduction of flavonoid content in the leaves. The microbial footprint of domestication revealed similar changes for different crops with a shift from slow-growing bacterial phyla such as *Bacteriodetes* on crop wild relatives and ancient cultivars to fast-growing phyla such as *Proteobacteria* on modern crop cultivars (Figure 1 above). This striking shift in microbiome composition has also been described for the human gut of lean and obese people (Turnbaugh, 2006; Berg and Raaijmakers, 2018).

New plant breeding programmes for organic systems should come back to the observation of *in situ* conservation of crop wild relatives in their centre of origin and the microbial associations that have co-evolved with the crop (Hohmann and Messmer (2017). In order to avoid the loss of co-evolved beneficial cooperation between plants and microbiomes we need to better understand the function and metabolism of native microbiota of crop wild relatives with respect to their contribution to plant adaptation and resilience. Thus, the genetic diversity of the associated microbiome should be safeguarded as well as crop wild relatives themselves (Berg & Raaijmakers, 2018).

What can we learn for organic seed production and plant breeding?

Looking back at the domestication process and development of agriculture, especially during the last century of industrial agriculture, the genetic diversity of the associated microbiome might have been reduced and shifted, through the selection of traits which are mainly focused on harvest index and yield increases to cover humans' calory demand. The consequences of modern agriculture and food systems are observed on the disorders of ecosystems and human health (malnutrition, obesity). In this paper we want to highlight how plant breeding can be more aligned with crop resilience and human health, even if reducing the performance in terms of yield, in order to favour a balance in microbiome and conserving some aspects of the robustness of wild plants.

3.2. About seeds and the complexity of microbial transmission

Considering the holobiont concept of plants, seeds constitute at least two pathways of heredity, in terms of transmitting information from one generation to the following: the DNA with the genetic and epigenetic information on the one hand, and the microorganisms inside and at the surface of the seed on the other hand. Differences in seed production conditions like pedo-climatic conditions and farming practise can result in altered seed germination characteristics and also in epigenetic and microbial changes. Until now such influences have not been taken into account in the global seed market, but they can help plant adaptation in the case of on-farm plant breeding and seed production.

Seeds are also a complex physical support of microorganisms. Figure 2 (next page) shows several transmission routes for microorganisms to reach the seed, either horizontally from the neighbouring environment or vertically from their parent (Gundel et al., 2011), both contributing to the composition of seed microbiota (Vandenkoorhuyse et al., 2015):

> "*Microbes may enter seeds maternally via the carbohydrate transport route from the leaves to the seed's outer coat, paternally via pollen grains or environmentally via penetration of the nectarthodes or the stigma style of the flowers. In addition to the vertical transmission via the parents, members of the plant microbiota are transmitted horizontally from the surrounding*

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environment or, for several wild plant species, possibly via passage through the gut of birds or other animals. Inside seeds, microbes have been localized in the seed coat but can also be found on the cotyledon as well as on the root hypocotyl embryo after seed germination (Berg and Raaijmakers, 2018*)".*

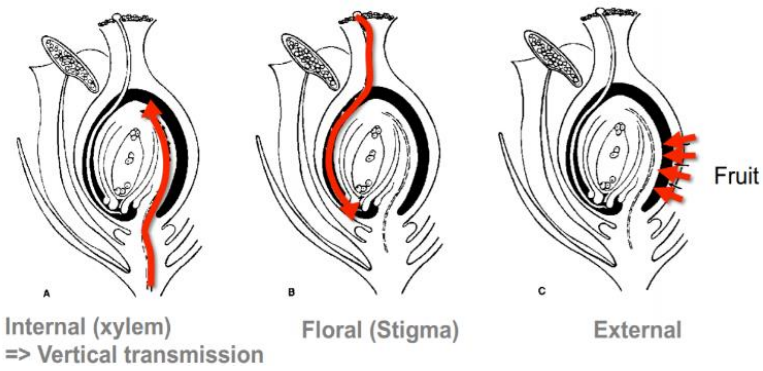

Figure 2. Three main pathways for the transmission of microorganisms on the seed, as currently described: (Maude, 1996): - internal (from plants to seeds), - floral (from stigma to seeds), - external (through the xylem)

Therefore, microorganisms can be found in every compartment of the seed (Shade et al., 2017): seed surface, embryo and storage tissues (endosperm and perisperm). During seed germination, some of the endophytic microorganisms living in the seed are preferentially inherited through distinct pathways to the seedling (Abdelfattah et al., 2020), as they have a competitive advantage because of their adaptation to living inside plant tissues (Kaga et al., 2009; Hardoim et al., 2012). These endophytes can either be neutral, pathogenic, or beneficial, the latest being preferably transmitted (Bergna et al., 2018). Moreover, their mode of action might depend on their relative abundance at a given developmental stage.

Among others, the seed microbiome supports:

- seed germination, seedling growth and establishment as well as the recruitment of other taxa by interacting with other microorganisms in the spermosphere (Nelson et al., 2018). These interactions between seed microbiota and soil microbiota are crucial for the plant fitness and are strongly impacted by plant species and soil type together (Berg & Smalla, 2009).
- disease resistance with the example of the role of endophytic microbiota of rice seed (Matsumoto et al., 2021)
- preservation of the continuity of transmission from one generation to another for plant adaptation (Berg & Raaijmakers, 2018).

What can we learn for organic seed production and plant breeding?

In modern plant breeding the crop health has mainly been thought in terms of management of genes of disease resistance (e.g., through pyramidising monogenic resistance genes) neglecting that the plant microbiome might play an important role for adaptation. Seed microbiome studies are stressing the importance of the seed as a true vector of adaptation and health and invite to better take into consideration the seed production stage as relevant means to improve efficiency and resilience of organic systems. Organic seeds carrying a diversified living microbiome, full of adapted microorganisms -instead of sterile seed free from pathogens-, are a means to enhance the development of organic agriculture and sustainable cropping systems in general. Thus, sterilization of seed by heat or steam treatment might even have detrimental effects on seedling resilience, as it might destroy the beneficial microorganisms (Morella et al. 2019).

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3.3. About plant adaptation and evolution for resilience

A main precondition of an ecosystem's resilience, including the resilience of agro-ecosystems, is the "health" of the living beings which compose it. Döring et al. (2012, 2013) have reviewed several ways to describe the notions of health and resilience. In organic agriculture, the term 'health' has been defined in the IFOAM principles and plays a central role for sustainable food systems, which aim at enhancing the "health of soil, plant, animal, human and planet as one and indivisible". The notion of resilience establishes links among the different domains of health. In that way, the microbial communities are prime candidates for linking the health of different organisms and systems. It is recognised as well for the agricultural soils as for the human gut. For instance, Western diets induced dysbiosis of the gut microbiota which has been shown to negatively impact human digestive physiology, to have pathogenic effects on the immune system, and, in turn, cause exaggerated neuroinflammation (Rinninella et al, 2019; González Olmo et al. 2021). Döring et al. (2012, 2013) concluded that resilience is a dynamic and relevant criterion of health across all levels and areas of agriculture and can be used as a link between the domains of soil, plant, animal, human and ecosystem health.

However, the global approach of health largely ignores the link between diversity and health, and therefore resilience. The management of biodiversity as a general approach to solve health problems in agriculture calls for greater biological diversity to promote simultaneously soil health, plant health, animal health, human health, and ecosystem health (reviewed by Vieweger & Döring, 2014). The soil biodiversity and crop diversity are vital. Large-scale, genetically-uniform, intensive monoculture production systems favour strong outbreaks and epidemics of pests and pathogens in agroecosystems (Zhang et al., 2020, Wei et al, 2020, Favela et al, 2021).

Along their evolution in natural systems, plants learned to adapt to the given environment and to interact with soil microbiota, extracting their utmost capacity to provide resources for plant development and successful colonization of terrestrial systems. Here, a large soil biodiversity is key for properly exerting this role and, thus, a prerequisite for plant adaptation in natural systems. Studies on the rhizosphere provide evidence of the powerful selection exerted by plants upon the living soil microbes. Starting from domestication in cultivated fields (Figure 3, next page), this symbiosis has been neglected, reducing both soil and crop biodiversity, and consequently, reducing plant development through the interference in their association with beneficial microbes (Dini Andreote and de Cassia Pereira e Silva, 2017).

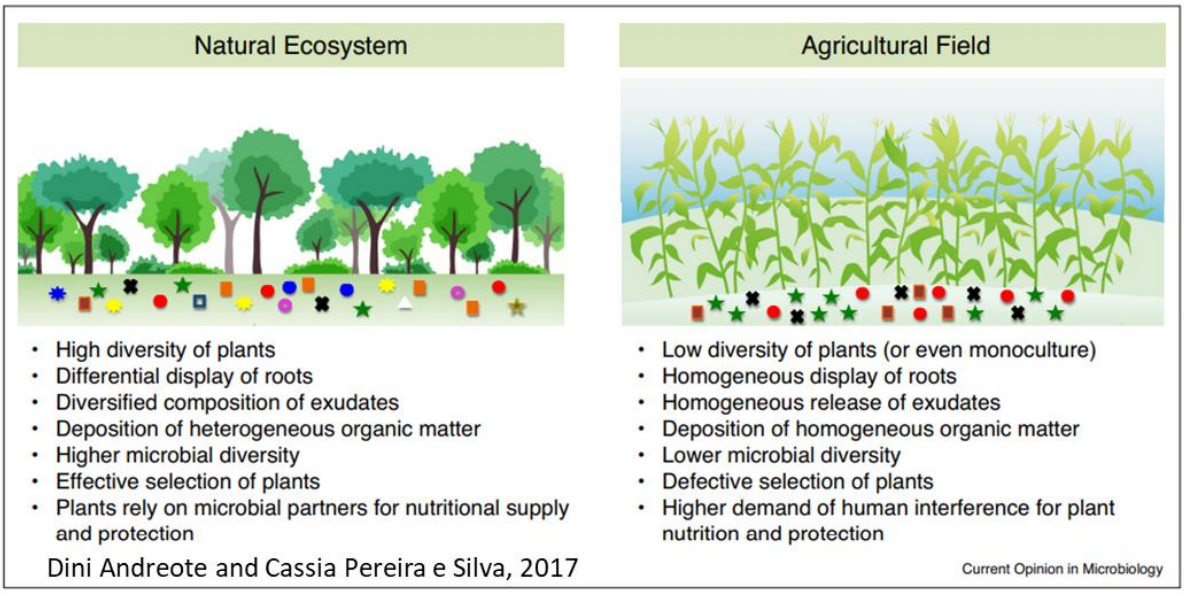

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Figure 3. Distinctive key characteristics and microbial interactions of plants and microbes in natural ecosystems and agricultural fields. Geometrical forms indicate the occurrence of microbial groups in soils (Dini Andreote and Cassia Pereira e Silva, 2017).

As domesticated species moved out of their original ranges and colonized new areas, they had to adapt to local environments and agronomical practices. Modern practices have increased the size of specific plant organs with the aim to increase crop production. But domestication and plant breeding shaped the crops without taking into account this dynamic of the relations between all components of an agroecosystem (Ottesen et al., 2013): (1) lack of co-evolution patterns, (2) reduced methodological concepts based on genetic knowledge and (3) lack of knowledge on microbial ecology.

In parallel, several biological concepts have created spaces for thought beyond the genotype / phenotype couple. Margulis (1981) has provided a very renewed vision of evolution with the ubiquity of symbiotic communities that reveals the closely intertwined relations between plants and-microorganisms which are inherent to plant life and its evolution. Bordenstein and Theis (2015) pointed out the limitations of the biological science in light of a new vision for the central importance of microbiology. Animals and plants are no longer considered as autonomous entities but rather as networks composed of the host and its associated microbes, i.e., "holobionts." Some authors have adapted a genetic theory, the hologenome, and suggested that the holobiont (the host and its symbiotic microbiota) is associated to its hologenome, acting in consortium, and should be considered a unit of selection in evolution, and that relatively rapid variation in the diverse microbial symbionts can have an important role in the adaptation and evolution of the holobiont (Zilber-Rosenberg & Rosenberg, 2008).

These concepts put a new perspective on the dogma about the plant's phenotype relying mostly on its own genotype, as there is a strong influence of microbial communities on the fitness of a plant. The equilibrium of these relations relies on the co-evolution of plants and microorganisms in given environments and these all have numerous implications for plant development and adaptation, and therefore for rethinking plant breeding (Vandenkoornhuyse et al., 2015).

What can we learn for organic seed production and plant breeding?

The recent understanding of the living beings as holobiont reinforces the necessity to re-think plant breeding for organic systems which calls for health, resilience, diversity, enhancing adaptation and co-evolution of crops to their environment. The organic sector has rapidly understood the interest of the holobiont concept to better integrate the plants in their environment and practices for good adaption. The dynamics of co-evolution, mainly in the context of climate instability, is perfectly included in the on-farm plant breeding activities but needs the conception of a new form of seed marketing in the formal plant breeding sector which, over time, has dissociated the environments of production from plant breeding and seed production.

4. Perspectives for organic seeds and organic plant breeding

*"The recent history of crop domestication and breeding has diverted crop plants from the evolutionary trajectories of their wild counterparts by selecting traits mainly associated with productivity under high-input conditions. This approach neglected the contribution of the microbiota to plant growth, development, and health. Thus, domestication and breeding have likely eroded the genetic diversity of the crop-associated microbial communities although the full impact of these processes on the crop microbiota remains to be fully elucidated" (*Escudero-Martinez and Bulgarelli, 2019).

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In this part, we will consider, in the light of the holobiont concept, some thoughts to boost an organic conception of plant breeding and seed production, and the consequences in terms of seed production environments.

4.1. Perspectives for organic systems' conception from soil to seed, and vice versa

No soil without plant!

The first concern of the organic pioneers such as Rudolf Steiner and Albert Howard was the soil and its degradation due to the chemical and industrial agriculture. Soil without plants is extremely rare: roots of higher plants and their associated microbes play a major role in soil formation processes; the choice of modern and homogeneous crops has thus also greatly modified the life within the soil. The coevolution of plants and soil is a complex process and an intermingled system that is often specific to context and history (Pierret and Moran, 2011). Microbiome alterations have the potential to affect subsequent generations of plants that germinate in the same soil with consequences for ecological and agricultural processes; inversely, recent observations stated that enrichment of protective microbes in the rhizosphere is associated with the development of disease-suppressive soils in which plants remain healthy even in the presence of pathogens (Teixeira, et al, 2019).

Understanding interdependencies between plants and soil should support the conception of new organic systems that can enhance interactions between living beings in a given agroecosystem, with the aim to reach resilience through dynamic equilibrium relations (Shade et al. 2019). First of all, we know that in the soil rhizosphere microbiota, several fungal and bacterial taxa were found to be farming system-specific, with an advantage in terms of the rhizosphere microbiota diversity for the organic farming system (Ares et al., 2021). Nevertheless, the composition, diversity, and function of beneficial rhizospheric microorganisms including Arbuscular Mycorrhizal Fungi (AMF) communities vary upon agronomic practices and soil conditions but also depend on the choice of cultivars: some modern crop cultivars are less responsive to AMF, since they are bred for highly intensive agricultural systems where there is sufficient supply of nutrients, especially P (Njeru, 2018). This implies that for different types of organic farming, different approaches towards diversification in organic farming systems should be considered.

Diversity needed for resilient agroecosystems

Field diversity as well as landscape heterogeneity are the most viable paths to increase productivity, sustainability, and resilience of agroecosystems (Nicolls et al. 2016). The higher the vegetational diversity of agroecosystems, the greater the capacity of the agroecosystem to buffer against pest and disease problems as well as to shifting climatic patterns (Figure 4, Zhang et al., 2020).

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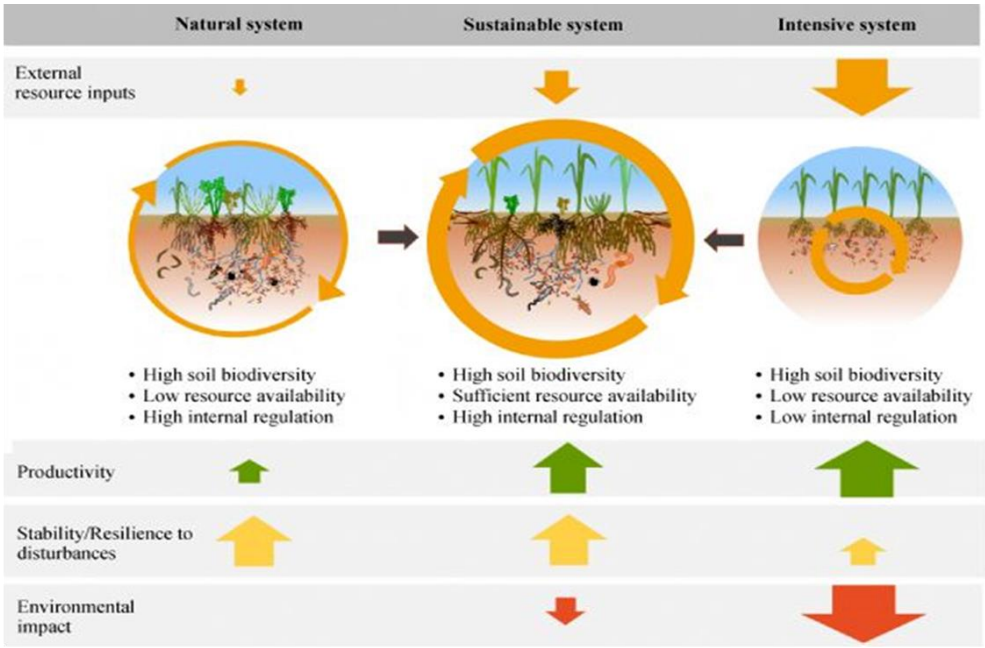

Zhang et al., 2020

Figure 4. Conceptualization of a sustainable cropping system combining features of natural ecosystems (high biodiversity and high level of internal regulatory processes) with features of intensive cropping systems (high productivity) to meet the challenge of producing sufficient yields of high quality with high resilience to disturbances (e.g., climate extremes, pest outbreaks), low external resource inputs and low environmental impact (Zhang et al., 2020)

Agriculture i.e., plant choice, changes the soil composition (Pérez-Jaramillo et al., 2016)

The transition from natural to agricultural systems may have hampered beneficial interactions between plants and microbes due to loss of soil microbial diversity. For instance, it was shown that long-term nitrogen fertilization resulted in the evolution of less-mutualistic rhizobia, providing fewer benefits to the host (Weese et al. 2015). In addition to changes in the production systems, domesticated lineages experienced a range of expansions far beyond their centres of origin due to human migrations and trade (Purugganan and Fuller 2009). Hence, the lack of a co-evolutionary trajectory between plants, microbial communities and pathogens in new agricultural landscapes made human interventions even more critical to maintain a healthy and productive crop. However, soil attributes can be affected by plant domestication, which in turn influence the soil microbial community composition. For example, García-Palacios et al. (2013) demonstrated, in microbial-rich and microbial-poor soils, that plant domestication increased litter quality, resulting in lower C:N ratio and higher NO3 availability.

Therefore, the responsibility of plant choice is high when it is known that fungal-bacterial diversity and microbiome complexity predict ecosystem functioning and indicate the importance of microbial interactions within and among fungal and bacterial communities for enhancing ecosystem performance (Wagg et al., 2019).

The choice of seeds' origins has responsibilities in the impoverishment of soil diversity

Considering the dominant seed systems, we have to consider that the international trade of seeds managed by international seed companies is by itself a factor of limitation for ecosystem performance. Seeds travel world-wide between major seed production areas and cropping areas in the Northern and Southern hemisphere to secure seed production all year round. This global trade of seeds has

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contributed to the homogeneity of the plant microbiome at a global scale but may also impact on soil microbial diversity and health (Berg and Raaijmakers, 2018).

4.2. Plant breeding for organic systems: which definition of heredity?

From the notion of "unit of selection"

Most studies on genome evolution focus only on the inheritance of DNA between parent and offspring. The overall plant breeding industry has been based on this hypothesis, in line with the widely accepted Neo-Darwinian framework that pairs Mendelian genetics with natural selection as explanations for the evolution of biodiversity on Earth (Collens, 2019).

The holobiont concept, with the association of genotypes of hosts and their microbiomes, has suggested that a new conceptual framework is needed with a new consideration of the appropriate unit of selection (Koskella and Bergelson, 2020). This "unit of selection" has been defined with three principles by Lewontin (1970), based on the observation of the phenotype: (i) that phenotypic variation among units exists; (ii) that this variation results in differential fitness (i.e., survival and reproduction); and (iii) that the traits underlying these fitness differences are heritable. In fact, the holobiont concept of the living beings helps us to go beyond this notion of "unit of selection" because different types of living beings (e.g., plants and their microbiome) are connected to each other.

> *"Modern plant breeding can no longer afford to ignore the interaction between plants and microbial key players. Increasing evidence suggests (i) that the expression of many plant traits (such as nutrient use efficiency or tolerances against biotic and abiotic stresses) is mediated by beneficial microorganisms and (ii) that there is an exploitable genetic base for the regulation of symbiotic relationships"* (Hohmann and Messmer, 2017).

Genetic, epigenetic and holobiont, three forms of hereditary information

Nevertheless, the recent concepts of phenotype variations question the gene centric conception of heredity. It is known that epigenetics provides also phenotypic variation in response to environmental conditions without individual genetic diversity for adaptive 'evolutionary' changes (Bossdorf et al., 2008). The links between genotype and phenotype are not that evident anymore when we include factors such as (i) epigenetic effects inducing modifications of gene expression, post-transcriptional and post-translational modifications, which allow a quick response to an environmental stress (Shaw and Etterson, 2012); and (ii) the adjustment of plant symbiotic microbiota dynamically recruited to adjust to environmental constraints (Vandenkoornhuyse et al., 2015). Some authors try to include the concept of the hologenome (genome of host plus symbiont) into an epigenetic phenomenon (Collens, 2019). Changes in DNA adenine methylation patterns could occur since the establishment of symbiosis, suggesting an effect of this interaction on the bacterial epigenome or at least, a role of epigenetic mechanisms in symbiosis development (Vanier et al. 2015*).* Observations indicates that histone acetylation is an important mechanism mediating inter-species or even inter-kingdom interactions: (1) several pathogens have been reported to affect the chromatin structure and transcriptional program of host cells by altering histone acetylation; and (2) histone acetylation was recently found to be involved in the bacteria–fungi interaction (Chen et al. 2018).

Considering the microbiome itself, individual symbionts or the whole microbiome can result from either vertical transmission of symbionts from parents to offspring or from specific host genetics that differentially 'filter' microbial communities through a horizontal acquisition. The "heredity" of microbiome information then is very complex and closely linked to the environmental complexity and its diversity of taxa.

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One of the key-issues of organic plant breeding is therefore to formulate new breeding objectives taking into account the complex plant-microbiota interactions and therefore to manage environmental conditions in order to select for the best associations between hosts and their microbial communities.

Adaptation, evolution, and plant breeding

Because epigenetic and plant-associated (micro-)organisms are both key sources of phenotypic variation allowing environmental adjustments, they must be considered in terms of short, medium, and long-time scale evolution for plant adaptation to changing environments (Vannier 2015). It is easy to conceive how the evolution of living beings is working in the "wild environment" in which all phenomena of adaptation may interact in complex ways, but how can this knowledge be put into practice in plant breeding and seed production?

In other words, if considering that host plants are able to stably transmit or recruit the required microbiota from generation to generation in natural contexts (Koskella and Bergelson, 2020), we may assume that this relationship remains the same in cropping systems when selection and seed saving is done on-farm in a given terroir. In contrast, such co-evolutionary dynamics are much more difficult to achieve when plant breeding, seed production and production are each managed in different environments.

4.3. Perspectives for integrating plant breeding and seed production

Over the last century, plant breeding has become more and more centred on plant genotypes and has supported a large market of seeds as they were not aware of epigenetic adaptation processes, nor microbiological interactions. Moreover, the objectives of selection related to stability and homogeneity of varieties in order to meet registration requirements has reinforced the genetic foundation of plant breeding in order to meet the uniformity and stability requirements, and finally led to the decrease of the diversity of the living beings (plants) and the resilience of the environment. Hence, the challenge for organic plant breeding is to emphasise the importance of the holobiont in terms of breeding procedures. It should result in a new model for the future, from new breeding practices to different marketing approaches and regulations that fit to these new breeding practices.

Managing plant breeding and seed production with the plant holobiont concept for better adaptation

Finally, microbes not only influence but are also part of many plant trait expressions such as nutrient use efficiency, drought and salt tolerance and disease resistance (Wille et al, 2020). When breeders select for these traits, they usually focus on their phenotypic expression without paying much attention to the role of associated microbes (Hohmann et al., 2016).

Currently, it is considered that there is an exploitable genetic base for the regulation of symbiotic relationships. The plant may play an active role in the process of mutualist-induced environment adaptation as it may be able to recruit microorganisms from soil (for review Vandenkoornhuyse et al., 2015) and preferentially promote the best co-operators through a nutrient embargo toward less beneficial microbes (Kiers et al., 2011).

In the perspective of better using the potentialities of microorganisms in agriculture, we need to elucidate the link between the beneficial functions of individual microorganisms or whole microbiomes with plant traits in order to (i) solve context dependency for microbial inoculation approaches, (ii) identify the resources which are still able to cooperate and (iii) identify the genetic determinants to allow breeding for improved beneficial plant–microbiome interactions (Hohman et al., 2020).

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In recent years, plant-associated microbes have received considerable attention in research for their ability to improve crop productivity and yield stability. Benefits include improved nutrient uptake and resistance against biotic and abiotic stresses. Influences of crop management, soil parameters and climatic effects are well documented. Knowledge on plant genetic determinants for beneficial interactions with individual microbes and entire communities is growing. Several reports indicate that not only the host species but also the host genotype play a significant role in driving microbial community composition and activity, with the host plant selecting for and against particular members of the microbial community. However, to what extent genetic factors are responsible for shaping beneficial plant microbiomes is still poorly understood. Similarly, seed or plant microbiome manipulation via the introduction of biologicals offers great promise, but still suffers from variable outcomes due to insufficient knowledge of the factors involved for a successful integration (Hohman et al., 2020). In conclusion, there are still many uncertainties on how to implement this knowledge into plant breeding and seed multiplication programmes.

This challenge goes beyond plant breeding and has to be included in seed production as well to preserve microbiome potential over plant generations (Groot & Raaijmakers, 2018). This implies high quality of organic seeds which can be reached through innovating in their production and storage (Groot et al., 2006).

It has become clear that the seed microbiome should also be taken into account when considering seed health. Until recently, seed health was almost exclusively oriented at controlling seed borne pathogens, but seeds can also contain beneficial micro-organisms that help the seedling against pathogens or abiotic stresses. In an up-to-date seed health strategy, the seed microbiome should be considered as part of seed health. A highly biodiverse microbiome seems to be advantageous for the seedling (Wassermann et al., 2019). Since the seed microbiome is partly originating from the soil and organic soils have a more biodiverse microbiome (Hartmann et al., 2015; Lupatini et al., 2017), this may give an advantage for organically produced seeds (Klaedtke and Groot, 2021).

Managing plant breeding and seed production at farm to favour co-evolution between plants and their ecosystems

Wild plants have evolved over time by selectively assembling plant-beneficial microbiota from the soil as their partners. The development of agriculture was based on the domestication of a large range of crop species based on plant phenotypes, but not consciously excluding the complexity of the holobiont interactions. Today, especially for major crop species, conventional plant breeding using molecular methods focused only on the plant genome in order to develop genetically homogenous crops, to be cultivated in a large range of ecological conditions. This conception of plant breeding has led not only to the erosion of genetic diversity of the plants; but also, to the extinction of huge microbial diversity in soils that would have been the source of several plant-beneficial microbiota (Perez-Jaramillo et al., 2016). Thus, we acknowledge that the plant seeds, the starting point for the next plant generation, have co-evolved with diverse microorganisms to assist seed preservation and germination, seedling growth and stress tolerance. Then the plant microbial assembly evolves from seedling to later developmental stages by adaptation. The microbial component of healthy seeds appears to be inherited between plant generations (Dai et al, 2020).

Nowadays, numbers of farmers in the world are reintroducing plant breeding and seed production on farm, thanks to decentralised plant breeding such as participatory plant breeding (PPB) or on farm selection (Chable et al, 2020). This brings the considerable advantage of benefiting from the entire range of epigenetic adjustments and microbial assemblies inherited from the previous generation: it allows a rapid adaptation thanks to co-evolution between the holobiont and its environment.

These alternative breeding initiatives are being used to develop more diverse cultivars better adapted to organic and low-input systems (Dawson et al, 2008). Decentralized breeding aims at developing

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cultivars adapted to the diversity of environments and farmers' practices, by breeding directly in the target environment with the farmers (van Franck et al, 2020) and by using seeds that have been produced where the crop will be cultivated. In parallel, we may imagine the positive feedback on the soil. We know that for nearly one century, modern agricultural practices have altered the interaction of crops with their root microbiome. An interesting option for further research would be to understand to which extend the consequences of a lack of co-evolution between plants and their microbiome are reversible by on-farm plant breeding and seed production, by reintroducing crop diversity and by cultivating plants fully able to interact with microorganisms.

5. What have we learnt from LIVESEED research?

LIVESEED activities have been conducted directly or indirectly activities associated to the holobiont hypotheses. The following table summarises these activities and provides the developed approaches with methods, data and results.

WP-Task	Partners	Questions/species	LIVESEED Documents
T2.3.1	Steven Groot (WR)	Effect seed production conditions on the seed microbiome; Ageing of the seed microbiome during storage on carrots	Deliverable D2.5 and Task 2.3 New Seed Health Strategy and Deliverable 6.3 Synthesis of the results, Chapter 4, p33[3],
T2.3.2	Stephanie Klaedtke (ITAB)	Common bunt on wheat and seed quality	Id.
T3.2.3	Solène Lemichez (INRAE)	Microbiome evolution through generations in roots for adaptation to agroforestry systems compared their shadeless control, on tomato	D2.6 and Annex 1
T3.3.1	Hélia Cardoso (UEV)	Usefulness of calorespirometry method to assess seed viability, on peas	Deliverable D3.2 and Annex 2A and 2B + publications
T3.3.3	Pierre Hohmann (FiBL-CH)	Plant health improvement by elucidating plant-microbe interaction using the case study of pea and root rot caused by a complex of soil-borne pathogens	Annex 3 and D3.3 + publications
T2.3.3	Pedro Mendes-Moreira (IPC)	Performance of maize varieties and their behaviour throughout selection, as well as their response to different environments	Annex 4 + publication

The activities associated with the holobiont approach have been introduced in five types of LIVESEED activities: exploration of new biological domains with the method of calorespirometry, plant breeding for disease resistance, understanding the microbiome evolution in on-farm plant breeding, managing seed production for seed health and resilient systems, and sharing actors' knowledge for holobiont management.

[3] Microsoft Word - _LIVESEED_D6.3__FINAL_20210727.docx

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5.1. Exploration of the interest of the alternative oxidase (AOX) activity as functional marker of seed vitality, experimentations on carrot and pea

Alternative oxidase (AOX) is a non-energy conserving terminal oxidase in the plant mitochondrial electron transport chain. AOX protein is located in the inner mitochondrial membrane and is encoded in the nuclear genome being involved in plant response upon a diversity of environmental stresses and also in normal plant growth and development. There is increasing evidence that AOX plays a role in molecular and metabolic cell reprograming under stress, and therefore, has been proposed as functional marker for multi-stress tolerance (Mohanapriya, 2019). In the LIVESEED project, we wanted to explore if this approach may help breeders and seed producers for the evaluation of the robustness of plant (Arnholdt-Schmitt, 2017). The question was whether AOX related traits during germination can be used as an indicator/a functional marker. To answer this question the methodology of isothermal calorespirometry was used to quantify both heat dissipation (specific respiratory heat rate, Rq) and oxygen consumption (respiratory CO2 rate).

The first phase was dedicated to adjusting the protocol to the different plant species from partners (carrot, broccoli, spinach and pea) and make a calibration to see if the identified markers can be linked to stress adaptation and robustness. The methodology is based on applying isothermal calorespirometry and consists of three steps: plant tissue preparation, calorespirometry measurements, and data processing.

Preliminary data on carrot revealed larger differences between the tested genotypes than between organically and conventionally propagated seed of the same cultivar. First results with broccoli seed also revealed differences between organic and conventional seed source indicating the possibility to discriminate organic versus conventional seed quality through calorespirometry. Nevertheless, the experience has often shown confounding effect of seed production management and genotypic effects for AOX related traits.

During 2020, the approach was reduced to pea seeds. Several restrictions of lab activities due to the sanitary situation led to focus on different lines of pea produced by LIVESEED partners FiBL, AREI and ITAB/UBIOS in three countries (Annex 2B). Moreover, Evora University proceeded to the evaluation of the expression of genes that encode the Alternative Oxidase Protein (AOX) in pea seeds during germination and establish the link between gene expression and calorespirometric parameters (Annex 2A). Respiratory heat rate (Rq) was significantly lower for the pea cvs G85, S91 and G78 and higher in the cvs EFB-2, S134, Respect-1 and EFB-1, but there was no correlation to root rot disease (Annex 2A). However, pea samples with higher values of Rq and RCO2 presented a high germination rate (Annex 2B). It was quite interesting that the seed samples of the susceptible cv. Respect-1 exhibited lower expression on the three AOX genes, was the cultivar exhibiting higher germination rates and higher values on the calorespirometric parameters.

Conclusions from holobiont perspectives

One major challenge for traits related to respiration and AOX as trigger for stress adaptation will be to disentangle environmental and epigenetic effects from genotypic effects. For breeding, it is important that the heritability of such AOX related traits as predictors for plant adaptation capacity are studied before recommendations are made to plant breeders and seed producers.

Another perspective questions the metabolic nature of AOX coordination and provides deeper phenotyping during the germination stage; for instance, AOX measurements can help to better observe microbial synergy with plants at the early stage comparing endophyte-free and microbiota-inoculated seeds (Revuru, submitted).

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An aspect that has not been investigated at the end of the project, which was inspired from a literature review, was to provide scientific evidence for the application of AOX as functional marker to study the impact of cytoplasmatic male sterility CMS on plant robustness. This topic is very relevant and timely in the context of organic seed and was mentioned in LIVESEED and BRESOV project consortia.

5.2. Plant breeding for disease resistance based on holobiont hypothesis

Breeding for the holobiont is based on the concept that the performance of a plant is not only determined by plant genes but also by the genes of the whole microbial community. The hypothesis is that plants that can attract a balanced microbial community in the soil will have a higher resilience against various stresses. LIVESEED subtask T3.3.2 has explored the importance of the holobiont as potential selection target to improve resilience and elucidate the concept of microbiome mediated breeding for disease resistance in pea (*Pisum sativum* L.). The main focus of this task was to improve plant health by elucidating plant microbe interactions using a case study on pea and root rot caused by a complex of soil borne pathogens. The root microbiome structure of adult pea plants at time of flowering was investigated in a multi-site field experiment over two years. Year, location and pea genotype showed an effect on taxa richness and diversity (*Annex 3*).

The LIVESEED approach for pea resistance aimed to design a screening system using the complexity of infested field soil; the screening system provided opportunities to study plant resistance in the light of diverse plant-microbe interactions occurring in the rhizosphere. These screening systems needed to include major factors of the target environment, e.g., the soil type and the microbiome composition of that particular soil, to provide reliable and field relevant data for selection. Significant genotype effect as well as significant soil x genotype interaction on microbial community composition was detected. Results of the phenotypic root rot assessment in the field trials in Switzerland, France and Latvia could verify the resistance level of the selected pea lines selected with the screening tool. First data on microbiomes of pea roots from field trials revealed that disease pressure and sampling time are main drivers of microbiome community diversity. Operational taxonomic units (OUT) richness was higher in infected soil than in healthy soil and generally higher at early sampling dates. Within the sites, sampling date had a big effect on both alpha and beta diversity. Different pea genotypes had only a minor effect on alpha and beta diversity in healthy soil. Due to COVID the microbiome sequencing was delayed and presently analysis is ongoing for root and seed microbiome data, which will be published.

Conclusions from holobiont perspectives

Can this result be further investigated and understood within the Anna Karenina principle[4], according to which "all healthy microbiomes are similar and each dysbiotic microbiome is dysbiotic in its own way"? A successful pathogen invasion can disrupt the plant microbiota and drive higher community heterogeneity (Zaneveld et al. 2017). Plant growth and health depend on their associations with a large number of microorganisms that interact with each other. Interactions between plant pathogens and other plant-associated microorganisms form networks that regulate disease are called pathobiomes by some authors (Pauvert et al. 2020). Some pathobiome members form a barrier that limits pathogen development through direct antagonistic interactions while others can prime the plant immune system. However, we also see that several taxa of microorganisms might be beneficial in some cases but pathogenic in other crops or circumstances. Therefore, the relative abundance of certain microorganisms and the balance among the microbiome community might be more important.

[4] The Anna Karenina principle is based on the first sentence of Leo Tolstoy's book: "Happy families are all alike; every unhappy family is unhappy in its own way". This can be transposed to microbiology as: "All healthy microbiomes are similar; each dysbiotic microbiome is dysbiotic in its own way" (Zaneveld et al. 2017).

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Deciphering microbial interactions within pathobiomes, and determining what environmental factors shape those interactions, will be an important step towards improved plant breeding for healthy crops.

5.3. Understanding the microbiome evolution in on-farm plant breeding

Co-evolutionary processes were investigated in an agroforestry system where on-farm plant breeding and seed production have been managed by farmers on tomatoes with two landraces of tomato in two farms (*Roumassouze* and *La ferme du Boulidou*). The seeds are harvested each year and then resown under two main conditions: sunny and shaded plots to observe how the tomato evolved (Annex 1; and Annex I of LIVESEED report on Enhancing resilience for organic plant breeding[5] - D3.6, p185).

Our results show that on farm selection maintains the genetic structure of varieties (observed by SSR markers). The very weak effect of variety on both bacterial and fungal communities of both varieties is surprising in regard of literature. Nevertheless, on-farm selection directly in an agroforestry context over two years has enlightened effects on microbiome structuration due to the strong environmental context: the shadow of trees is not a common habitat for the species. Bacteria tend to segregate according to the light gradient while fungi tend to differentiate among extreme environments (deep shade or sunny conditions).

In another context (task 3.2.1, Annex 4) for maize by IPC in Portugal, a study aimed to unravel the effect of genotype and farming system on structural diversity and putative functions of the microbial communities in the rhizosphere of 16 open-pollinated maize populations and CCPs to verify the performance of the varieties and their behaviour through selection, as well as their response to different environments. Results are still pending due to the delay in laboratory analyses because of the sanitary situation.

The results of the first trials in 2019 were presented in a LIVESEED interim report (Deliverable D3.3) and was published in 2021 (Ares et al. 2021). IPC characterized the structural composition of fungal and bacterial communities present in the soil rhizosphere associated with the two maize populations and CCPs cultivated under organic and conventional farming systems. The farming system had a statistically significant impact on the soil rhizosphere microbiota, and several fungal and bacterial taxa were found to be farming system specific. The rhizosphere microbiota diversity in the organic farming system was higher than that in the conventional system for both varieties. This study has underlined the potential of organic practices to improve the microbial quality of the soil. Arbuscular mycorrhizae (phyla *Glomeromycota*) were among the most important functional groups in the fungal microbiota and *Achromobacter, Burkholderia, Erwinia, Lysinibacillus, Paenibacillus, Pseudomonas,* and *Stenotrophomonas* in the bacterial microbiota (Ares et al. 2021). Further research is ongoing (Annex 4).

Conclusions from holobiont perspectives

Our LIVESEED approach to on-farm plant-breeding and seed production has maximised the contrast between sunny and shady conditions of tomato crops in both farms to observe the microbiome evolution within a short period of time. Other approaches on the evolution of other traits always showed a rapid evolution in only few generations (Serpolay-Besson, 2013; Dawson et al. 2012, 2013; Kahn et al., 2020).

[5] https://www.liveseed.eu/wp-content/uploads/2021/08/LIVESEED_D3.6_Enhancing-resilience-at-systems-level-1-min-compressed-compressed.pdf

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Questions now emerge about how to combine plant diversity and evolutionary processes in order not only to provide adapted cultivars, but how they contribute to the revival of the soil, and therefore organic farming efficiency, especially thanks to genetic resources that have conserved their "interactivity" with the micro-organisms.

5.4. Managing seed production for seed health and resilient systems

Organic seed health issues in LIVESEED have been illustrated with 2 case studies, carrot with *Alternaria ssp*. and soft wheat with common bunt (*Tilletia spp*.). The studies have been reported in the LIVESEED Report "Inventory of scientific, legal, and technical measures to improve the quality of seed health in organic[6]" in which an inventory of current problematic issues for the quality of organic seed had been collected.

Based on these findings and on a review of scientific literature, a strategy for organic seed health and quality has been designed. In the LIVESEED project, we have particularly illustrated and confirmed that seed vigour and microbial activity on seeds and seedlings are inter-related: high seed vigour not only provides more tolerance to abiotic stresses as drought and cold but can also make seedlings more tolerant to pathogens. For carrots, high vigour un-aged seeds were more tolerant to damping-off caused by *Alternaria radicina* than artificially degraded, low-vigour seeds. The findings indicated that seed health and seed quality are intimately intertwined. The management of seedborne plant diseases in organic systems requires not only investigating seed health and sanitation per se, but also extending the view to the system in which the seed is embedded, as it was particularly discussed with the actors for common bunt.

All the practical recommendations that arise from this organic seed health and quality system integrate the role of diversity and the seed microbiome in seed quality aspects such as: (i) harness the potential of optimised seed microbiomes to aid in the protection of the seedling towards biotic (pathogens) and abiotic (e.g., climate) stresses, going towards more resilient cropping systems; (ii) investigate into optimised seed microbiomes and their implications, taking into account local variation and adaptation; (iii) investigate the effect of seed production conditions, harvesting, treatments and seed storage; (iv) place more emphasis on producing and maintaining high seed vigour to further improve stress resilience of seedlings; (v) study the interactions between crop genetics, the seed microbiome and seed vigour, in particular the role of crop diversity and overall diversity in production systems and incorporate this in breeding programs; (vi) train seed producers, seed companies and farmers on the role of the seed microbiome and seed vigour.

Conclusions from holobiont perspectives

The microbiome altered perspectives on seed health and may respond to demands for complementary approaches in plant breeding, seed production and treatments. It suggests taking another perspective on seed transmitted microorganisms. Ridding seeds from pathogens by disinfection treatments likely also removes micro-organisms that can offer support to the germinating seed and emerging seedling. Pathogenicity is also a matter of concentration and absence or presence of antagonistic factors, including other micro-organisms. More attention should be directed at the effect of seed production. Firstly, the potential of using organic produced seeds with a likely more adapted microbiome should be studied, as compared to using non-chemically treated conventional seeds. Secondly, the contribution of locally adapted microbiomes to seed and plant health should also be elucidated, in contexts such as on-farm breeding and seed production, where microbiomes have co-evolved with crops.

[6] https://www.liveseed.eu/wp-content/uploads/2021/02/LIVESEED_D2.5-Inventory-of-scientific-legal-and-technical-measures-to-improve-seed-quality-in-organic.pdf

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To support this paradigm shift, during the EUCARPIA-LIVESEED scientific conference, as well as during a LIVESEED workshop held in June 2021 with seed companies to discuss a 'New Organic Seed Health Strategy', it was proposed to disseminate the concept of "salutogenesis", the dynamic process allowing living organisms to evolve towards health[7]. The concept of salutogenesis was initially described in the context of human health (Antonovsky, 1996) and taken up by (Döring et al., 2012) in the context of plant health. They proposed that the science concerned with the health of plants may evolve from a stance of plant pathology – focussing on plant diseases – to a stance of plant salutology – focussing on health-sustaining processes. For the organic sector, this position will reinforce the IFOAM principles application, e.g., in particular the principle of health, but also the principles of ecology, care and fairness.

5.5. Sharing actors' knowledge for holobiont management

During the LIVESEED project, several workshops were centred on the consideration of the microbiome for organic plant breeding and seed production. It is important to mention them in the context of a multi-actor project. We will mention two particular meetings in 2019.

The workshop on Implementing Plant-Microbe Interactions in Plant Breeding

This event was co-organized by LIVESEED and ECO-PB from 2 to 5 December 2019 in Tulln, Austria, as a satellite workshop of the miCROPe 2019 symposium (www.microp e.org). The booklet is available at the Organic Eprints repository (Hohmann et al. 2019 https://orgprints.org/36920/); see also Annex 3 from FiBL and Hohman et al, 2020).

The miCROPe and the satellite workshop brought together researchers from academia and industry, to discuss about the benefit of targeted microbiome-based genotype selections. Plant breeding for beneficial plant microbiome interactions was highlighted as an underutilised and promising approach to improve crop resilience and yield stability. The key messages from the symposium with common views and thoughts on emerging research priorities have focussed on the challenges of (i) successful applications of microorganisms for crop production and (ii) plant breeding towards improved interaction with beneficial microorganisms (Hohman et al., 2020).

The workshop aimed at strengthening a network of plant breeders and scientists of different disciplines and exploring the integration of knowledge on plant-microbe interactions in plant breeding. In recent years, plant-associated microbes have received considerable attention in research for their ability to improve crop productivity and yield stability.

From literature, we know that not only the host species but also the host genotype plays a significant role in driving microbial community composition and activity, However, there are still many uncertainties on how to implement this knowledge into plant breeding and seed multiplication programmes.

Opportunities were particularly seen in yield stability (increased resilience for challenging conditions) and productivity (maintaining yield while reducing fossil-based inputs). Emphasised tools and applications were high-throughput microbiome-based phenotyping, machine learning and modelling approaches, novel seed treatments and the focus on endophytes, plant genetic markers, gene editing, and monitoring and decision tools for agricultural practice and crop/genotype selection in general. Methodology approaches have been discussed to promote breeding programmes that allow high-throughput selection of plant genotypes that enable beneficial microbe interactions. The need to work

[7] FROM SEED TO PLANT HEALTH – A BROADER PICTURE Stephanie M. KLAEDTKE[1], Emma FLIPON[1], Frédéric REY[1], Steven P.C. GROOT[2] EUCARPIA_2021_Abstract_e-Book_final_web3.pdf (liveseed.eu), page 97.

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closely with farmers and to link controlled experiments with field conditions was highlighted. Figure 5 (next page) illustrates the main keywords pointing out the opportunities and challenges for the future.

This workshop fostered the dialogue and collaboration between the different actors in order to develop advanced breeding strategies for the future.

Figure 5 (from annex 3): Word cloud of written highlights after the group work session on opportunities and challenges of implementing plant-microbe interactions in plant breeding

Workshops on Common Bunt Management

A series of three workshops (described also in the LIVESEED report on seed health - D2.58) were held in crop diversity associations in France as well as, for instance during the LIVESEED Cross Visit for seed production professionals in Italy (June 2019).

Although organic common bunt management is heavily reliant on preventive seed treatments (often a disinfection with white vinegar), questions concerning the role of seeds and, especially, soil microbiota for reducing the risk of bunt were recurring, and also, questions about further elucidating biological interactions affecting bunt at field level. For instance, do certain previous crops reduce the risk? Does soil microbiota affect the speed at which bunt spores are eliminated in soils? How do interactions between seed treatments and resistance breeding affect bunt development over time? How is the distribution and interaction of common bunt with draft bunt in farmers' field? How fast can races of common bunt evolve and overcome dominant resistance genes? To develop a deeper understanding of the interactions between bunt and wheat plants, beyond monogenic resistance, are there other plant defence mechanisms that come into play (field resistance, tolerance, seed microbiome)? How strongly does seed vigour affect bunt infection under field conditions (the Alternaria / carrot case, described also in deliverable D2.5, provides relevant methods for this)? The actors wished to continue disseminating available knowledge to farmers and seed industry, especially in countries with little focus on bunt.

According to participant feedback, the workshops offered valuable learning opportunities. They are an effective way both to disseminate knowledge on bunt management and to allow for practitioners' experience and questions to feed into the research and development conducted on common bunt at ITAB. Figure 6 on next page shows the broad approach of the disease by the farmers and LIVESEED partners, in an organic farming system. The complexity of the interactions between plants and their environments, including management practices, calls for more detailed study. With farmers, the

[8] IVESEED_D2.5-Inventory-of-scientific-legal-and-technical-measures-to-improve-seed-quality-in-organic.pdf

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keywords highlighted a broader vision embracing complex interactions between the components of farming systems, way beyond genetics and resistance breeding.

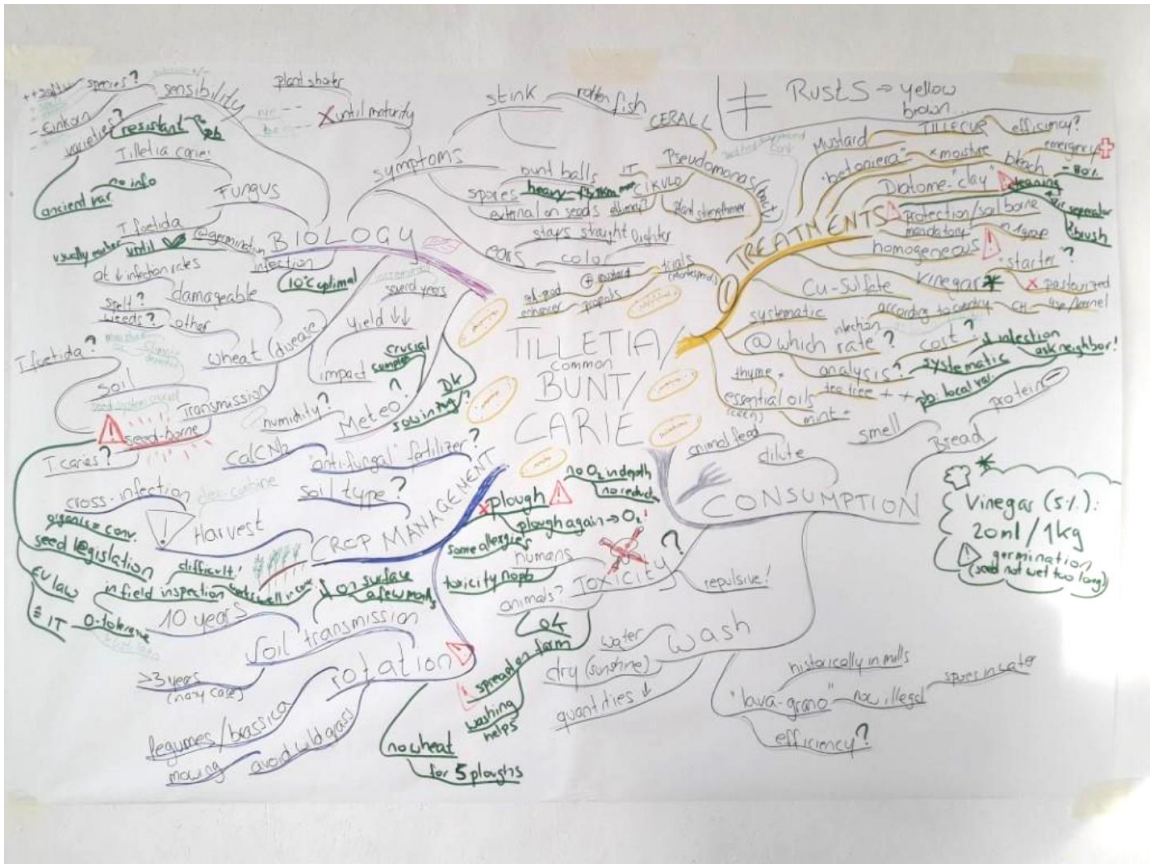

Figure 6. Mind-map constructed at a bunt workshop with local participants at the LIVESEED Cross Visit in Italy, June 2019 (ITAB)

6. Synthesis, conclusions and future perspectives

We propose a conceptual scheme which aims to summarise different types of organic plant breeding and seed production (on-farm, small scale breeding, large companies) and how hereditary information may be passed on (genetic, epigenetic and microbiome) in each situation, as we now have a better understanding of how the environment, farming practices, plant breeding and seed production may impact these different types of hereditary information and as a consequence also the resilience of a farming system (Figure 7).

The most important shift to mention is the co-evolution phenomenon; it constitutes a qualitative break between on-farm plant breeding and seed production and other schemes where plant breeding is disconnected from agricultural production. In the first case of on-farm plant breeding, the hereditary information can evolve from one generation to the other and contributes to the finetuning the adaptation of the crop from year to year, mainly through epigenetic and microbiological dynamics. When plant breeding is performed outside the site of production, and moreover when varieties should meet the DUS criteria (Distinctness, Uniformity, Stability) for registration and seed marketing, co-evolution between crops and their environment is impossible. In this case, plant breeding strategies aim to offer the crops the best capabilities to adapt to a large range of environments which ascertains the profitability of the breeders and seed producers.

To optimise on-farm adaptation, a great challenge is to harness interactions between plants and micro-organisms. Opportunities have been particularly seen in the area of yield stability (increased

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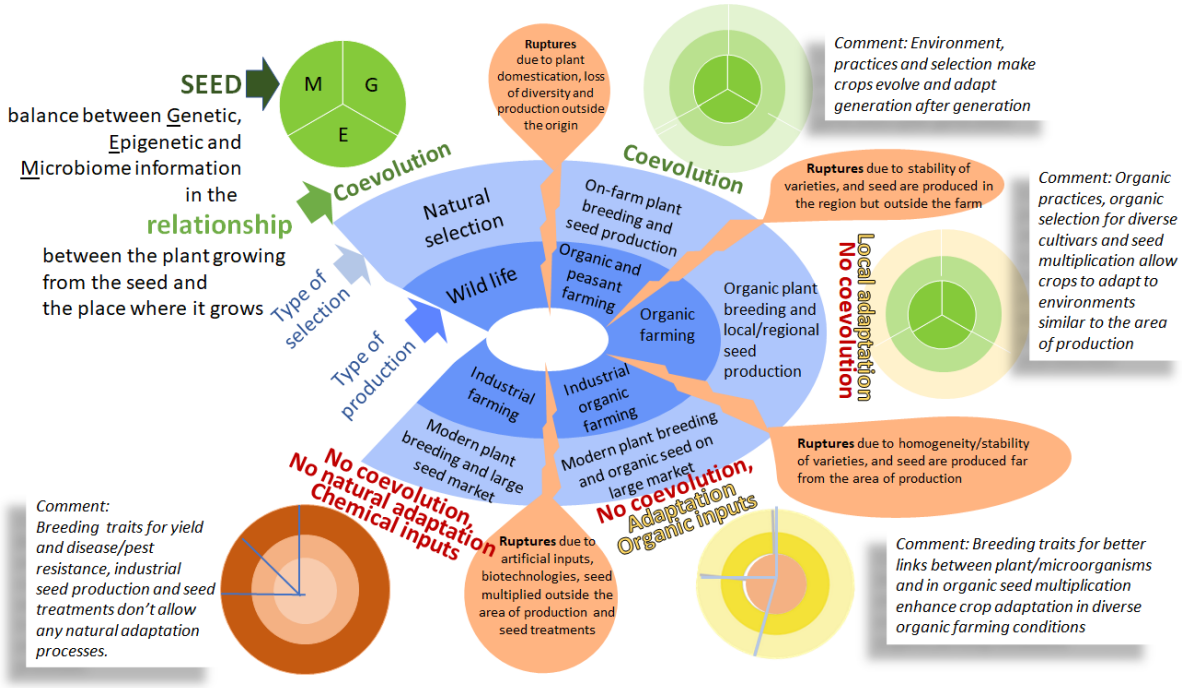
resilience for challenging conditions) and productivity (maintaining yield while reducing fossil-based inputs) exploring the potential of and implementing the growing knowledge on plant-microbe interactions in plant breeding in order to improve stress resistance, plant nutrition, plant health and general adaptability.



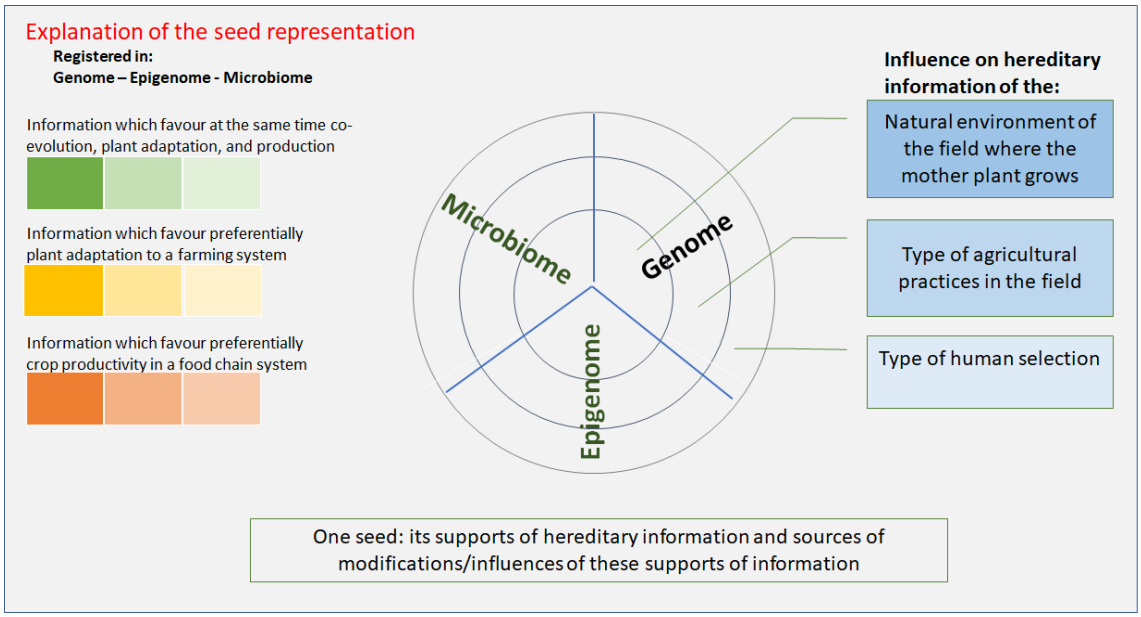


Figure 7. Conceptual scheme on the transfer of hereditary information (genetic, epigenetic and microbial) and the influence of various sources of modifications on several schematic situations of plant breeding and seed production.

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We may conclude that the recent discoveries and understanding of the co-evolution between plant and the micro-biome and the dynamic interactions of plants with micro-organisms invite a holistic conception of plant breeding and seed production, especially for organic farming, that should boost the adaptation of the plant within its agroecosystem. Organic plant breeding should be a holistic strategy that includes crop performances but also the improvement of the agroecosystem. Diversified cultivars, with an efficient interaction with soil microbial diversity, will also enhanced soil life and its biodiversity.

To what extent genetic factors are responsible for shaping beneficial plant microbiomes is very complex and still poorly understood. The interaction with the environment seems often more relevant, and ongoing research will bring more insight. Can this present lack of knowledge become an invitation to relativise the supremacy of gene-based approaches of the selection in crops and instead to broaden the question of plant adaptation and co-evolution with its environment and with farming practices? The complexity of the question also invites to conceive the answer collectively with organic farmers and other value chain actor, and to reconsider the place of genetics for plant breeding and seed production. The holobiont concept calls for a better balance and collaboration between different scientific approaches to improve the resilience of our food systems.

Finally, considering another dimension of organic agriculture, concerning values and the conception of life, the holobiont definition of plants could redefine our vision of plants as sensitive and intelligent organisms (Trewavas, 2003; Bais et al., 2004; Gagliano, 2018) since they are able to make active choices in the recruitment of microorganisms (Bais et al., 2004).

Our future research activities may help to develop and answer questions that are not only very important for organic agriculture, but for conventional agriculture as well. Can this conception help us to escape from the dominant consideration of “plant material” leading too often to a materialist management of production? Can awareness raising of the respect of the living dimensions of the agroecosystem help to also consider the co-evolutionary processes better, and its importance and efficiency for food production? Finally, may the thought of the co-evolution between living beings help to foster the principle of fairness in the seed system, and bring it to the same level as the principles of ecology, care and health of organic food systems as outlined by IFOAM?

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References

Abdelfattah, A., Wisniewski, M., Schena, L., & Tack, A. J. (2021). Experimental evidence of microbial inheritance in plants and transmission routes from seed to phyllosphere and root. *Environmental Microbiology* 23(4), 2199–2214, doi:10.1111/1462-2920.15392

Antonovsky, A. (1996) The salutogenic model as a theory to guide health promotion. *Health promotion international* 11, 11-18. https://doi.org/10.1093/heapro/11.1.11

Ares, A., Costa, J., Joaquim, C., Pintado, D., Santos, D., Monika M. Messmer, M.M., Mendes-Moreira, P.M. (2021), Effect of Low-Input Organic and Conventional Farming Systems on Maize Rhizosphere in Two Portuguese Open-Pollinated Varieties (OPV), "Pigarro" (Improved Landrace) and "SinPre" (a Composite Cross Population), *Front. Microbiol*., 26 February 2021, https://doi.org/10.3389/fmicb.2021.636009

Arnholdt-Schmitt, B. (2017) Respiration Traits as Novel Markers for Plant Robustness Under the Threat of Climate Change: A Protocol for Validation. In: Jagadis Gupta K. (eds) Plant Respiration and Internal Oxygen. Methods in Molecular Biology, vol 1670. Humana Press, New York, NY, doi.org/10.1007/978-1-4939-7292-0_15

Bais, H. P., Park, S. W., Weir, T. L., Callaway, R. M., & Vivanco, J. M. (2004). How plants communicate using the underground information superhighway. *Trends in plant science, 9*(1), 26-32.

Bakker, M.G., Manter, D.K., Sheflin, A.M. *et al.* (2012). Harnessing the rhizosphere microbiome through plant breeding and agricultural management. *Plant Soil* **360,** 1–13. https://doi.org/10.1007/s11104-012-1361-x

Berg, G., & Raaijmakers, J. M. (2018). Saving seed microbiomes. *The ISME journal*, *12*(5), 1167-1170

Berg, G., & Smalla, K. (2009). Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. *FEMS microbiology ecology*, *68*(1), 1-13.

Bergna, A., Cernava, T., Rändler, M., Grosch, R., Zachow, C., & Berg, G. (2018). Tomato seeds preferably transmit plant beneficial endophytes. *Phytobiomes Journal*, *2*(4), 183-193.

Bordenstein SR, Theis KR (2015) Host Biology in Light of the Microbiome: Ten Principles of Holobionts and Hologenomes. PLoS Biol 13(8): e1002226. doi:10.1371/journal.pbio.1002226

Bossdorf, O., Richards, C. L., & Pigliucci, M. (2008). Epigenetics for ecologists. *Ecology letters*, *11*(2), 106–115. https://doi.org/10.1111/j.1461-0248.2007.01130.x

Chable, V.; Nuijten, E.; Costanzo, A.; Goldringer, I.; Bocci, R.; Oehen, B.; Rey, F.; Fasoula, D.; Feher, J.; Keskitalo, M.; Koller, B.; Omirou, M.; Mendes-Moreira, P.; van Frank, G.; Naino Jika, A.K.; Thomas, M.; Rossi, A. Embedding Cultivated Diversity in Society for Agro-Ecological Transition. *Sustainability* **2020**, *12*, 784. https://doi.org/10.3390/su12030784

Chen, Y., Wang, J., Yang, N., Wen, Z., Sun, X., Chai, Y., Ma, Z. (2018) Wheat microbiome bacteria can reduce virulence of a plant pathogenic fungus by altering histone acetylation. Nat Commun, 9:3429

Collens, A; Kelley, E; Katz, LA (2019) The concept of the hologenome, an epigenetic phenomenon, challenges aspects of the modern evolutionary synthesis, Journal of Experimental Zoology Part B-Molecular and Developmental Evolution, 332, 8: 349-355, DOI: 10.1002/jez.b.22915

LIVESEED is funded by the European Union's Horizon 2020 under grant agreement No 727230 and by the Swiss State Secretariat for Education, Research and Innovation (SERI) under contract number 17.00090.

Compant, S., Samad, A., Faist, H., & Sessitsch, A. (2019). A review on the plant microbiome: ecology, functions, and emerging trends in microbial application. *Journal of advanced research*, *19*, 29-37.

Dai, Y.; Li, X.-Y.; Wang, Y.; Li, C.-X.; He, Y.; Lin, H.-H.; Wang, T.; Ma, X.R. (2020) The differences and overlaps in the seed-resident microbiome of four Leguminous and three Gramineous forages. Microb. Biotechnol., 13, 1461–1476

Dawson, J.C., Murphy, K.M., Jones, S.S. (2008) Decentralized selection and participatory approaches in plant breeding for low-input systems. E*uphytica* 160(2) 143–154

Dawson, J. C., Serpolay, E., Giuliano, S., Schermann, N., Galic, N., Berthellot, J.-F., Chesneau, V., Ferté, H., Mercier, F., Osman, A., Pino, S., Goldringer, I. (2013) Phenotypic diversity and evolution of farmer varieties of bread wheat on organic farms in Europe. *Genet. Resour. Crop Evol.* 60: 145-163

Dawson, J.C., Serpolay, E., Giuliano, S., Schermann, N., Galic, N., Chable, V., Goldringer, I. (2012) Multi-trait evolution of farmer varieties of bread wheat after cultivation in contrasting organic farming systems in Europe. *Genetica* 140: 1-17

Döring, T.F., Pautasso, M., Finckh, M.R. and Wolfe, M.S. (2012) Concepts of plant health – reviewing and challenging the foundations of plant protection. *Plant Pathology* 61, 1-15. https://doi.org/10.1111/j.1365-3059.2011.02501.x

Döring T.F., Vieweger A., Pautasso M., Vaarst M., Finckh M.R. & Wolfe M.S., (2013). Resilience as a universal criterion of health. *J Sci Food Agric*, 95, 455–465.

Favela, A., O. Bohn, M. & D. Kent, A. Maize germplasm chronosequence shows crop breeding history impacts recruitment of the rhizosphere microbiome. I*SME J* (2021). https://doi.org/10.1038/s41396-021-00923-z

Fierer, N. (2017). Embracing the unknown: disentangling the complexities of the soil microbiome. *Nature Reviews Microbiology*, *15*(10), 579-590.

Gagliano, M. (2018). Inside the Vegetal Mind: On the Cognitive Abilities of Plants. In *Memory and Learning in Plants* (pp. 215-220). Springer, Cham.

García-Palacios, P., Milla, R., Delgado-Baquerizo, M., Martín-Robles, N., Álvaro-Sánchez, M., & Wall, D. H. (2013). Side-effects of plant domestication: ecosystem impacts of changes in litter quality. *New Phytologist*, *198*(2), 504-513.

Lupatini, M., Korthals, G.W., de Hollander, M., Janssens T.K.S., Kuramae, E.E., (2017) Soil Microbiome Is More Heterogeneous in Organic Than in Conventional Farming System Front. *Microbio*l., 04 January 2017, https://doi.org/10.3389/fmicb.2016.02064

González Olmo, B.M.; Butler, M.J.; Barrientos, R.M. (2021) Evolution of the Human Diet and Its Impact on Gut Microbiota, Immune Responses, and Brain Health. *Nutrients* 13, 196.

Groot, S. P. C., & Raaijmakers, M. H. J. E. (2018). Organic seed production, certification and availability: Corp breeding and cultivation. In *Improving organic crop cultivation*. Burleigh Dodds Science Publishing Limited.

LIVESEED is funded by the European Union's Horizon 2020 under grant agreement No 727230 and by the Swiss State Secretariat for Education, Research and Innovation (SERI) under contract number 17.00090.

Groot, S. P. C., Jalink, H., Köhl, J., Langerak, C. J., Michta, A., Werner, S., ... & van den Bulk, R. W. (2006, b). The need for a supply of high quality organic vegetable seeds. In *Proceedings of the European Joint Organic Congress, Odense, Denmark, May 30-31, 2006* (pp. 382-383).

Gundel, P. E., Rudgers, J. A., & Ghersa, C. M. (2011). Incorporating the process of vertical transmission into understanding of host–symbiont dynamics. *Oikos*, *120*(8), 1121-1128.

Hardoim, P. R., Hardoim, C. C., Van Overbeek, L. S., & Van Elsas, J. D. (2012). Dynamics of seed-borne rice endophytes on early plant growth stages. *PloS one*, *7*(2), e30438.

Hartmann, M., Frey, B., Mayer, J., Mäder, P., & Widmer, F. (2015). Distinct soil microbial diversity under long-term organic and conventional farming. *The ISME journal*, *9*(5), 1177-1194.

Hohmann, P., & Messmer, M. M. (2017). Breeding for mycorrhizal symbiosis: focus on disease resistance. *Euphytica*, *213*(5), 113.

Hohmann, P., Backes, G., Thonar, C., & Messmer, M. (2016). Breeding for symbioses–Mycorrhizae as a case study. In *Proceedings of the 20th EUCARPIA General Congress. Plant Breeding: the Art of Bringing Science to Life, Aug 29-Sep 1, 2016, Zürich, Switzerland* (p. 406). Agroscope.

Hohmann, P., Schlaeppi, K., and Sessitsch, A. (2020). miCROPe 2019 - emerging research priorities towards microbe-assisted crop production. *FEMS Microbiol Ecol* 96, 1-7.

Kaga, H., Mano, H., Tanaka, F., Watanabe, A., Kaneko, S., & Morisaki, H. (2009). Rice seeds as sources of endophytic bacteria. *Microbes and environments*, 0904220080-0904220080.

Khan, A.R., Goldringer, I., Thomas, M. (2020) Management Practices and Breeding History of Varieties Strongly Determine the Fine Genetic Structure of Crop Populations: A Case Study Based on European Wheat Populations. *Sustainability* 12, 613; doi:10.3390/su12020613

Kiers ET, Duhamel M, Beesetty Y, Mensah JA, Franken O, Verbruggen E, Fellbaum CR, Kowalchuk GA, Hart MM, Bago A et al.2011. Reciprocal rewards stabilize cooperation in the mycorrhizal symbiosis. *Science* 333: 880–882

Klaedtke, S., Groot, S. (2021) Seed for a new seed health strategy. LIVESEED Task 2.3 New Seed Health Strategy- Version 20210608

Kong, H. G., Song, G. C., & Ryu, C. M. (2019). Inheritance of seed and rhizosphere microbial communities through plant–soil feedback and soil memory. *Environmental microbiology reports*, *11*(4), 479-486.

Koskella, B., Bergelson, J., 2020, The study of host-microbiome (co)evolution across levels of selection, *Philosophical Transactions of The Royal Society B-Biological Sciences* 375, 1808, Article Number: 20190604, DOI: 10.1098/rstb.2019.0604

Lewontin, R.C. (1970) The units of selection. *Annu. Rev. Ecol. Syst*. 1, 1–18. (doi:10.1146/annurev.es.01. 110170.000245)

Margulis, L. (1981). Symbiosis in cell evolution: Life and its environment on the early earth. *Boston University Boston, MA, United States*

Margulis L. (1993) Symbiosis in Cell Evolution. New York: W.H. Freeman

LIVESEED is funded by the European Union's Horizon 2020 under grant agreement No 727230 and by the Swiss State Secretariat for Education, Research and Innovation (SERI) under contract number 17.00090.

Matsumoto, H., Fan, X., Wang, Y., Kusstatscher, P., Duan, J., Wu, S., ... & Wang, M. (2021). Bacterial seed endophyte shapes disease resistance in rice. *Nature Plants*, 1-13.

Mohanapriya G, Bharadwaj R, Noceda C, Costa JH, Kumar SR, Sathishkumar R, Thiers KLL, Santos Macedo E, Silva S, Annicchiarico P, Groot SPC, Kodde J, Kumari A, Gupta KJ, Arnholdt-Schmitt B. Alternative Oxidase (AOX) (2019) Senses Stress Levels to Coordinate Auxin-Induced Reprogramming From Seed Germination to Somatic Embryogenesis-A Role Relevant for Seed Vigor Prediction and Plant Robustness. *Front Plant Sci. 2019 Sep 20;10:1134*. doi: 10.3389/fpls.2019.01134. PMID: 31611888; PMCID: PMC6776121.

Morella, N.M., Zhang, X., Koskella B (2019) Tomato seed-associated bacteria confer protection of seedlings against foliar disease caused by Pseudomonas syringae. *Phytobiomes Journal 3:177-190.*

Maude, R. B. (1996). *Seedborne diseases and their control: principles and practice*. CAB international.

Nelson, E. B. (2018). The seed microbiome: origins, interactions, and impacts. *Plant and Soil*, *422*(1), 7-34.

Nicholls C., Altieri M., Vazquez L, 2016, Agroecology: Principles for the Conversion and Redesign of Farming Systems, *J Ecosys Ecograph* 2016, S5:1 http://dx.doi.org/10.4172/2157-7625.S5-010

Ottesen, A. R., Peña, A. G., White, J. R., Pettengill, J. B., Li, C., Allard, S., ... & Brown, E. (2013). Baseline survey of the anatomical microbial ecology of an important food plant: Solanum lycopersicum (tomato). *BMC microbiology*, *13*(1), 1-12.

Pauvert, C., Fort, T., Calonnec, A., Faivre d'Arcier, J., Chancerel, E., Massot, M., Chiquet, J., Robin, S., Bohan, D.A., Vallance, J., Vacher, C. (2020) Microbial association networks give relevant insignhts into plant pathobiomes, February 23, 2020. Preprint BioRxiv doi: https://doi.org/10.1101/2020.02.21.958033

Peiffer, J. A., Spor, A., Koren, O., Jin, Z., Tringe, S. G., Dangl, J. L., ... & Ley, R. E. (2013). Diversity and heritability of the maize rhizosphere microbiome under field conditions. *Proceedings of the National Academy of Sciences*, *110*(16), 6548-6553.

Perez-Jaramillo JE, Carrion VJ, de Hollander M, Raaijmakers JM: The wild side of plant microbiomes. *Microbiome* 2018, 6.

Perez-Jaramillo, J. E., Mendes, R., and Raaijmakers, J. M. (2016). Impact of plant domestication on rhizosphere microbiome assembly and functions. *Plant Mol. Biol*. 90, 635–644. doi: 10.1007/s11103-015-0337-7

Pierret A., Moran C.J. (2011) Plant Roots and Soil Structure. In: Gliński J., Horabik J., Lipiec J. (eds) Encyclopedia of Agrophysics. Encyclopedia of Earth Sciences Series. Springer, Dordrecht. https://doi.org/10.1007/978-90-481-3585-1_121

Purugganan, M.D. (2019), **Evolutionary Insights into the Nature of Plant Domestication**, Current Biology 29, R705–R714, July 22, 2019, https://doi.org/10.1016/j.cub.2019.05.053

Revuru, B., Noceda, C., Gunasekaran, M., Kumar, S.R., K.L., Costa, J.H., Elisete Santos Macedo, E., Aprajita Kumari, A., Gupta, K.J., Srivastava S., Adholeya, A., Oliveira, M., Velada, I., Sircar, D., Sathishkumar, R., Arnholdt-Schmitt, B. (2021) Adaptive reprogramming during early seed germination requires temporarily enhanced fermentation – a critical role for alternative oxidase

LIVESEED is funded by the European Union's Horizon 2020 under grant agreement No 727230 and by the Swiss State Secretariat for Education, Research and Innovation (SERI) under contract number 17.00090.

(AOX) regulation that concerns also microbiota effectiveness
bioRxiv 2021.06.08.447570; doi: https://doi.org/10.1101/2021.06.08.447570

Rinninella, E.; Cintoni, M.; Raoul, P.; Lopetuso, L.R.; Scaldaferri, F.; Pulcini, G.; Miggiano, G.A.D.; Gasbarrini, A.; Mele, M.C. Food Components and Dietary Habits: Keys for a Healthy Gut Microbiota Composition. *Nutrients* **2019**, *11*, 2393. https://doi.org/10.3390/nu11102393

Saleem, M., Law, A. D., Sahib, M. R., Pervaiz, Z. H., & Zhang, Q. (2018). Impact of root system architecture on rhizosphere and root microbiome. *Rhizosphere*, *6*, 47-51.

Serpolay-Besson, E., Giuliano, S., Schermann, N., Chable, V. (2014) Evaluation of Evolution and Diversity of Maize Open-Pollinated Varieties Cultivated under Contrasted Environmental and Farmers' Selection Pressures: A Phenotypical Approach. *Open Journal of Genetics*, **4**, 125-145. doi: 10.4236/ojgen.2014.42014

Shade, A., Jacques, M. A., & Barret, M. (2017). Ecological patterns of seed microbiome diversity, transmission, and assembly. *Current opinion in microbiology*, *37*, 15-22.

Shaw, R. G., & Etterson, J. R. (2012). Rapid climate change and the rate of adaptation: insight from experimental quantitative genetics. *The New phytologist*, *195*(4), 752–765. https://doi.org/10.1111/j.1469-8137.2012.04230.x

Simonin, M., Dasilva, C., Terzi, V., Ngonkeu, E. L., Diouf, D., Kane, A., ... & Moulin, L. (2020). Influence of plant genotype and soil on the wheat rhizosphere microbiome: Evidences for a core microbiome across eight African and European soils. *FEMS microbiology ecology*, *96*(6), fiaa067.

Teixeira, P.J.P.L., Colaianni, N.R., Fitzpatrick, C.R., Dangl, J.L. (2019) Beyond pathogens: microbiota interactions with the plant immune system, Current Opinion in Microbiology 2019, 49:7–17

Trewavas, A. (2003). Aspects of plant intelligence. Annals of botany, 92(1), 1-20.

Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. Nature, 444:1027–31. doi: 10.1038/nature05414

Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., & Dufresne, A. (2015). The importance of the microbiome of the plant holobiont. *New Phytologist*, *206*(4), 1196-1206.

van Frank, G.; Rivière, P.; Pin, S.; Baltassat, R.; Berthellot, J.-F.; Caizergues, F.; Dalmasso, C.; Gascuel, J.-S.; Hyacinthe, A.; Mercier, F.; Montaz, H.; Ronot, B.; Goldringer, I. Genetic Diversity and Stability of Performance of Wheat Population Varieties Developed by Participatory Breeding. *Sustainability* **2020**, *12*, 384. https://doi.org/10.3390/su12010384

Vannier, N., Mony, C., Bittebiere, AK, Michon-Coudouel, S., Marine Biget, M., Vandenkoornhuyse, P. (2018) A microorganisms' journey between plant generations. *Microbiome* **6,** 79 https://doi.org/10.1186/s40168-018-0459-7

Vieweger A. & Döring T.F. (2014). Assessing health in agriculture – towards a common research framework for soils, plants, animals, humans and ecosystems. *J Sci Food Agric*, 95, 438–446

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Wagg, C., Schlaeppi, K., Banerjee, S., Kuramae, E.E., van der Heijden M.G.A., 2019, Fungal-bacterial diversity and microbiome complexity predict ecosystem functioning, NATURE COMMUNICATIONS, 10:4841, https://doi.org/10.1038/s41467-019-1279

Wassermann B, Cernava T, Müller H, Berg C, Berg G. Seeds of native alpine plants host unique microbial communities embedded in cross-kingdom networks. Microbiome. 2019 Jul 24;7(1):108. doi: 10.1186/s40168-019-0723-5

Weese DJ, Heath KD, Dentinger BT, Lau JA (2015) Long-term nitro gen addition causes the evolution of less-cooperative mutualists. Evolution 69(3):631–642. doi:10.1111/evo.12594

Wille L, Messmer MM, Studer B et al. Insights to plant-microbe interactions provide opportunities to improve resistance breeding against root diseases in grain legumes. Plant Cell Environ 2019; 42:1–21.

Wille, L., Messmer, M.M., Bodenhausen, N., Studer, B., Hohmann, P., 2020, Heritable Variation in Pea for Resistance Against a Root Rot Complex and Its Characterization by Amplicon Sequencing, Front. Plant Sci., 03 November 2020 | https://doi.org/10.3389/fpls.2020.542153

Wei Z., Friman V-P., Pommier T., Geisen S., Jousset A., Shen Q., (2020). Rhizosphere immunity: targeting the underground for sustainable plant health management[J]. *Front. Agr. Sci. Eng*. 7(3), 317-328.

Xiong, C., Zhu, Y. G., Wang, J. T., Singh, B., Han, L. L., Shen, J. P., ... & He, J. Z. (2021). Host selection shapes crop microbiome assembly and network complexity. *New Phytologist*, *229*(2), 1091-1104.

Zaneveld, J.R., Ryan McMinds, R., Thurber, R.V. (2017) Stress and stability: applying the Anna Karenina principle to animal microbiomes, Nature Microbiology 2, 17121, DOI: 10.1038/nmicrobiol.2017.121

Zhang J., Van der Heijden M.G.A, Zhang F., Dender S.F., (2020). Soil biodiversity and crop diversification are vital components of healthy soils and agricultural sustainability. *Front. Agr. Sci. Eng,* 7(3), 236-242.

Zilber-Rosenberg, I., & Rosenberg, E. (2008). Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. *FEMS microbiology reviews*, *32*(5), 723-735.

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Annex 1. Understanding the microbiome evolution in on-farm plant breeding (tomatoes in agroforestry system, task 3.2.3)

Author: Solene Lemichez (INRAE)

Summary

In the light of new eco-evolutive knowledge, considering the holobiont as a selection target is crucial for organic breeding (Duhamel & Vandenkoornhuyse, 2013). On-farm selection, as it relies on the multiplication of the fittest plants in a given environment based on their phenotype, already makes the most of plant / micro-organisms interactions. This allows a combination all forms of heredity (genetic, epigentic and microbiotic) and their synergetic interactions. Understanding how the plant microbiome evolves through on farm selection is a way to inspire organic selection by unravelling the dynamics linking plants, soil and micro-organisms in their terroir.

This study was set in France on two farms of the Cévennes region, with contrasted agroforestrial environments. Both farms integrate market gardening in agroforestrial systems, with different pruning intensities of trees and display a shade less control plot as well. The focal point was the fungal and bacterial microbiote of the root endosphere of two stabilised populations of Rose de Berne and Coeur de Boeuf varieties selected on-farm. Tomato fine roots were sampled at the end of their vegetative cycle in October 2019 and 2020 to undergo total DNA extraction and specific PCRs (Lê Van et al. 2017). Up to date sequencing methods were used, as well as bioinformatic pipeline FROGS (Find Rapidly OTUs with Galaxy Solution) (Escudié et al., 2017) to reconstruct the targeted microbiota. Statistical analyses were then performed at the community and main phyla scales, as well as on diversity indexes (Shannon's index, Simpson's index, Pielou's equitability and species richness) to assess the microbiome structure.

Over two years, our results show that the environment (agroforestrial modality, year and sampling site) has a major impact for both fungi and bacteria at community and phyla levels. However, varietal effect was scarcely significant despite their genetic differentiation, which was surprising in regard of current molecular ecology literature. This result could indicate that the genetic component of heredity is balanced in on-farm selection with epigenetic and microbiotic processes, and that co-evolution between plants and micro-organisms is fully embodied. Rather unconsciously, on-farm selection takes its strength from the holobiont concept of plants, explaining their constant and rapid adaptation as well as agro-ecosystem resilience (Serpolay-Besson et al., 2015; van Frank et al., 2020)

Link to other tasks

The results of this study were also developed in LIVESEED Report on Enhancing resilience at the systems level (D.3.6), focusing on agroforestry impact on chemotype, yield and quality of fruits. Links with microbiome structure are established as well, taking into account the unbalanced sampling design.

1. Methods

1.1. Experimental site

The main experimental site, *les Terres du Roumassouze*, is located in Vézénobres (44° 30' 11'' North, 4°08' 10'' East) in the Cévennes area in France. It consists of 11 hectares of organic agroforestrial

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market gardening, open field cultures and sylviculture. On the agroforestrial area, hybrid walnut (Juglans nigra L. x Juglans regia L.) planted in 1996 are interspaced with market gardening crops, with 10m space between each tree.

In 2015, 4 agroforestrial modalities are defined, corresponding to different pruning intensities: Light pruning (L), Medium pruning (M), Pollard (T) and Shade less control (TS). The volume (m3) of wood removed from trees in each modality was 0.015 (L), 0.084 (M) and 0.3 (T). A second pruning was done in 2018 to maintain the desired shade intensities. Each modality corresponds to a 30*40m surface (1200 m²), including cultivation beds (3 rows) and grass strips between trees. Cultivation beds are 25m long, 1m wide and separated by a 0.8m pathway. The different agroforestrial modalities are separated by a one meter large a grass strip. Cultural practices are similar between the different agroforestrial modalities. Only plants in the central row are sampled to ensure a homogenous relative distance to the trees (Figure 1).

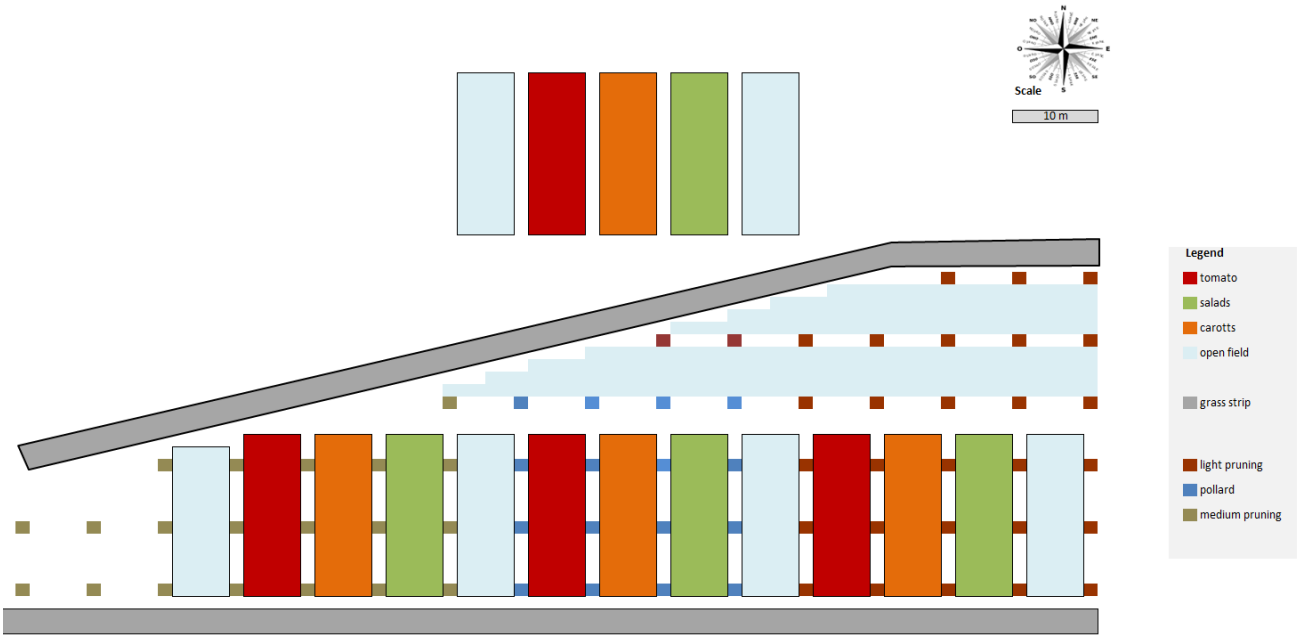

Figure 1. Experimental site at les Terres de Roumassouze.

A second site located in Cazilhac, *la Ferme du Boulidou*, also in the Cévennes region is taken as a comparison point for microbial communities. On this farm, diversified fruit trees grow in a permacultural design with market garden crops. The variety Rose de Berne is also cultivated there and selected on-farm. A control plot without trees is also established.

1.2. Genetic analysis

Molecular marking of 12 plants per combination of variety and modality was performed by VEGENOV. It targeted 8 SSR markers usually used in varietal diagnostic. This genetical analysis was performed on plants of the second selection generation in 2020.

1.3. 3.Microbiome characterization

For each variety*agroforestrial modality combination, the roots of 12 plants were sampled, for a total of 96 samples. The fine roots (<1mm) were taken at a depth of 10 to 20cm in the immediate vicinity of the plant studied, and the weight of a sample was calibrated between 80 and 100 mg. The samples were rinsed with a 5/1000 Triton solution and then with sterile water before being dried and stored

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at -80°C before DNA extraction. The extraction was performed by the GENTYANE platform (UMR GDEC, INRAE) using a Sbeadex™ kit (LGC) automated on an oKtopur™ machine (LGC). The DNA assay was performed with the Hoechst 33258 dye and an Infinite® M1000 automaton (TECAN). Extracted DNA was stored at -20°C before being standardised to a concentration of 10 ng/µl. A PCR (illustra™ puReTaq Ready-To-Go™ PCR Beads, GE healthcare®) was performed with the fungal primer pair NS22B (5'-AATTAAGCAGACAAATCACT-3') and SSU0817 (5'-TTAGCATGGAATAATRRAATAGGA-3') targeting a region of approximately 550 bp of the 18S rRNA (Borneman & Hartin, 2000; Lê Van et al., 2017). The amplification protocol included 35 cycles of denaturation (95°C for 30s), hybridisation (54°C for 30s) and elongation (72°C for 1min), with an initial denaturation (95°C for 4min) and a final elongation (72°C for 7min). The 16S rRNA gene was amplified using bacterial primers 799F (5'-AACMGGATTAGATACCCKG-3') and 1223R (5'-CCATTGTAGTACGTGTGTA-3'). The conditions for this PCR consisted of an initial denaturation step at 94 °C for 4 min followed by 32 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 1 min with a final extension step at 72 °C for 10 min. The products of these first PCR were then purified (Agencourt® AMpureXP Magnetic Beads) with a robotic pipettor (Bravo-Agilent®), quantified by fluorimetry (Quant-iT PicoGreen™ dsDNA Assay Kit) and standardised to the same concentration (0.5 ng/µL) for the preparation of the sequencing library. A second PCR reaction was performed on a Smartchip instrument (Takara) to allow individual labelling of the samples (Illumina multiplexing) and the final production of the sequencing library. This library was then purified (AMpureXP Agencourt® magnetic beads) and quantified (Kapa Library Quantification Kit-Illumina®) before being sequenced with a MiSeq Illumina® instrument (Paired-end 2x300 cycles kit). The production of the libraries and the sequencing were carried out by the Environmental and Human Genomics platform (GEH, Rennes, France).

The sequencing data were processed with the FROGS (Find, Rapidly, OTU, with Galaxy Solution) pipeline (Escudié et al., 2017). The clustering method used was SWARM (Mahé et al., 2014), which generates sequence clusters (~OTUs) but with the advantage of not using an arbitrary identity threshold (which produces OTUs). In order to avoid the creation of artificial sequence clusters, only those containing sequences from at least 3 independent samples were kept. The fungal sequence database PHYMYCO-DB (Mahé et al., 2012) was used for BLAST taxonomic affiliation of sequence clusters in FROGS. The one used for the affiliation of bacterial sequences was SYLVA 16S. These clusters were then filtered according to the quality of the affiliation with a threshold of 95% in coverage and 95% in identity. The data were organised in a contingency table and the number of sequences per sample was normalised using the 'vegan' package in R. This number of sequences allowed to accurately describe the composition of the root microbiome, verified by rarefaction curves for each sample with the 'vegan' module in R. These normalised data were used for statistical analysis. The assembly of the root microbiota was studied at the community and main phyla levels.

1.4. Statistical methods

An ANOVA was performed on the genetic data to assess the genetic structure at the level of varieties and agroforestrial modalities. Linear models were constructed to to test the effect of variety and pruning intensity on microbiota structuration (Shannon index, Simpson index and equitability). Sampling year, variety and pruning modality were fixed factors, and the individuals were included as a random effect. A Poisson model was used to test the effect of the variety, pruning modality and year on the number of microorganism species (species richness). For each model, outliers were removed and when necessary, and variables are log-transformed to ensure the normality of the residuals. The significance of each factor in the model was determined by an ANOVA. Interactions between factors were not taken into account. To represent the composition of the microbiota according to the variety or pruning modality effect, a PLS-DA regression on the abundance (number of sequences) of each cluster per individual was performed. All the statistical analysis were performed with R.

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2. Results

2.1 On-farm selection maintains a genetic structure at the variety level but does not create structuration at the level of agroforestrial modalities.

The ANOVA results show a significant genetic structure (p= 9.999e-05) at the level of variety (Figure 2). On the other hand, differentiated selection between agroforestrial modalities dos does not induce a new structuration for the tested markers (p=0.19) (Figure 3).

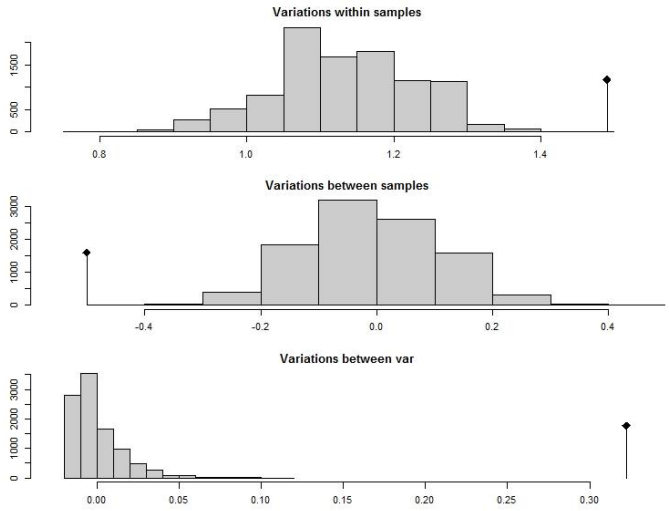

Figure 2. ANOVA results showing the variation within samples, between samples and between varieties for 8 SSR markers. Quasi-gaussian pattern reveals no structuration within and between samples, while it can be observed at the level of varieties. This variation is significant (p= 9.999e-05).

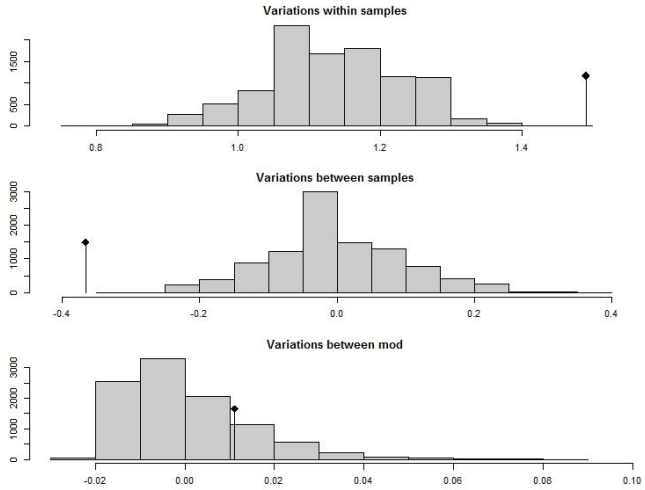

Figure 3. ANOVA results showing the variation within samples, between samples and between agroforestrial modalities for 8 SSR markers. No significant structuration is observed, even at the level of agroforestrial modalities (p=0.19).

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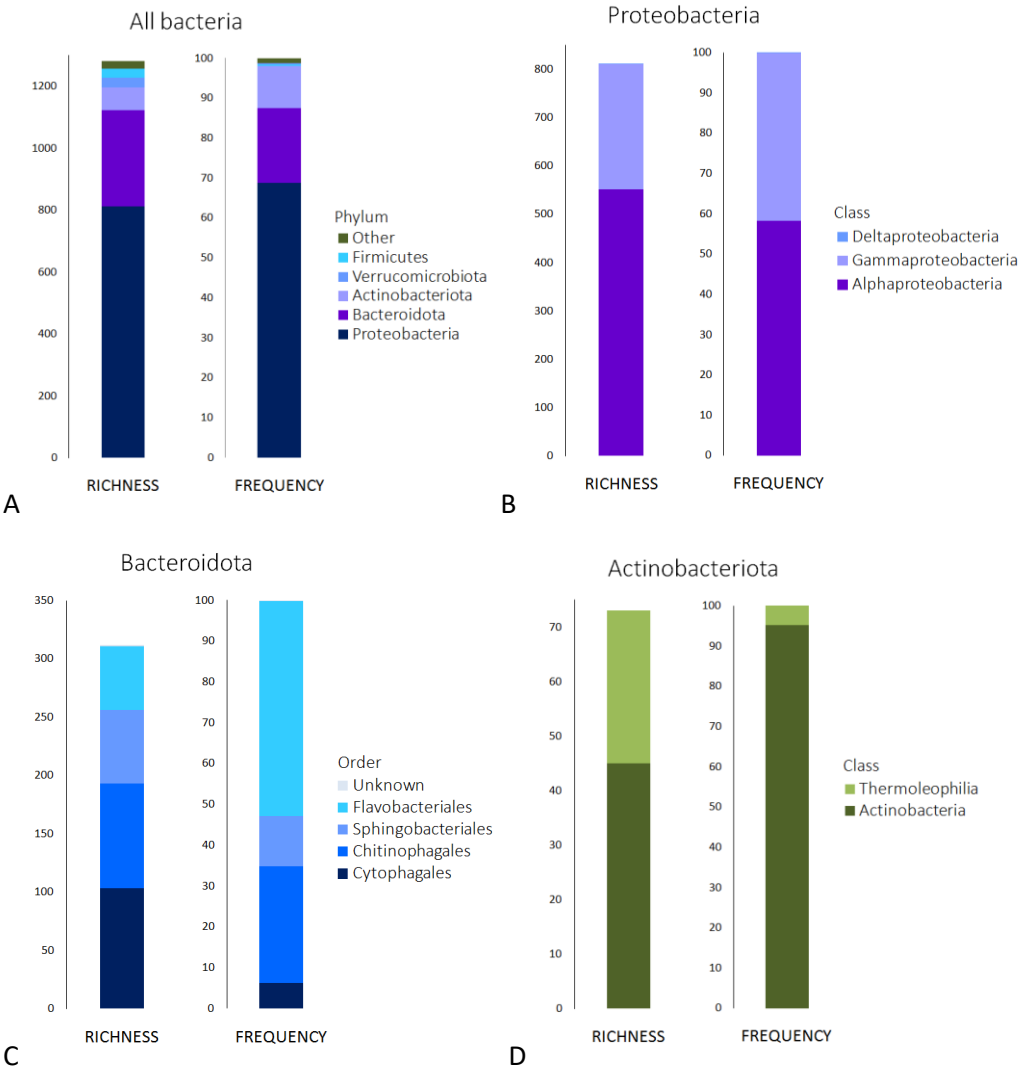
2.2. Characterisation of fungal and bacterial communities

At the community scale, 568479 sequences were distributed in 1317 clusters for bacteria. At the γ-diversity scale (total microbiota diversity), the tomato root endospheric bacterial community was mainly composed by Proteobacteria (811 sequence-clusters, 68% of the sequences) and the Bacteroidota (312 sequence-clusters, 18% of the sequences) phyla. Actinobacteria, Firmicutes and Verrucomicobiota were less represented with a richness of 73, 31 and 29 sequence-clusters respectively (Figure 4).



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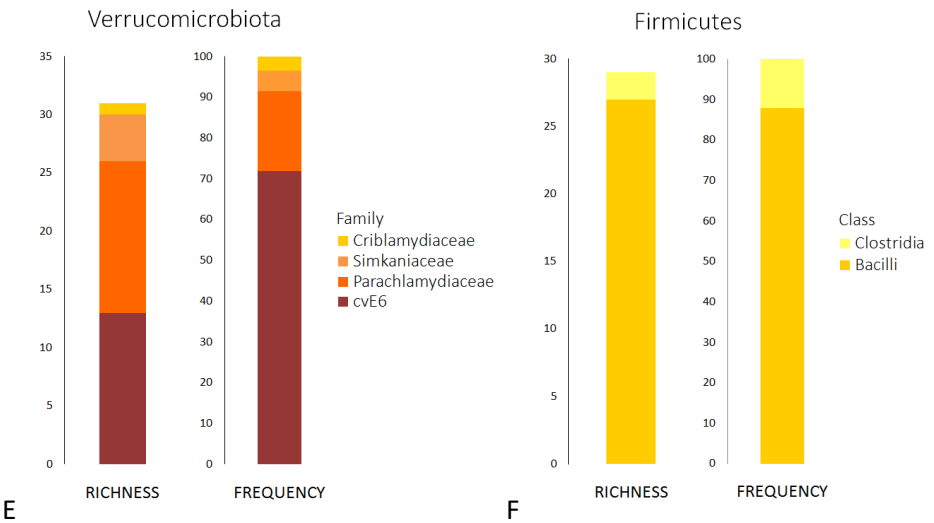
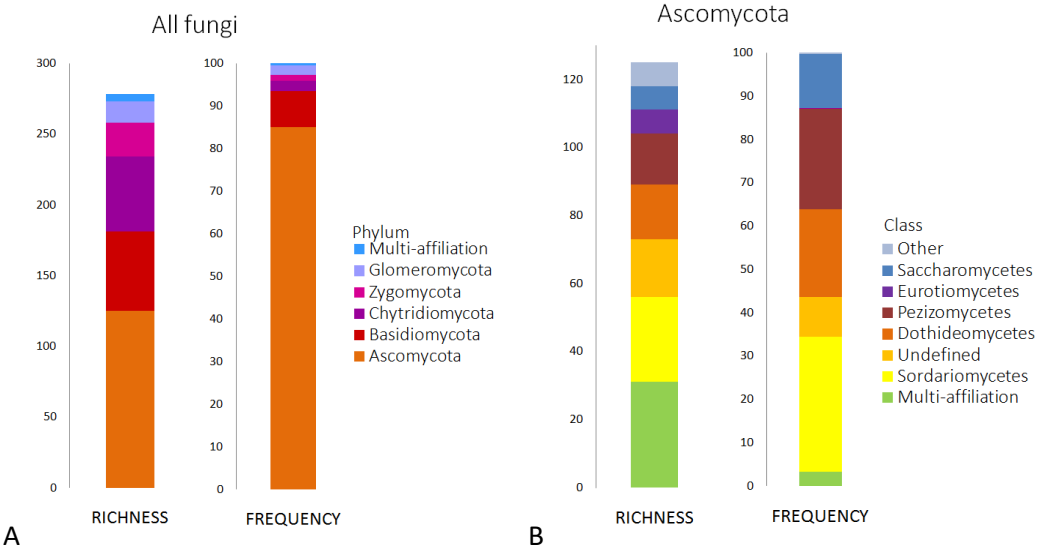


Figure 4. Sequence-clusters richness and relative abundance of the tomato root endospheric bacterial microbiota (γ-diversity) and within the most represented phyla.

Considering fungi, 5727061 sequences were distributed in 278 clusters. The tomato endospheric microbiota was composed by 85.0% of Ascomycota (125 sequence-clusters), 8.4% of Basidiomycota (56 sequence-clusters), 2.4% of Chytridiomycota (53 sequence-clusters), 2.2% of Glomeromycota (15 sequence-clusters) and 1.4% of Zygomycota (24 sequence-clusters) (Figure 5).



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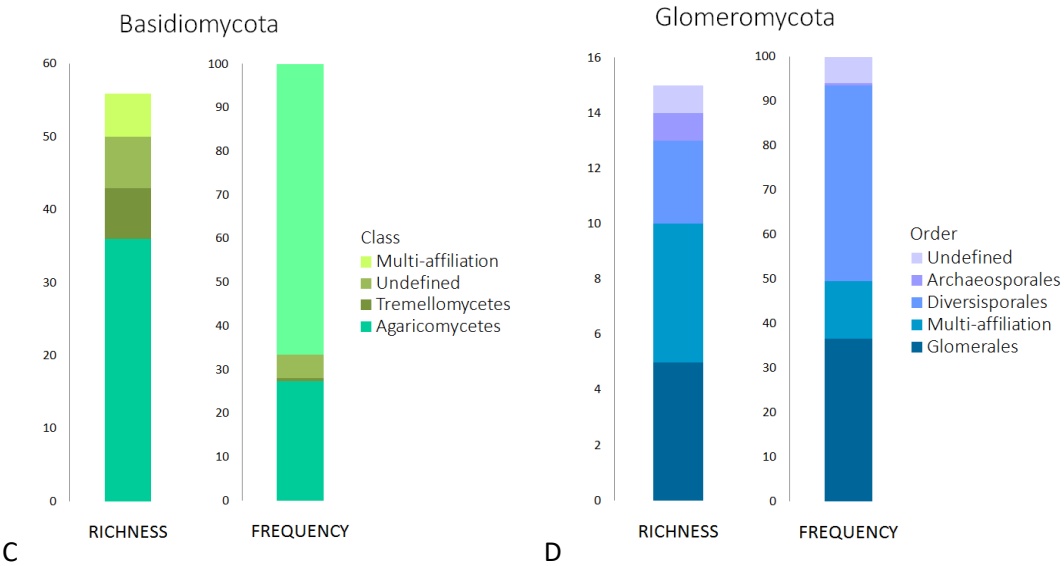

Figure 5. Sequence-clusters richness and relative abundance of the tomato root endospheric fungal microbiota (γ-diversity) and within the main phyla.

2.3. Year and agroforestrial modality have a strong impact on fungal and bacterial microbiome composition, but variety has a lower effect

ANOVA results show a predominance of year and agroforestrial modality on both bacterial (Table 1) and fungal (Table 2) microbiota. On the other hand, variety only has a significant effect on Firmicutes' Shannon index (Table 1), fungal community specific richness as well as Ascomycota and Zygomycota specific richness (Table 2).

Variable		Year		Farm		Agroforestrial modality		Variety	
		F	p-value	F	p-value	F	p-value	F	p-value
Community	Specific richness	0.4359	0.510001	0.9548	0.32981	3.3383	**0.006692** **	0.2608	0.610242
	Shannon index	24.7945	**1.571e-06** ***	0.0022	0.96285	2.8841	**0.01588** *	0.0007	0.97842
	Pielou's equitability	35.9339	**1.211e-08** ***	0.2873	0.59261	1.9721	0.08524 .	0.0200	0.88760
	Simpson index	6.6151	**0.01098** *	0.0008	0.977026	1.6852	0.14074	1.3927	0.23961
Acidobacteria	Specific richness	17.9039	**3.812e-05** ***	0.7998	0.37236	0.9476	0.4518	0.2305	0.6318
	Shannon index	20.3717	**1.195e-05** ***	0.4392	0.5084	1.6182	0.1578	0.3684	0.5447
	Pielou's equitability	18.4052	**3.696e-05** ***	0.2769	0.59967	1.4123	0.2249	0.1598	0.6901
	Simpson index	1.2075	0.27340	0.2687	0.6049	0.3107	0.90603	0.0300	0.86267
Actinobacteria	Specific richness	5.9914	**0.015404** *	0.9240	0.33771	1.4605	0.205374	0.2341	0.629097
	Shannon index	1.3729	0.242968	0.3730	0.5421570	2.9516	**0.013980** *	0.0485	0.825898
	Pielou's equitability	9.0106	**0.0030938** **	0.0213	0.8840252	4.3917	**0.0008764** ***	0.4263	0.5147030
	Simpson index	0.6207	0.43192	0.7430	0.38986	2.9615	**0.01372** *	0.0323	0.85753
Bacteroidota	Specific richness	49.9925	**3.954e-11** ***	9.5243	**0.002348** **	10.9491	**3.904e-09** ***	0.0948	0.75858
	Shannon index	3.2854	0.07168 .	3.9941	**0.04717** *	9.3735	**6.78e-08** ***	0.0997	0.75253
	Pielou's equitability	1.2463	0.2659	0.8735	0.35123	5.8966	**4.763e-05** ***	0.1663	0.6839
	Simpson index	0.1167	0.7331	1.6605	0.1992	5.8297	**5.418e-05** ***	0.9968	0.3195
Firmicutes	Specific richness	35.7055	**1.334e-08** ***	0.6789	0.411054	10.8686	**4.509e-09** ***	3.1779	0.07645 .
	Shannon index	27.2058	**5.328e-07** ***	0.1460	0.70287	5.8034	**5.700e-05** ***	4.9556	**0.02734** *
	Pielou's equitability	0.9811	0.32359	5.2189	**0.02370** *	1.8711	0.10299	3.6221	0.05903 .
	Simpson index	8.8171	**0.003421** **	0.1746	0.6765719	2.3181	**0.045613** *	0.5183	0.472549
Proteobacteria	Specific richness	3.8993	**0.049945** *	6.2996	**0.01296** *	3.2480	**0.007954** **	0.1039	0.747585
	Shannon index	55.4869	**4.705e-12** ***	0.1809	0.67109	1.2047	0.30908	0.0532	0.81788
	Pielou's equitability	65.7108	**1.035e-13** ***	0.4037	0.5260	0.6230	0.68242	0.0302	0.86223
	Simpson index	28.6002	**2.873e-07** ***	0.0271	0.8694	0.8511	0.5155	0.1278	0.7211
Verrucomicrobiota	Specific richness	116.4313	**< 2.2e-16** ***	17.6966	**4.081e-05** ***	8.0754	**7.541e-07** ***	2.6814	0.10340
	Shannon index	84.7341	**< 2.2e-16** ***	13.5646	**0.0003046** ***	7.4961	**2.244e-06** ***	1.3648	0.24437
	Pielou's equitability	3.2720	0.07275 .	3.1242	0.07925 .	2.4206	**0.03898** *	0.5067	0.47782
	Simpson index	23.1327	**3.343e-06** ***	3.6142	0.05888 .	3.1789	**0.009075** **	1.6591	0.199500

Table 1. ANOVA results for bacterial community and main phyla structuration characterised by diversity indexes, depending on year, farm, agroforestrial modality and tomato variety.

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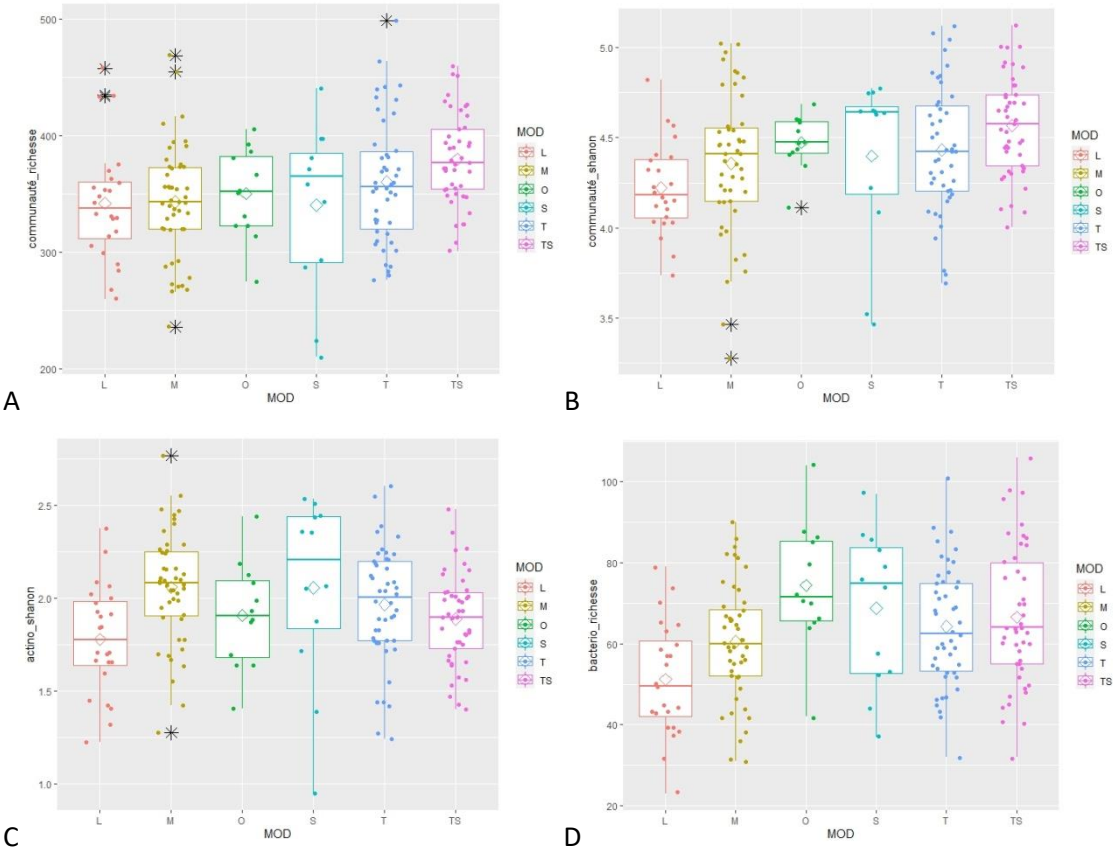
Variable		Year		Farm		Agroforestrial modality		Variety	
		F	p-value	F	p-value	F	p-value	F	p-value
Community	Specific richness	585.0295	**< 2.2e-16** ***	2.0564	0.1532741	8.4073	**3.937e-07** ***	11.7541	**0.0007607** **
	Shannon index	0.7281	0.3947	0.9630	0.3277	1.7355	0.1290	0.0648	0.7994
	Pielou's equitability	54.5152	**6.46e-12** ***	0.4962	0.4821	0.6926	0.6297	0.1462	0.7027
	Simpson index	0.0240	0.8770291	0.0759	0.7832	0.5287	0.7543747	0.2022	0.6534981
Ascomycota	Specific richness	799.6651	**< 2.2e-16** ***	0.6646	0.415997	2.6211	**0.0259703** *	11.2447	**0.0009833** **
	Shannon index	0.8802	0.3495	0.0334	0.8553	0.4684	0.7994	0.0044	0.9470
	Pielou's equitability	48.6902	**6.309e-11** ***	0.0163	0.8985	0.8204	0.536671	0.5515	0.458710
	Simpson index	0.6582	0.418307	0.4475	0.5044	0.8863	0.491552	0.0006	0.980057
Basidiomycota	Specific richness	166.2795	**< 2.2e-16** ***	6.6564	**0.01066** *	6.7313	**9.419e-06** ***	2.7321	0.10018
	Shannon index	0.0014	0.9701110	5.9530	**0.01565** *	5.1450	**0.0002012** ***	0.0016	0.9686109
	Pielou's equitability	30.2988	**1.363e-07** ***	4.7318	**0.03091** *	4.2238	**0.001213** **	0.2154	0.643198
	Simpson index	1.1949	0.2758716	7.9816	**0.00525** **	4.4992	**0.0007055** ***	0.0844	0.7717091
Glomeromycota	Specific richness	8.5429	**0.003938** **	18.4563	**2.819e-05** ***	21.5421	**< 2.2e-16** ***	2.2179	0.138264
	Shannon index	11.1460	**0.001034** **	36.9470	**6.898e-09** ***	17.8638	**3.138e-14** ***	0.0603	0.806295
	Pielou's equitability	46.1628	**2.166e-10** ***	16.4508	**7.629e-05** ***	4.0362	**0.001796** **	2.7286	0.100576
	Simpson index	1.1949	0.2758716	40.4139	**1.592e-09** ***	4.4992	**0.0007055** ***	0.0844	0.7717091
Zygomycota	Specific richness	142.1083	**< 2.2e-16** ***	0.5286	0.46812	2.0436	0.0749324 .	5.9875	**0.0154196** *
	Shannon index	0.3869	0.534748	0.6073	0.4368172	1.6567	0.147647	1.8100	0.180290
	Pielou's equitability	71.7006	**1.916e-14** ***	0.3819	0.537449	1.5282	0.18418	0.0001	0.99185
	Simpson index	2.1166	0.14754	1.0331	0.310777	2.2581	0.05082 .	0.4279	0.51392
Chytridiomycota	Specific richness	8.5429	**0.003938** **	18.4563	**2.819e-05** ***	21.5421	**< 2.2e-16** ***	2.2179	0.138264
	Shannon index	11.1460	**0.001034** **	36.9470	**6.898e-09** ***	17.8638	**3.138e-14** ***	0.0603	0.806295
	Pielou's equitability	46.1628	**2.166e-10** ***	16.4508	**7.629e-05** ***	4.0362	**0.001796** **	2.7286	0.100576
	Simpson index	17.6343	**4.299e-05** ***	40.4139	**1.592e-09** ***	14.8188	**4.518e-12** ***	0.0000	0.998523

Table 2. ANOVA results for fungal community and main phyla structuration characterised by diversity indexes, depending on year, farm, agroforestrial modality and tomato variety.

When looking at diversity indexes depending on agroforestrial modalities, distinct patterns between bacterial and fungal communities appear; bacteria tend to be more diversified in terms of sequence-clusters, with a more equitable repartition following the light gradient in a linear relation (Figure 6); on the other hand, fungi tend to react to "extreme" environments (Light pruning, Sun Control TS and S) (Figure 7).



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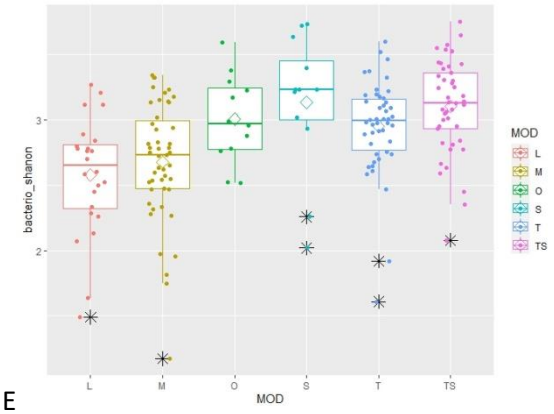

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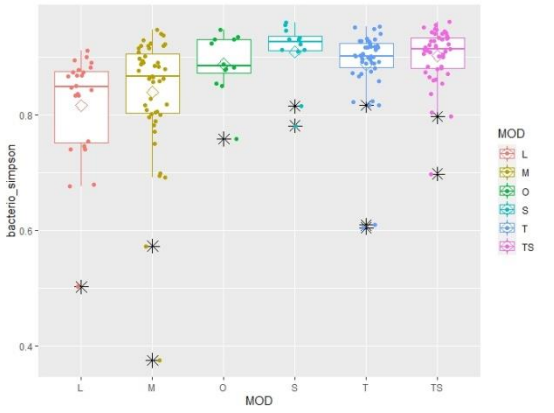

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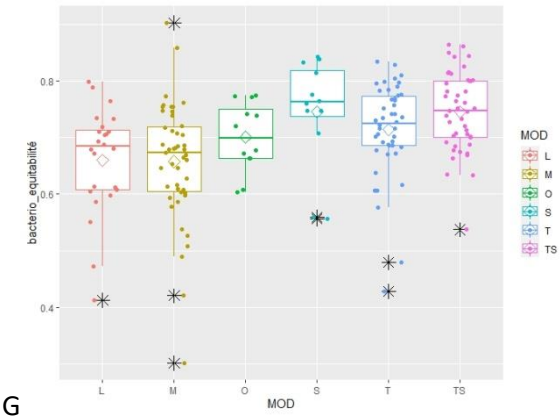

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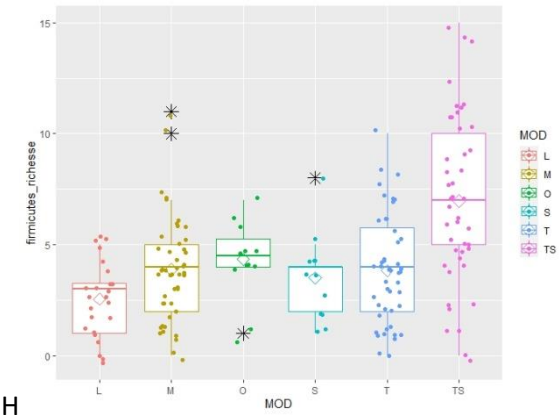

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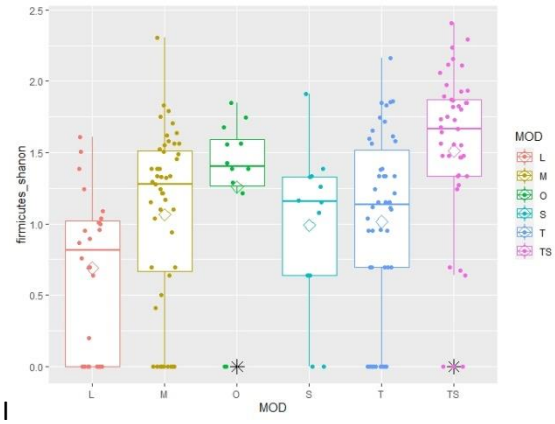

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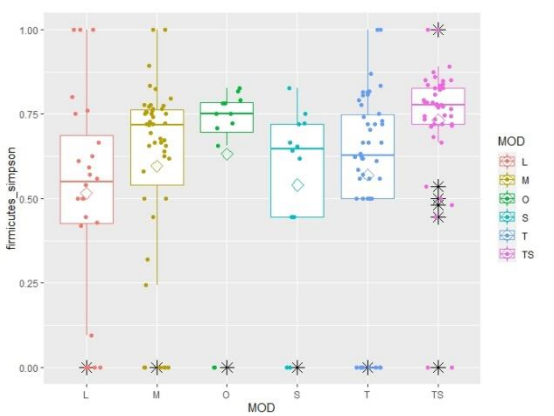

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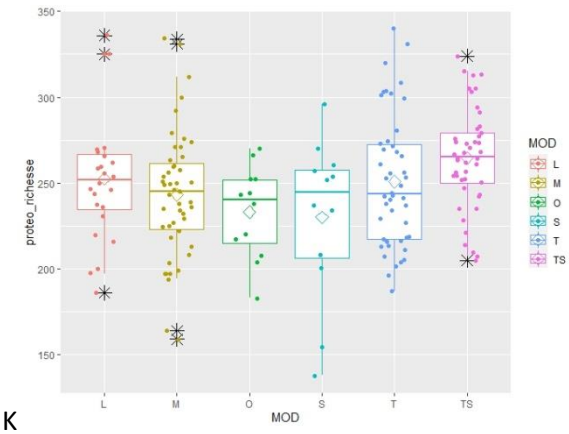

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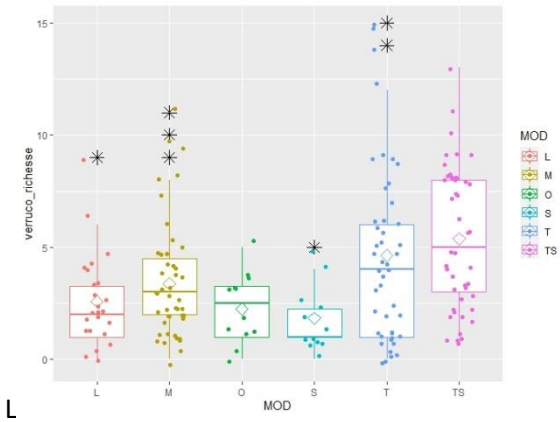

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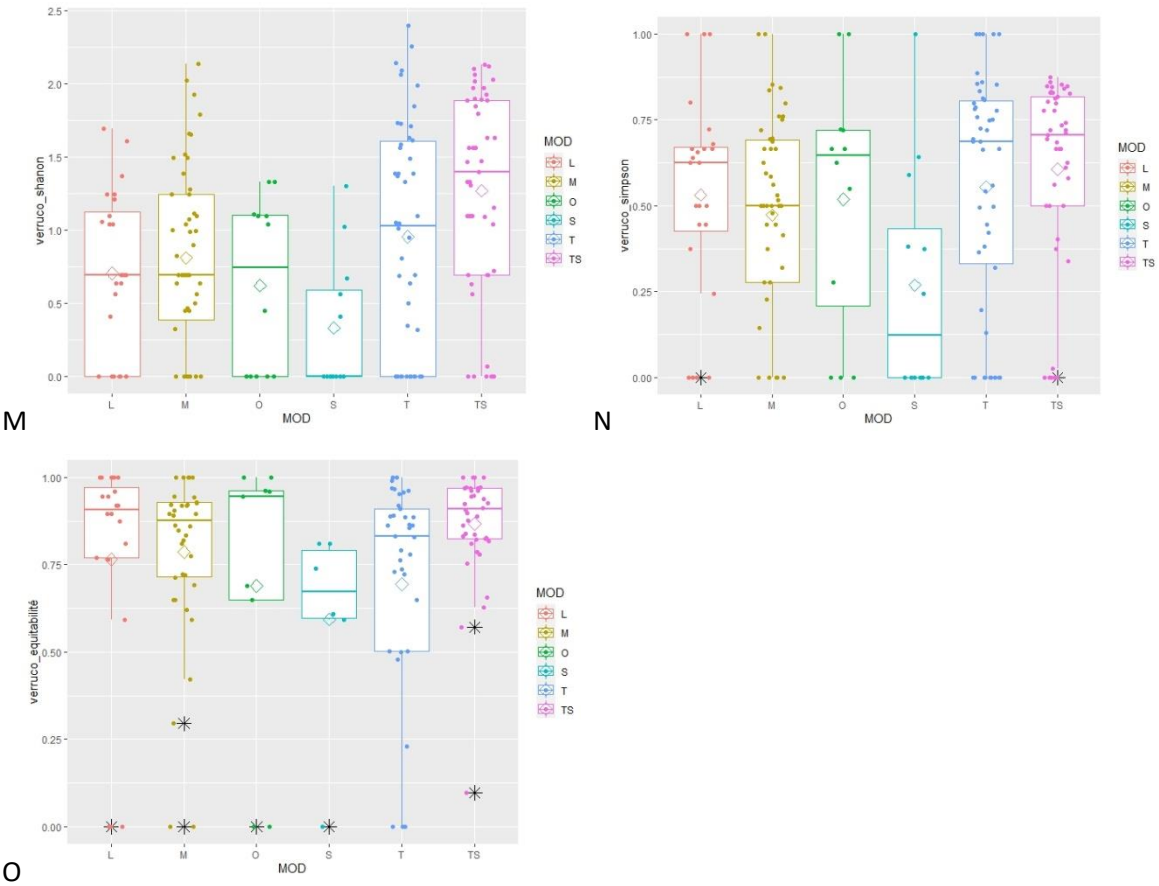
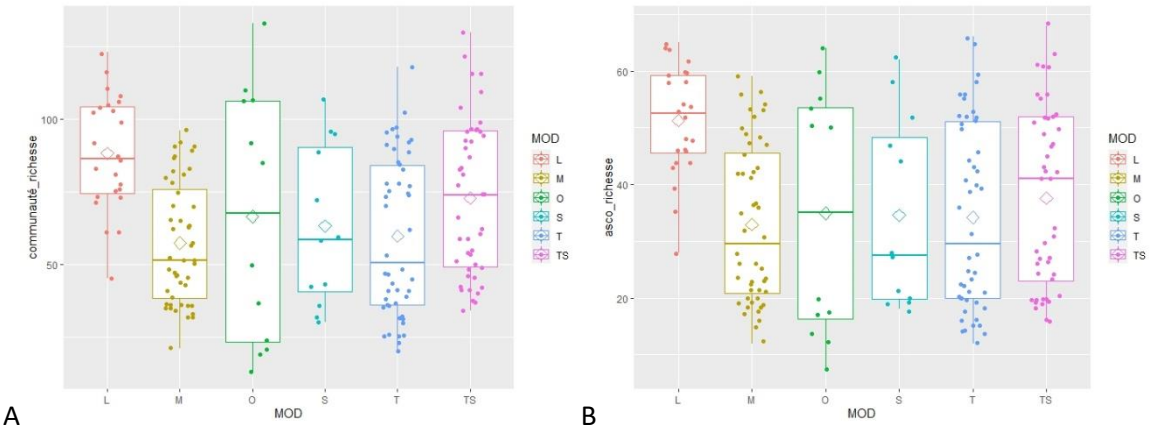


Figure 6. diversity indexes of bacterial communities for which a significant effect of agroforestrial modality is observed. L Light pruning; M medium pruning; T pollard; TS sun control; O shade modality at Boulidou farm; S sun control at Boulidou farm. A Community specific richness; B Shannon index of community; C Shannon index of actinobacteria; D Bacteroidota specific richness; E Bacteroidota Shannon index; F Bacteroidota Simpson index; G Bacteroidota equitability; H Firmicutes specific richness; I Firmicutes Shannon index; J Firmicutes Simpson index; K Proteobacteria specific richness; L Verrucomicrobiota specific richness; M Verrucomicrobita Shannon index; N Verrucomicrobitota Simpson index; O Verrucomicrobiota equitability.



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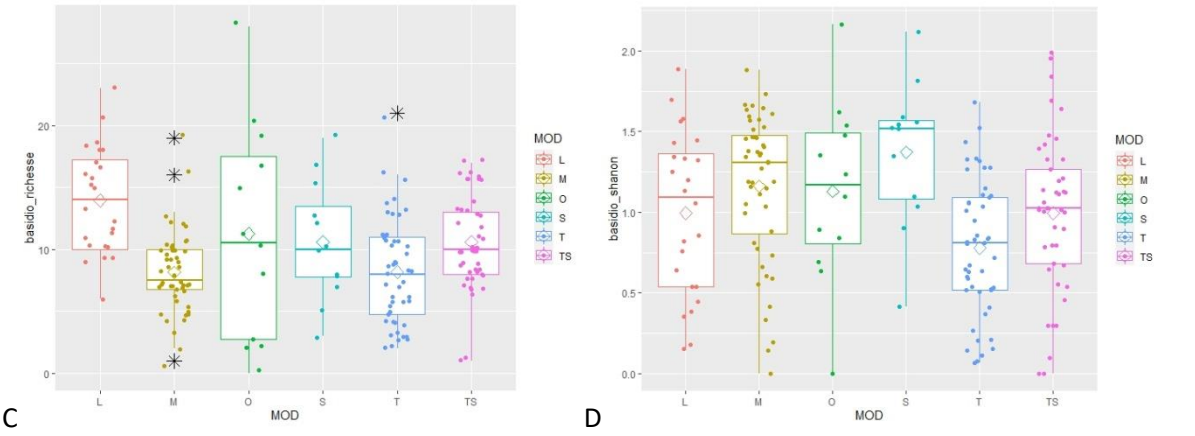

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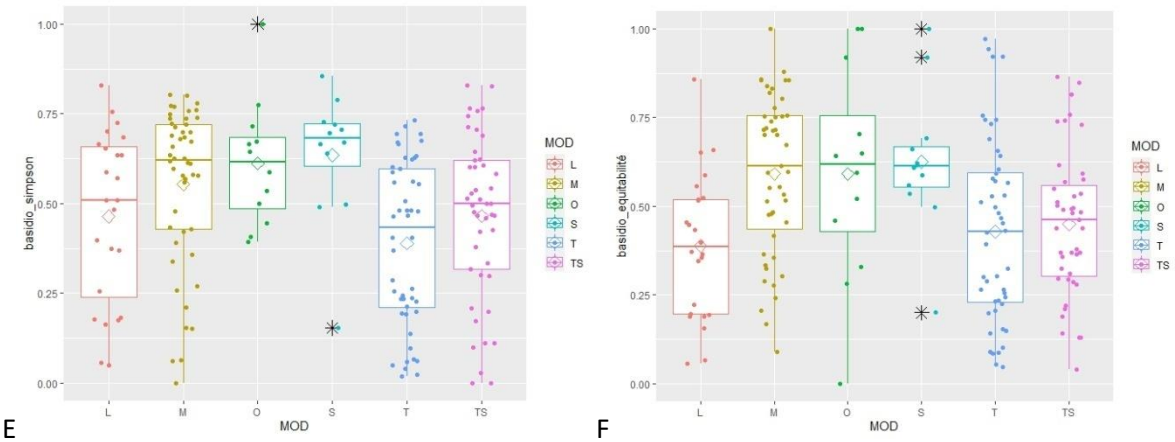

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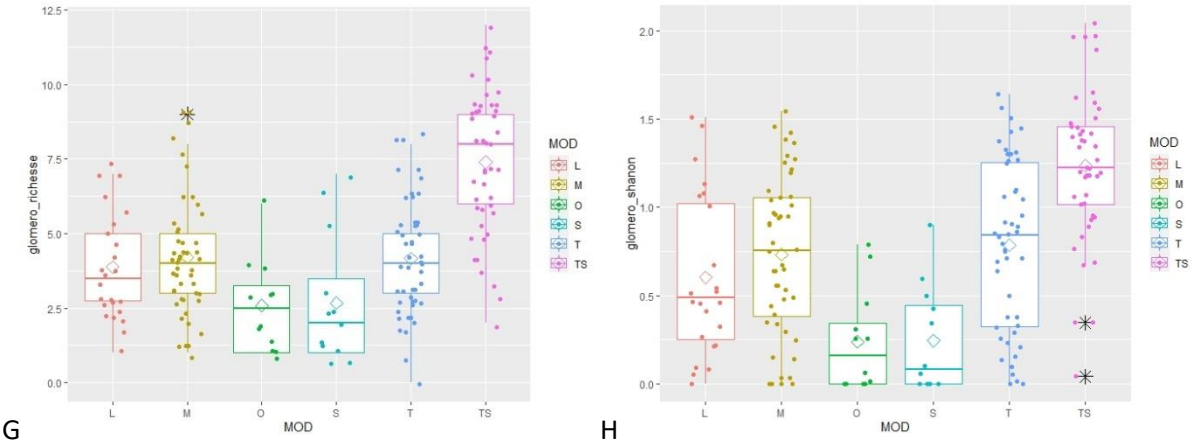

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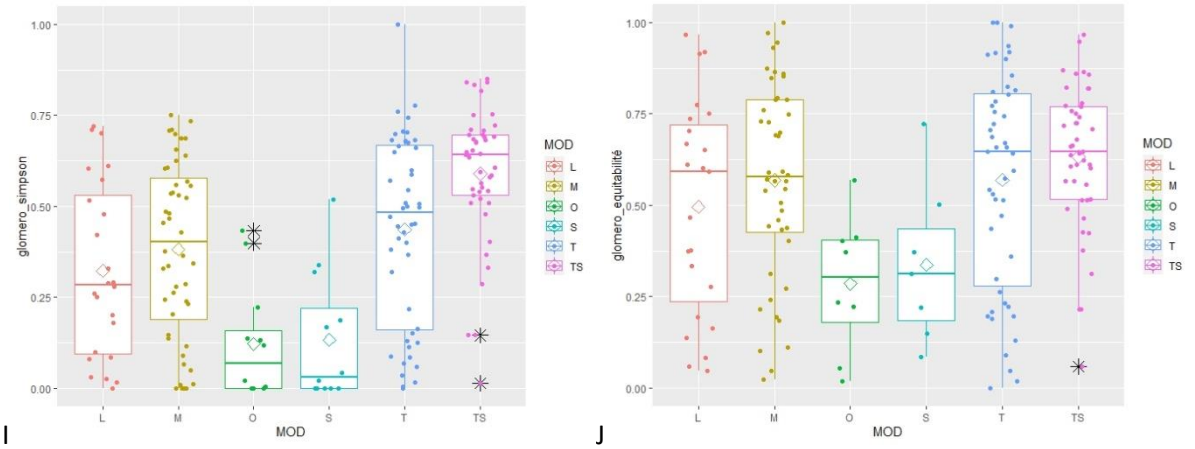

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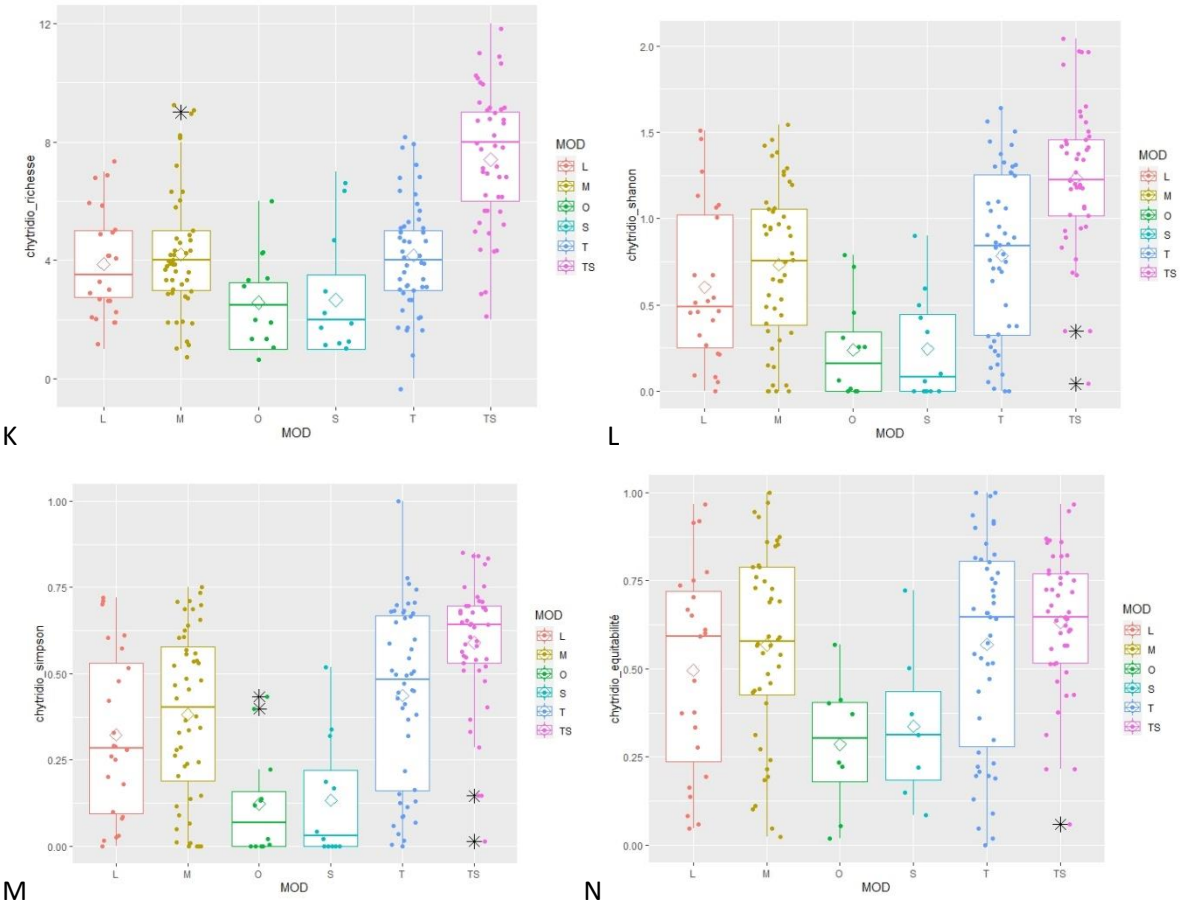

Figure 7. diversity indexes of fungal communities for which a significant effect of agroforestrial modality is observed. L Light pruning; M medium pruning; T pollard; TS sun control; O shade modality at Boulidou farm; S sun control at Boulidou farm. A Community specific richness; B Ascomycota Shannon index; C Basidiomycota specific richness; D Basidiomycota Shannon index; E Basidiomycota Simpson index; F Basidiomycota equitability; G Glomeromycota specific richness; H Glomeromycota Shannon index; I Glomeromycota Simpson index; J Glomeromycota equitability; K Chytridiomycota specific richness; L Chytridiomycota Shannon index; M Chytridiomycota Simpson index; N Chytridiomycota equitability.

2.4. Most of sequence clusters are shared among agroforestrial modalities, but their structuration varies between them.

Venn diagram showing shared and unique sequence-clusters between agroforestrial modalities display that clusters are mainly shared between at least 4 modalities. They are very few unique sequence clusters (Figure 8- 9).

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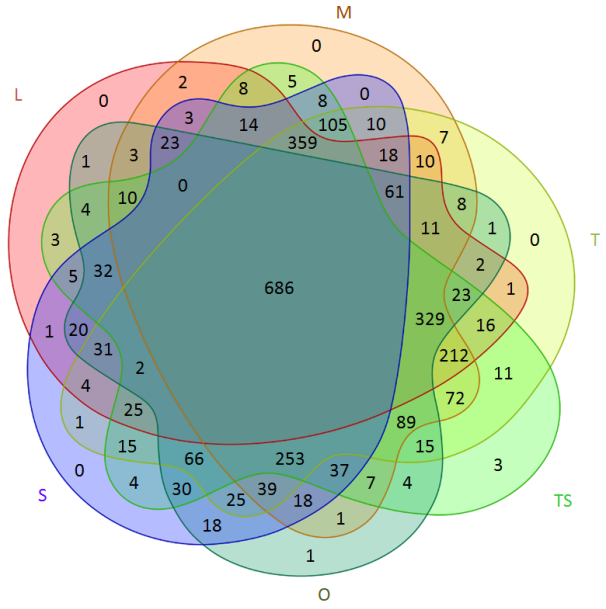

Figure 8. Venn diagram showing bacterial sequence-clusters repartition

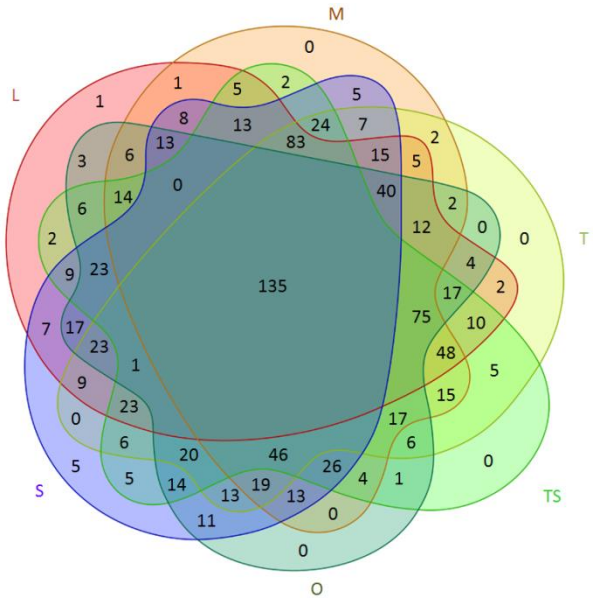

Figure 9. Venn diagram showing fungal sequence-clusters repartition

Partial Least Squares Discriminant Analysis (PLS-DA) ordination of the bacterial and fungal communities depending on agroforestrial modalities represent these contrasted colonization patterns (Figure 10). Bacteria tend to segregate accordingly to the light gradient, with a sensitivity to the sampling site, while fungi tend to differentiate among extreme environments (deep shade L or sun control TS and S).

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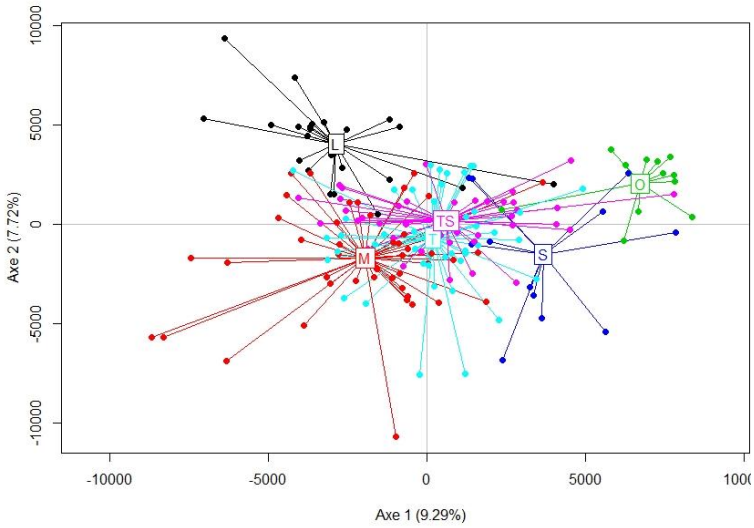

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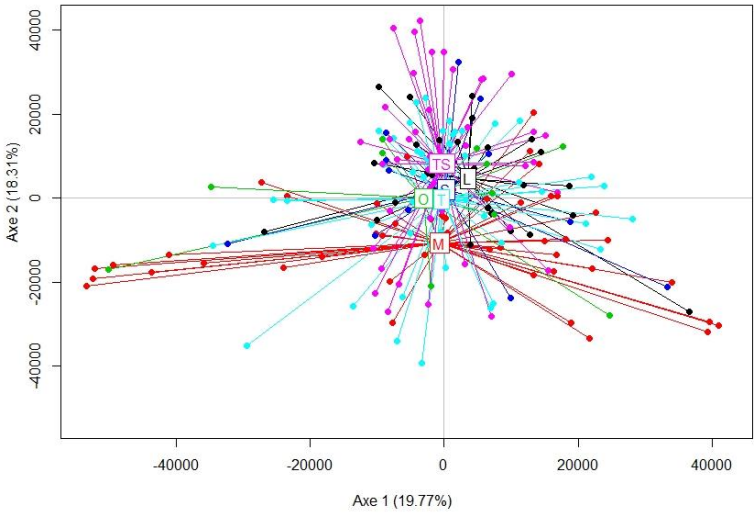

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Figure 10. Partial Least Squares Discriminant Analysis (PLS-DA) ordination of the bacterial (A) and fungal (B) community in Light pruning (L), medium pruning (M), pollard (T), sun control (TS at Roumassouze and S at Boulidou farms), and agroforestrial modality at Boulidou (O) on the total community (1317 sequence-clusters for the bacteria and 278 for the fungi).

3. Discussion, outcomes, and conclusion

Our results show that on farm selection maintains the genetic structure of varieties. This allow us to assess that we are working on two distinct populations, with a potential effect on microbiome assembly. Speaking of on-farm selection, we cannot directly assess its effect on microbiome structuration, as we do not have a "non-selected" control. The experiment being on-farm, having such a population could impair the farmer's work. However, the very weak effect of variety on both bacterial and fungal communities is surprising in regard of literature (Bergna et al., 2018; Bulgarelli et al., 2012; Bulgarelli et al., 2015; Kwak et al., 2018; Lebeis et al., 2015; Lundberg et al., 2012; Peiffer et al., 2013; Rasche et al., 2006) despite their difference. This result could be an indication of co-evolution

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between plants and micro-organisms being supported by on-farm selection, with possible epigenetic regulations as well (Vannier et al., 2015). Indeed, on-farm selection involves all 3 types of heredity: genetic, epigenetic and microbiotic. In our study, the genetic component seems to be balanced at least by the microbiotic one, as the epigenetic regulation has not been explored.

Our results are in accordance with the holobiont hypothesis framework (Margulis, L., 1981; Vandenkoornhuyse et al., 2015) associated to the hologenome theory (Zilber-Rosenberg, I., & Rosenberg, E., 2008). These paradigm shifts in the plants' conception are necessary in organic plant breeding aiming at resilience, in the way that they contribute to a holistic, system-based approached. On-farm selection, rather unconsciously, already makes the most of these interactions, as it considers plants phenotype as an expression of genetic, epigenetic and microbiotic adaptation to a given terroir.

The results of this study, even with some partial data from one year to another, give a first idea the drivers of plants and microbiome co-evolution through on-farm selection in an agroforestrial context. These results could inspire organic breeding to integrate different levels of heredity when willing to select for resilient cropping systems and are a first step toward a more holistic agriculture.

References

Bergna, A., Cernava, T., Rändler, M., Grosch, R., Zachow, C., & Berg, G. (2018). Tomato seeds preferably transmit plant beneficial endophytes. *Phytobiomes Journal*, *2*(4), 183-193.

Bulgarelli, D., Garrido-Oter, R., Münch, P. C., Weiman, A., Dröge, J., Pan, Y., & Schulze-Lefert, P. (2015). Structure and function of the bacterial root microbiota in wild and domesticated barley. *Cell host & microbe*, *17*(3), 392-403.

Bulgarelli, D., Rott, M., Schlaeppi, K., van Themaat, E. V. L., Ahmadinejad, N., Assenza, F. & Peplies, J. (2012). Revealing structure and assembly cues for Arabidopsis root-inhabiting bacterial microbiota. *Nature*, *488*(7409), 91-95.

Duhamel, M., & Vandenkoornhuyse, P. (2013). Sustainable agriculture: possible trajectories from mutualistic symbiosis and plant neodomestication. *Trends in plant science*, *18*(11), 597-600.)

Escudié, F., Auer, L., Bernard, M., Mariadassou, M., Cauquil, L., Vidal, K., ... & Pascal, G. (2018). FROGS: find, rapidly, OTUs with galaxy solution. *Bioinformatics*, *34*(8), 1287-1294.

Kwak, M. J., Kong, H. G., Choi, K., Kwon, S. K., Song, J. Y., Lee, J., ... & Jung, E. J. (2018). Rhizosphere microbiome structure alters to enable wilt resistance in tomato. *Nature biotechnology*, *36*(11), 1100-1109.

Lebeis, S. L., Paredes, S. H., Lundberg, D. S., Breakfield, N., Gehring, J., McDonald, M. & Dangl, J. L. (2015). Salicylic acid modulates colonization of the root microbiome by specific bacterial taxa. *Science*, *349*(6250), 860-864.

Lê Van, A., Quaiser, A., Duhamel, M., Michon-Coudouel, S., Dufresne, A., & Vandenkoornhuyse, P. (2017). Ecophylogeny of the endospheric root fungal microbiome of co-occurring Agrostis stolonifera. *PeerJ*, *5*, e3454.

Lundberg, D. S., Lebeis, S. L., Paredes, S. H., Yourstone, S., Gehring, J., Malfatti, S., ... & Edgar, R. C. (2012). Defining the core Arabidopsis thaliana root microbiome. *Nature*, *488*(7409), 86-90.

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Margulis, L. (1981). Symbiosis in cell evolution: Life and its environment on the early earth. *Boston University Boston, MA, United States*

Peiffer, J. A., Spor, A., Koren, O., Jin, Z., Tringe, S. G., Dangl, J. L., ... & Ley, R. E. (2013). Diversity and heritability of the maize rhizosphere microbiome under field conditions. *Proceedings of the National Academy of Sciences*, *110*(16), 6548-6553.

Rasche, F., Hödl, V., Poll, C., Kandeler, E., Gerzabek, M. H., Van Elsas, J. D., & Sessitsch, A. (2006). Rhizosphere bacteria affected by transgenic potatoes with antibacterial activities compared with the effects of soil, wild-type potatoes, vegetation stage and pathogen exposure. *FEMS microbiology ecology*, *56*(2), 219-235.

Serpolay-Besson, E., Giuliano, S., Schermann, N., & Chable, V. (2014). Evaluation of Evolution and Diversity of Maize Open-Pollinated Varieties Cultivated under Contrasted Environmental and Farmers' Selection Pressures: A Phenotypical Approach.

Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., & Dufresne, A. (2015). The importance of the microbiome of the plant holobiont. *New Phytologist*, *206*(4), 1196-1206.

van Frank, G., Rivière, P., Pin, S., Baltassat, R., Berthellot, J. F., Caizergues, F., ... & Montaz, H. (2020). Genetic Diversity and Stability of Performance of Wheat Population Varieties Developed by Participatory Breeding. *Sustainability*, *12*(1), 384.

Vannier, N., Mony, C., Bittebière, A. K., & Vandenkoornhuyse, P. (2015). Epigenetic mechanisms and microbiota as a toolbox for plant phenotypic adjustment to environment. *Frontiers in plant science*, *6*, 1159.

Zilber-Rosenberg, I., & Rosenberg, E. (2008). Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. *FEMS microbiology reviews*, *32*(5), 723-735.

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Annex 2a. Analysis of the trade-off between selection for improved resilience and product quality in vegetable crops linked with mitochondrial activities - Pea (task 3.3.1)

Authors: Hélia Cardoso and Lénia Rodrigues (UEV)

1. Introduction

Calorespirometry, a technique that simultaneously measures heat and CO2 rates, has been proposed as a screening tool to assess metabolic and respiratory changes associated with cell reprogramming events. Considering that seed germination involves the activation of several metabolic pathways, including cellular respiration to provide the required energy, the objective of this work was to prove the usefulness of calorespirometry method to assess seed viability by monitoring cellular respiration associated with the germination process, and to explore the link between calorespirometry and alternative respiration.

During 2020 pea seeds from different lines were produced by LIVESEED partners FiBL, AREI and ITAB/UBIOS in three countries, for most lines with multiple replicates. Samples from these seed productions were sent to the University of Évora (UEV), and the seven genotypes received at UÉVORA were evaluated using calorespirometry: EFB-1, EFB-2, G78, Respect-1, G85, S134, S91. Each genotype was characterized by three biological replicates, named Rep.1, Rep.2, Rep.3. To evaluate the germination efficiency, seeds used for analysis int the calorimeter were placed under dark at 25˚C. Germination was evaluated six days after calorespirometric measurements. The pea lines were selected by FiBL-CH based on their differentiation for root rot resistance (resistant: EFB, G78, S91, S134, versus susceptible: Respect, G85).

To establish the link between the results of calorespirometry and alternative pathway, AOX was investigated in two genotypes, selected based on calorespirometric data, at transcript by evaluating AOX gene expression by RT-qPCR, and at protein level by western-blot analysis.

Statistical analyses were performed by SPSS version 22.0. Normality of variances and homoscedasticity were checked for all data and mean comparisons was performed by one-way ANOVA followed by a Tukey HSD test. When the data do not meet the assumptions for performing parametric tests, mean comparisons was performed by Kruskal-Wallis nonparametric test. Statistical significance was considered for $P<0.05$.

2. Metabolic changes associated with differences in seed viability assessed by calorespirometry

Calorespirometric measurements were performed 16h after seed imbibition period running at isothermal mode at 25°C. Calorespirometric measurements were performed in a Multi-Cell Differential Scanning Calorimeter (TA Instruments).

The final analysis was carried out with 9 replicates of each genotype. After statistical analysis we observed that Respiratory heat rate (*Rq*) was significantly lower in the cvs G85, S91 and G78 and higher in the cvs EFB-2, S134, Respect-1 and EFB-1 (Figure 1A). There was no correlation to root rot disease.

CO2 production rate (*RCO2*) exhibited a similar pattern than the observed in *Rq* (Figure 1B), except with cultivar G85 that present lower values of *Rq* and higher values of *RCO2*. There was also no distinction between resistant and susceptible genotypes.

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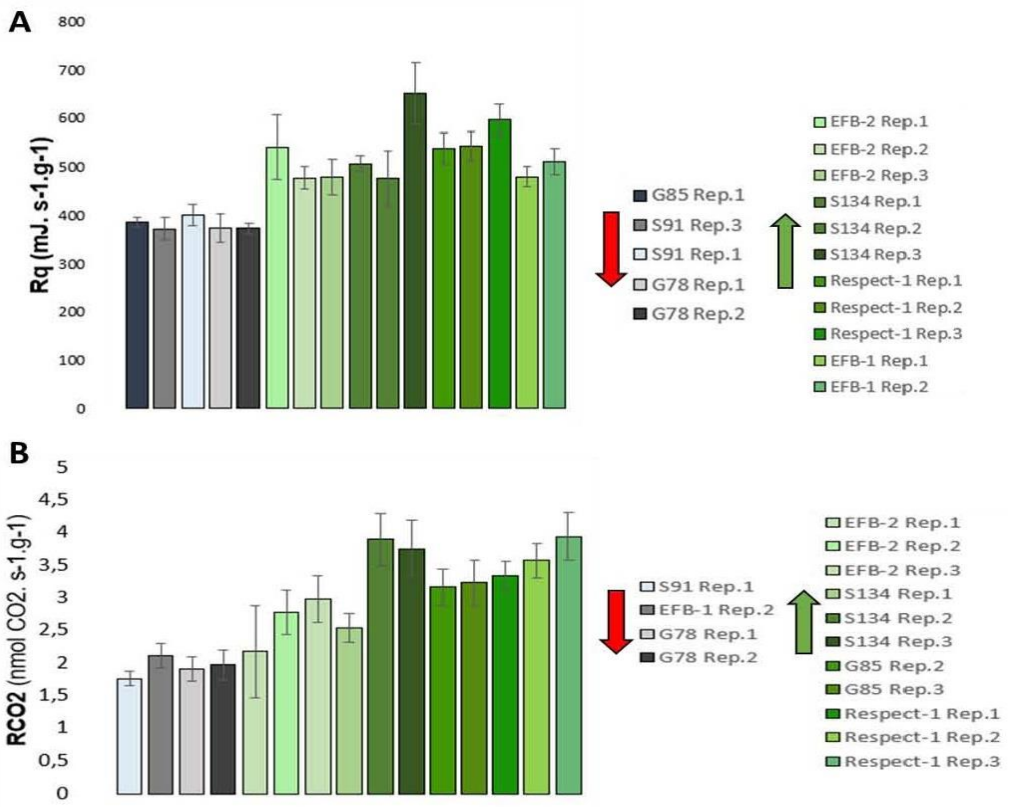

Figure 1. Genotypes with significant differences on calorespirometric parameters Rq (A) and RCO2 (B). Red arrows indicate significantly lower values and green arrows indicate significantly higher values of calorespirometric parameters.

Comparing the results of germination with the calorespirometric parameters Rq and RCO2, it was observed that the cultivars with higher values of Rq and RCO2 presented a high germination rate.

3. Exploring the involvement of alternative pathway by AOX protein analysis

To establish the link between AOX protein expression and calorespirometric parameters, the alternative oxidase was validated by Western blot technique. For this study, four cultivars that presented extreme values of calorespirometric parameters were selected: Respect-1, S134, S91, G78.

To perform the western blot, proteins were extracted by phenol extraction procedure after cell homogenization using liquid nitrogen. The measurement of total protein concentration was performed using the PierceTM 660nm Protein Assay Reagent. Indeed, it was observed that the cvs. Respect-1 Rep.1 and Respect-1 Rep.3 have significantly higher total protein concentration values compared to cvs. G78 Rep.3, S91 Rep.1, S134 Rep.1 and S134 Rep.3. The S91 Rep.1 and G78 Rep.3 were the cultivars with the lowest concentration values, compared to the other cultivars. The Respect-1 Rep.1, Respect-1 Rep.2 and G78 Rep.1 were the cultivars with the higher concentration values, compared to the other cultivars (Figure 2).

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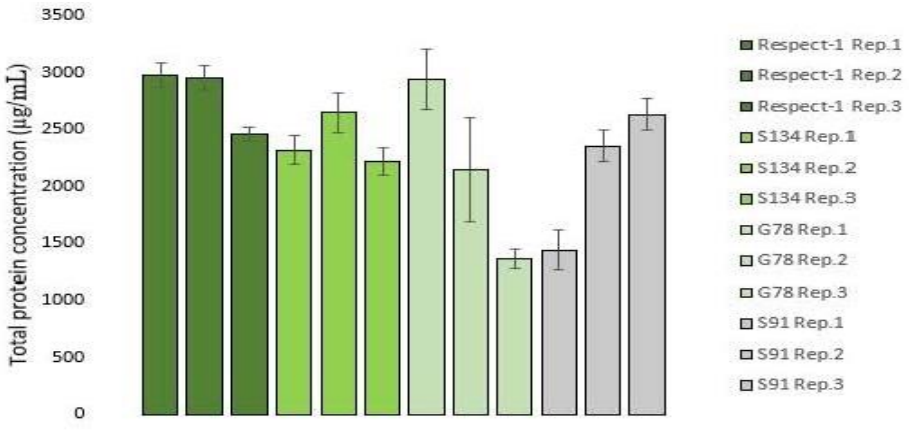

Figure 2. Total of protein concentration achieved in the different genotypes after protein extraction following the phenol extraction procedure and further quantification using the PierceTM 660nm Protein Assay Reagent.

The expression analysis of AOX protein between different cultivars using the Western blot technique is still in progress. Below on Figure 3 it is presented an example, which shows the result of a gel exhibiting differential expression of AOX.

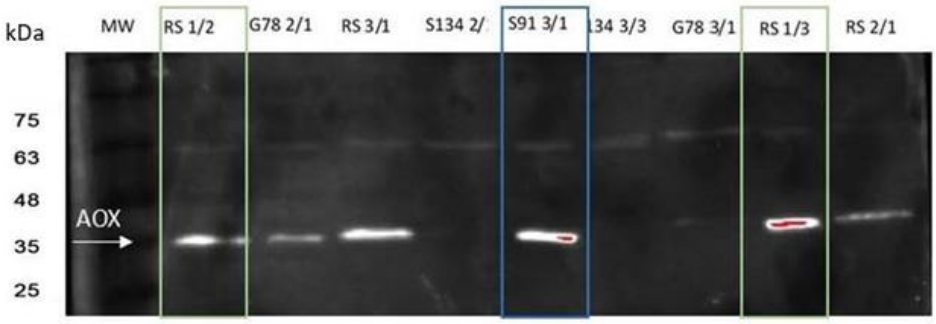

Figure 3. Western-blot analysis for the AOX protein, achieved using an AOX-specific primary antibody. On the left side of the image, part of the molecular mass marker is represented.

4. Exploring the involvement of alternative pathway by AOX gene expression analysis

For gene expression analysis two genotypes with the most different behavior on the calorespirometric measurements were selected: the Respect-1 and the S91. For each genotype three biological replicates were considered, and from each one four replicates (four sets of four seeds) were carried out.

Before transcript analysis of the three AOX genes, named as *PsAOX1*, *PsAOX2a* and *PsAOX2b*, selection of the most reliable set of genes to be used as reference genes was carried out. From a set of three candidate reference genes (*PsUBI*, *PsPOB* and *PsSAR1*), the *PsPOB* and *PsSAR1* were selected as the most appropriate set of genes based on geNorm software tool.

In Figure 4 it is presented the results achieved on each cultivar for the three AOX gene members. A significant difference is visible between both genotypes (Figure 4A). The cv. S91 presents a higher transcript level in comparison with Respect-1. However, when the analysis is made considering each biological replicate, a significant effect was detected (Figure 4B).

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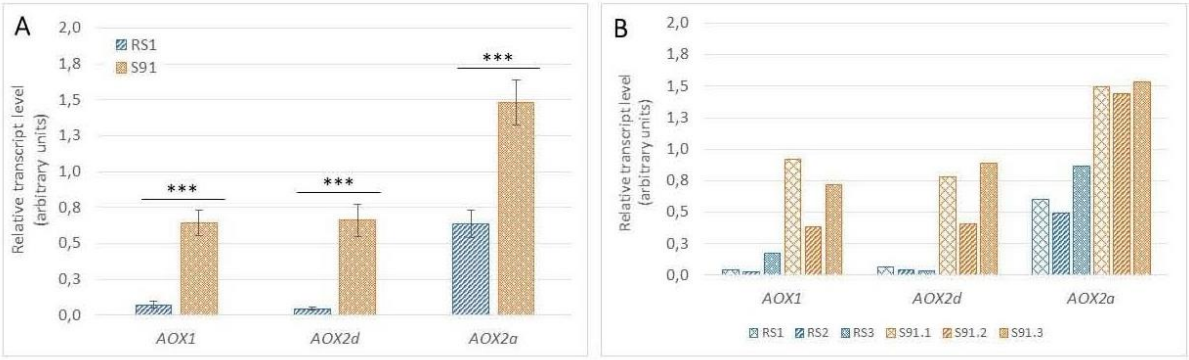

Figure 4. Relative quantification of PsAOX1, PsAOX2a and PsAOX2d in three biological replicates of two pea genotypes, the cv. Respect-1 and the S91. RS1, RS2 and RS3 are the three biological replicates of Respect-1; S91.1, S91.2 and S91.3 are the three biological replicates of S91. From each biological it was considered four replicates of four seeds each. RT-qPCR was performed in duplicate for each sample.

Conclusions

The cv. Respect-1, which exhibits lower expression on the three AOX genes, was the cultivar exhibiting higher germination rates and higher values on the calorespirometric parameters. This was also the cultivar that was more susceptible to root rot disease compared to the resistant S91 (task 3.3.2).

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Annex 2b. Pea seed quality testing (task 2.3)

Authors: Jan Kodde and Steven Groot (WR)

1. Introduction

During 2020 pea seeds from different lines were produced by LIVESEED partners FiBL-CH in Switzerland, AREI in Latvia, and ITAB/UBIOS in France, for most lines with multiple field replicates. The pea genotypes were selected by FiBL-CH for their contrasting level to root rot disease, i.e., resistant: EFB, G78, S64, S91, S127, S134, S199 and susceptible: Respect, G85, S118 (task. 3.3.2). Samples from these seed productions were sent to WR for extraction of DNA and seed quality analysis. Not from all lines samples were received from all three locations, and the number of replicates per production location varied from one till eight. After arrival, the pea seeds have been stored in a storage room at 13 °C and 30% RH. The pea seed samples from AREI were packed in plastic bags and had not been equilibrated to the low humidity during the storage till DNA extraction. In May 2021 a germination test has been performed with the pea seed samples, using 90 seeds per sample. The samples represented replicate seed lots produced from these lines at different locations. The number of seed lots per line and seed production varied between one and eight, with on average four. Thereafter a second germination test was started to determine the frequency of normal seedlings.

2. First germination test, evaluation of germination parameters

The seeds were first germinated on paper blotters in stacked germination boxes placed in an incubator at 20 °C and eight hours light (Figure 1). Germination of the seeds, defined as root protrusion of at least 2 mm, was scored daily, to determine also the germination speed, an indicator for seed vigour. To enable daily scoring of newly germinated seeds, the germinated seeds were removed every day. Final germination was scored after 10 days (Figure 2). The germination speed is characterised by T_{50}, defined as the period until 50% of the final germination was reached (Figure 3). Area under the germination curve (AUC) till 200 hours can also be used as indicator for seed vigour, combining the speed of germination and the number of germinated seeds reached after 200 hours (Figure 4).

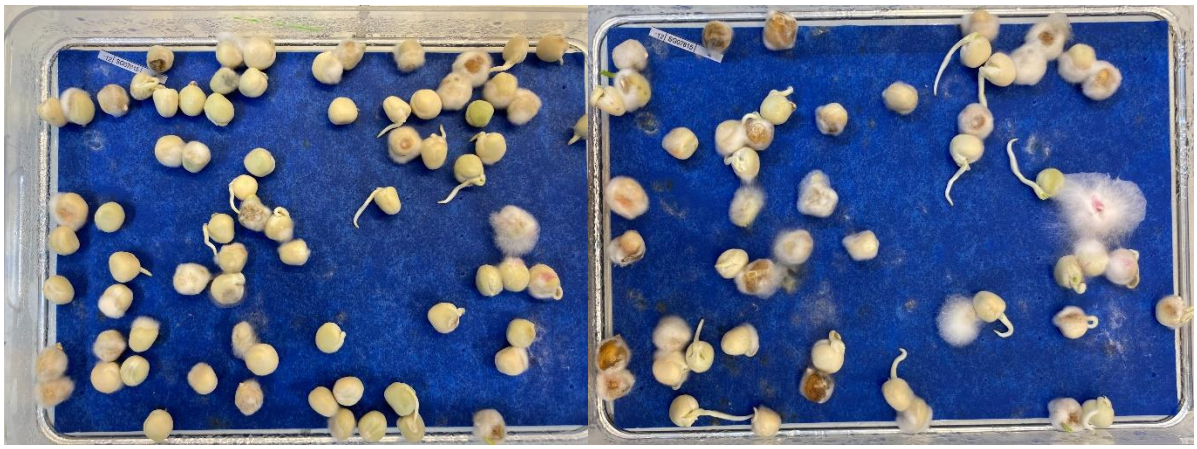

Figure 1. Example of the germination test in stacked boxes. Her sample SG07815 (G85 AREI) six or eight days after start of the test. Seeds with protruded radicle were counted and removed daily.

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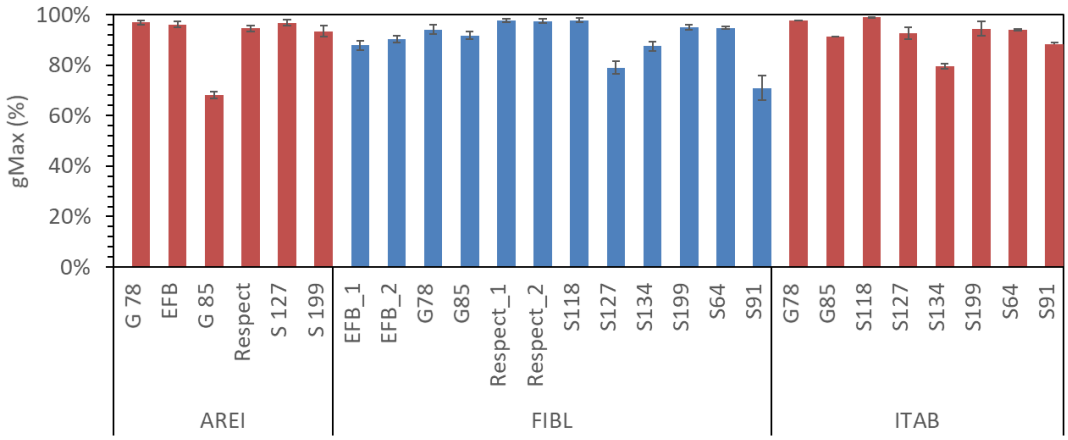

Figure 2 Maximum germination with the pea seed samples after ten days (g^{Max}). Each bar represents the average of tests with 90 seeds per seed lot. The number of seed lots per line and production location varied between one and eight. The error bar represents the SE.

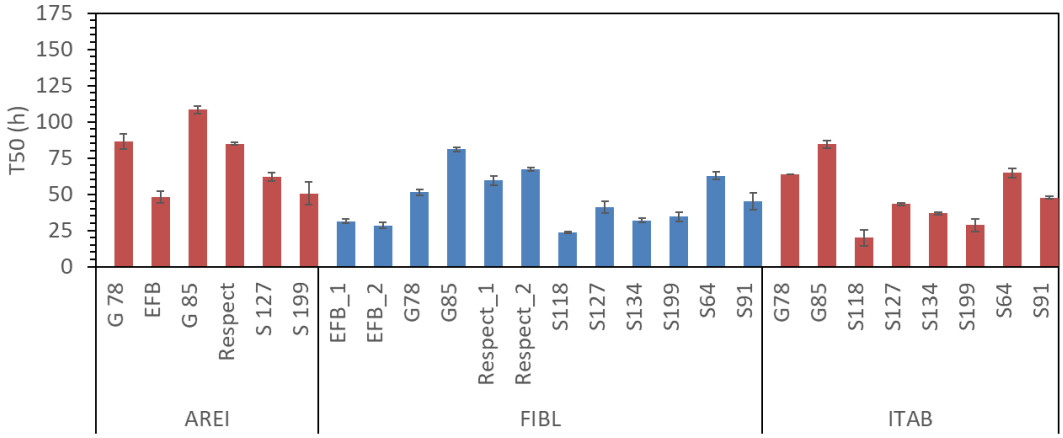

Figure 3. Speed of germination with the pea seed samples, indicated by T50, the time to reach 50% of the final germination. Each bar represents the average of tests with 90 seeds per seed lot. The number of seed lots per line and production location varied between one and eight. The error bar represents the SE.

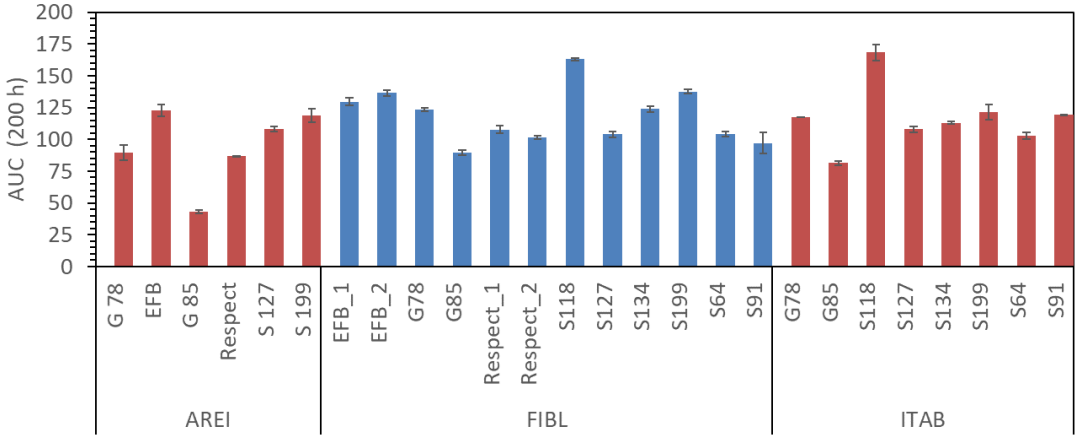

Figure 4. Performance of the pea seed lots indicated by the Area under the germination curve till 200 hours. Each bar represents the average of tests with 90 seeds per seed lot. The number of seed lots per line and production location varied between one and eight. The error bar represents the SE.

In general, the germination performance of the seed lots is rather good, most reaching 88% or higher germination levels. The small error bars indicate a low variance between the replicate harvests. Also,

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between locations the differences are small, indicating that for the cultivars tested the location effect on germination is minimal. In general, the germination of the AREI seed lots underperform those of the other two locations, but this could be caused by the packaging in plastic bags which resulted in the maintenance of a relative high humidity during the 6 months of storage.

3. Second germination test, evaluation of seedling quality

The first germination test only evaluated germination parameters, counted as root protrusion from the seed. To evaluate seedling quality a second test was performed. This was done on a so-called Jacobsen table with 16 h light, where seeds are placed on a moist blotter, covered with a transparent cup and receive light from the top. With this test the surface for placing seeds is much smaller and it involves much more labour. First a pilot test was performed. Here seeds were germinated on a filter placed on a so-called Copenhagen-table. This system is meant to evaluate seedling quality. The two seed lots that performed extreme in the first germination test were used in this pilot. We compared the effect of 32 or 44 seeds per blotter and two blotter colours (Figure 5).

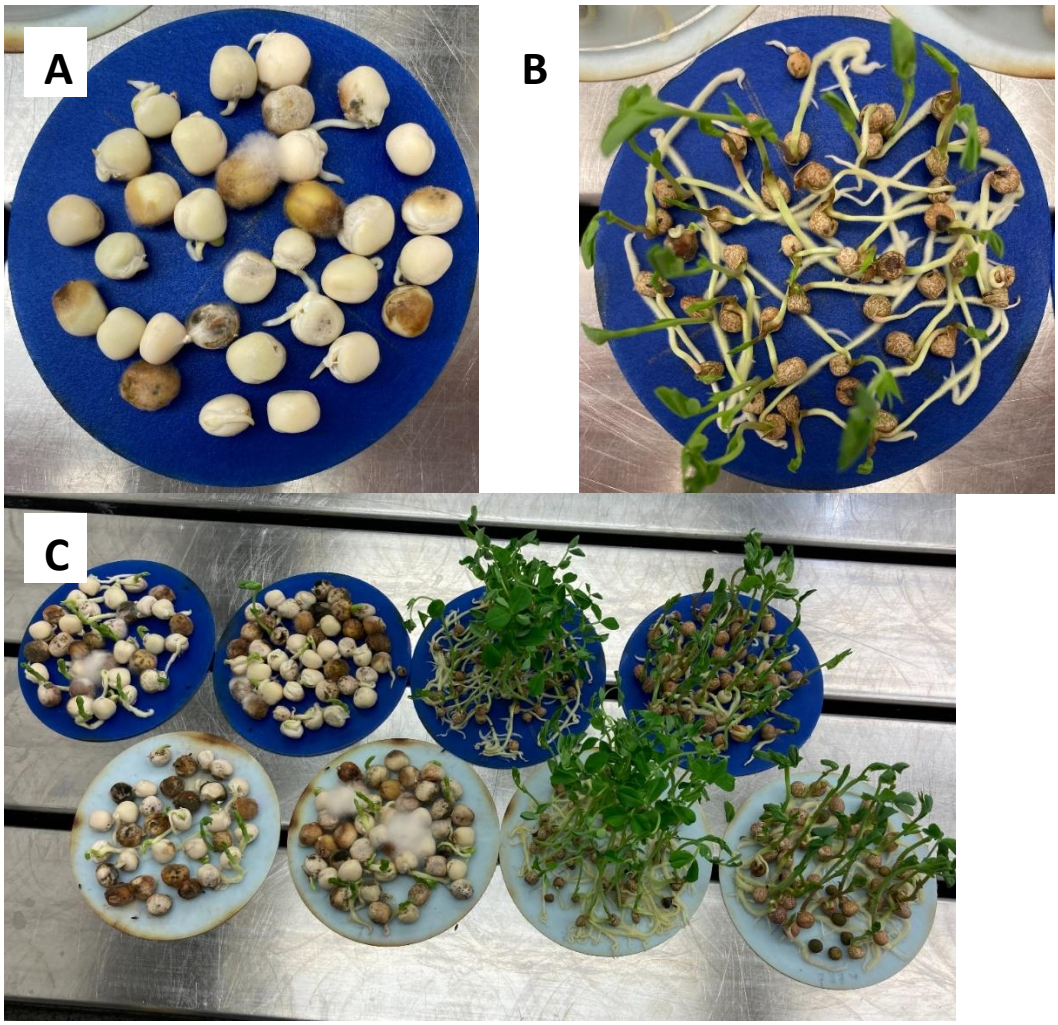

Figure 5. Pilot germination test on the Copenhagen-table to evaluate seedling quality, testing also two blotter colours. A: Picture after eight days of SG07783 (G85 AREI) with 44 seeds. B: Picture after eight days of SG07850 (S118 KWS) with 44 seeds. C: Picture made 11 days after start of the test. From left to right: SG07783 (G85 AREI) 32 seeds.SG07783 (G85 AREI), SG07850 (S118 KWS), SG07874 (S134, KWS).

This pilot clearly showed the effect of seed quality. Lot SG07783 (G85 AREI) performed clearly less compared to lot SG07850 (S118 ITAB). The performance is in agreement with the observations made

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in the first germination test in boxes, where G85 AREI germinated much slower compared to lot S118 ITAB. With seed lot SG07874 (S134, ITAB) a number of 44 seeds per blotter is too much to evaluate seedling quality, especially with lines producing large seeds. With 32 seeds per blotter, it is possible. Seedlings can be evaluated according to certain criteria, e.g., a normal seedlings on day 8, according to rules of the International Seed Testing Association. But it is often somewhat subjective method when the seed quality is rather good. In the Figure 5, SG07783 (G85 AREI) has no good seedlings, but for SG07874 (S134, ITAB) the seedlings have to be evaluated one by one, which involves much labour. For the eye this image displayed in Figure 5 is more illustrative compared to those in Figures 2-4, but the challenge is the amount of work involved. The first germination test already showed significant differences in seed lot quality.

Based on the pilot a seedling evaluation test was performed with all seed lots in a single sample of 28-32 seeds (Figure 6). At nine days after imbibition a large variation in speed of germination is observed and many seed lots could not be evaluated. Therefore, evaluation was delayed till 12 days after imbibition.

Figure 6. Overview of half of the pea samples germinated on the Jacobsen table to evaluate seedling quality. The picture was taken nine days after the start of imbibition.

Seedlings were divided into eight classes: (1) Normal seedlings with at least 2 cm shoot, (2) Small seedlings with a healthy root while shoot is not present or smaller than 2 cm, (3) Seedlings with fungal infection, (4) Seedlings with a brown root, (5) Root visible with brown colour but no shoot, (6) No shoot but root protruded and infected by fungi, (7) non germinated clean seeds and (8) non-germinated seeds with fungal growth. Examples of these seedlings are provided in Figures 7 and 8.

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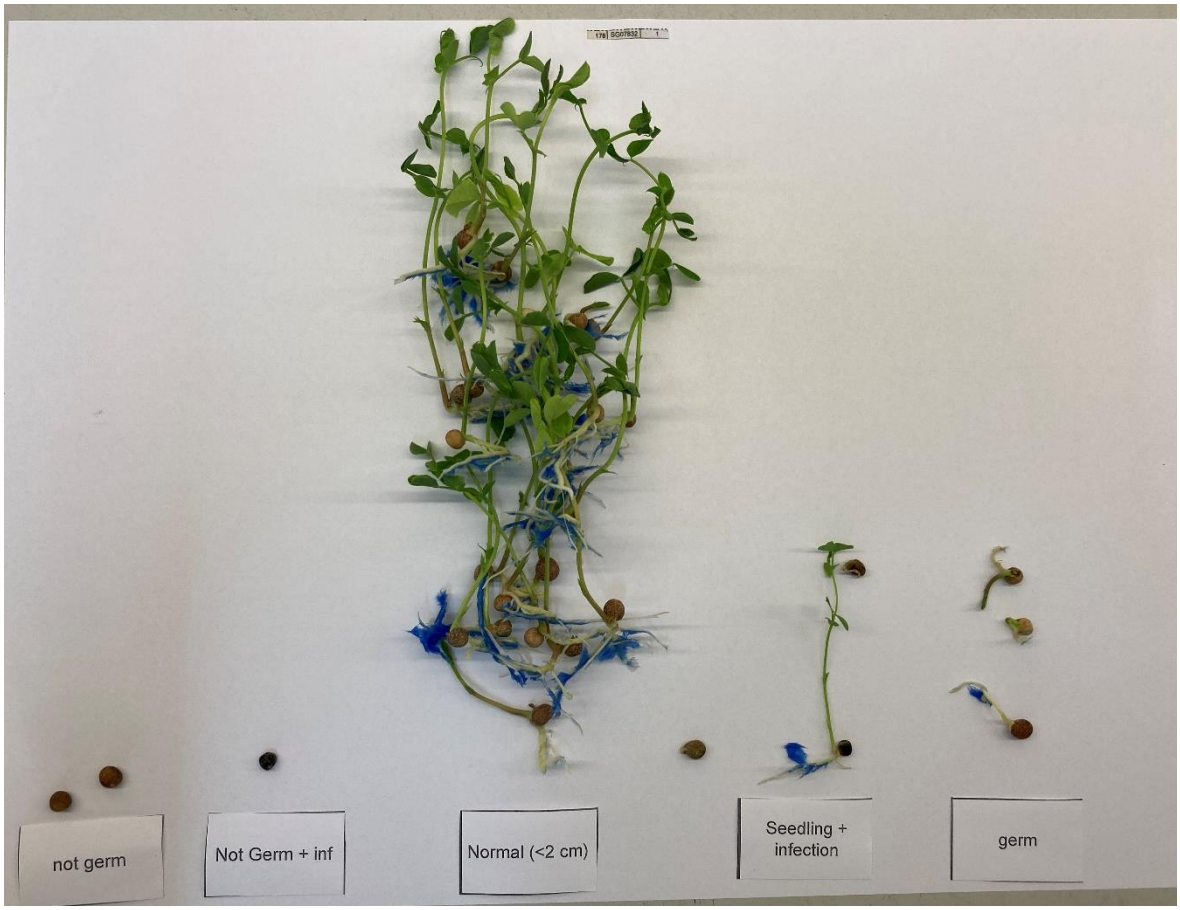

Figure 7. Seedling evaluation from sample, SG07832 = FiBL S134 rep 3. Note <2 cm should be read as >2 cm

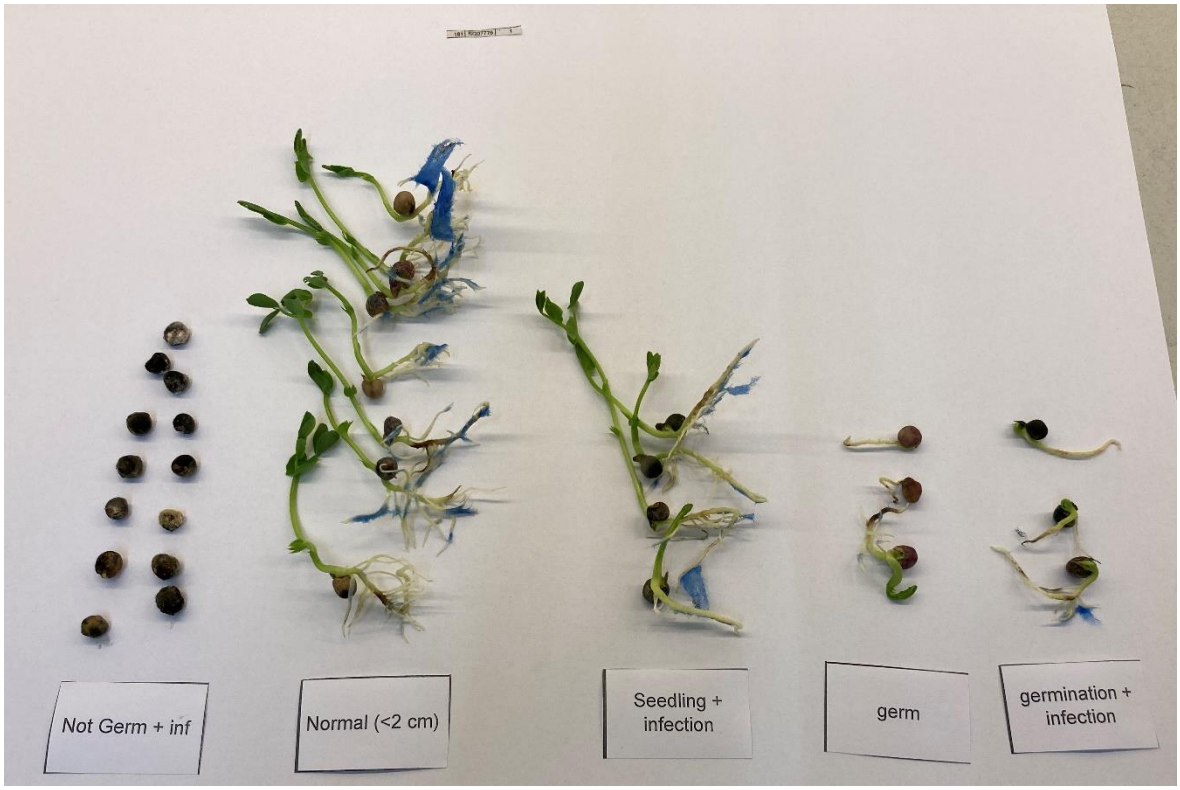

Figure 8. Seedling evaluation from sample, 181 SG0776 = FiBL S91 rep 1. Note <2 cm should be read as >2 cm

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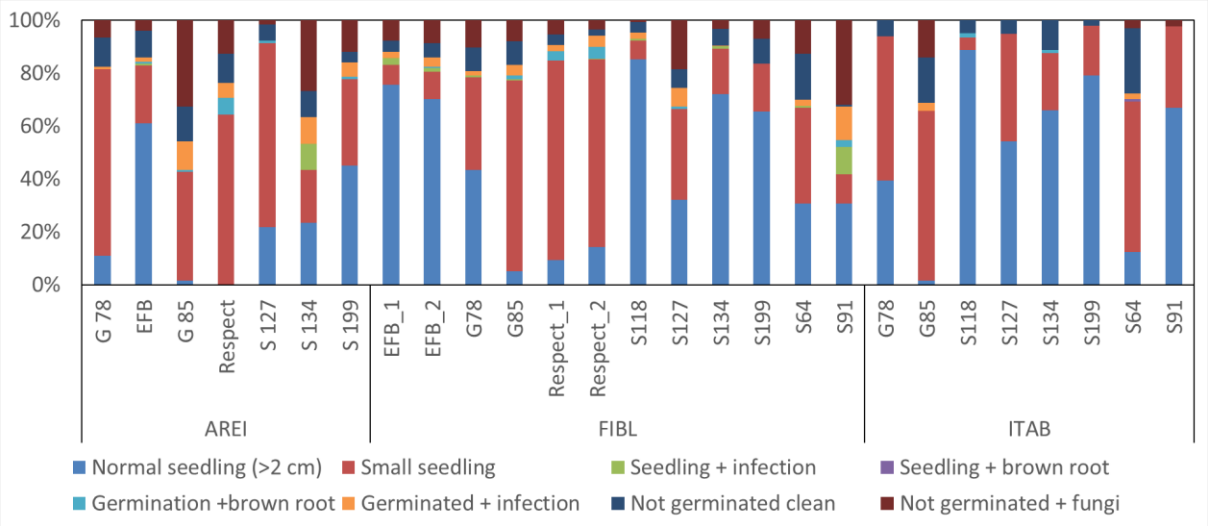

Figure 9. Evaluation of seedling quality for the different pea lines produced at three locations in Europe. Data are the average for one to eight biological replicates. The number of seeds per replicate was between 28 and 32.

Table 1. Frequency of Normal seedlings and Small seedlings obtained from the different lines produced at the three locations. Combined these give the total frequency of healthy seedlings. Data are the average for one to eight biological replicates. The number of seeds per replicate was between 28 and 32.

Normal seedlings (> 2cm)			
	AREI	FiBL	ITAB
EFB	61%		
EFB_1		76%	
EFB_2		70%	
G78	11%	43%	39%
G85	2%	5%	2%
Respect	0%		
Respect_1		9%	
Respect_2		14%	
S118		85%	89%
S127	22%	32%	54%
S134	23%	72%	66%
S199	45%	65%	79%
S64		31%	12%
S91		31%	67%
Average	23%	45%	51%

Small seedlings			
	AREI	FiBL	ITAB
EFB	22%		
EFB_1		8%	
EFB_2		10%	
G78	70%	35%	55%
G85	41%	72%	64%
Respect	64%		
Respect_1		75%	
Respect_2		71%	
S118		7%	5%
S127	70%	34%	41%
S134	20%	17%	22%
S199	33%	18%	19%
S64		36%	57%
S91		11%	31%
Average	46%	33%	37%

Total healthy seedlings			
	AREI	FiBL	ITAB
EFB	83%		
EFB_1		83%	
EFB_2		80%	
G78	82%	78%	94%
G85	43%	77%	66%
Respect	64%		
Respect_1		85%	
Respect_2		85%	
S118		92%	93%
S127	91%	66%	95%
S134	43%	89%	88%
S199	78%	83%	98%
S64		67%	69%
S91		42%	98%
Average	69%	77%	88%

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Due to the slow germination more than half of the samples had a low number of normal seedlings with at least 2 cm shoot on day eight, the standard day for seedling evaluation. The evaluation was therefore delayed till day twelve. Still a considerable number of samples had a low number of normal seedlings with a shoot of at least 2 cm (Figure 9, Table 1, Table 2). The slow germination can be considered a sign of seed vigour. In the field also these small seedlings will likely have resulted in establishment, but they will have more challenges in the competition with weed seeds. On average the frequency of normal seedlings larger than 2 cm was higher with the seed production at FiBL and ITAB compared to those produced by AREI. A reason can be that those AREI pea seeds were not dried at 30% RH, packaged in plastic. This resulted in six months storage at a relative higher moisture level, compared to the seeds produced at FiBL or ITAB. The frequency of seed lots produced at all three locations is too low to conclude on a potential line effect for the frequency of normal or small seedlings.

A few seed lots showed a high frequency fungal infection on the seedlings, protruded roots or non-germinated seeds (G85 and S134 from AREI and S92 from FiBL). It was not determined if this concerned pathogenic or saprophytic fungi. The latter group will grow easily on seeds that failed to germinate and died.

Table 2. Statistical analyses of the variation in seedling quality with pea seed samples produced at three locations. The replicates concerned replicate productions for those lines at the site of seed production, varying between one and eight. The column for total frequency of fungi contaminated infected) seedlings and seeds is colour formatted to visualise the relative effect of contamination.

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Seed producer	Line	Replicates	Average									Standard Error								
			Normal seedling (>2 cm)	Small seedling	Seedling + infection	Seedling + brown root	Germination +brown root	Germinated + infection	Not germinated clean	Not germinated + fungi	Infected (total)	Normal seedling (>2 cm)	Small seedling	Seedling + infection	Seedling + brown root	Germination +brown root	Germinated + infection	Not germinated clean	Not germinated + fungi	Infected (total)
AREI	EFB	4	61%	22%	1%	0%	1%	2%	10%	4%	7%	6%	4%	1%	0%	1%	1%	4%	1%	2%
	G 78	4	11%	70%	0%	0%	0%	1%	11%	6%	7%	4%	5%	0%	0%	0%	1%	3%	3%	3%
	G 85	4	2%	41%	0%	0%	1%	11%	13%	33%	44%	2%	2%	0%	0%	1%	2%	6%	7%	7%
	Respect	4	0%	64%	0%	0%	6%	6%	11%	13%	24%	0%	7%	0%	0%	2%	2%	6%	2%	3%
	S 127	4	22%	70%	0%	0%	1%	0%	6%	2%	2%	3%	3%	0%	0%	1%	0%	2%	1%	1%
	S 134	1	23%	20%	10%	0%	0%	10%	10%	27%	47%									
	S 199	4	45%	33%	0%	0%	1%	5%	4%	12%	18%	8%	7%	0%	0%	1%	4%	2%	6%	11%
FIBL	EFB_1	8	76%	8%	2%	0%	0%	2%	4%	8%	13%	2%	1%	1%	0%	0%	1%	1%	2%	2%
	EFB_2	8	70%	10%	2%	0%	0%	4%	5%	9%	14%	4%	2%	2%	0%	0%	1%	2%	2%	3%
	G78	4	43%	35%	1%	0%	0%	2%	9%	10%	13%	4%	2%	1%	0%	0%	2%	6%	2%	4%
	G85	8	5%	72%	0%	0%	2%	4%	9%	8%	14%	2%	4%	0%	0%	1%	1%	2%	1%	2%
	Respect_1	8	9%	75%	0%	0%	4%	2%	4%	5%	11%	3%	3%	0%	0%	1%	1%	1%	1%	2%
	Respect_2	8	14%	71%	0%	0%	4%	4%	2%	4%	13%	3%	4%	0%	0%	2%	1%	1%	1%	2%
	S118	4	85%	7%	1%	0%	0%	2%	4%	1%	4%	3%	4%	1%	0%	0%	1%	2%	1%	2%
	S127	4	32%	34%	0%	0%	1%	7%	7%	18%	26%	8%	7%	0%	0%	1%	2%	3%	3%	3%
	S134	8	72%	17%	1%	0%	0%	0%	6%	3%	4%	1%	3%	1%	0%	0%	0%	2%	1%	1%
	S199	4	65%	18%	0%	0%	0%	0%	9%	7%	7%	10%	6%	0%	0%	0%	0%	3%	3%	3%
	S64	4	31%	36%	1%	0%	0%	2%	17%	13%	16%	5%	4%	1%	0%	0%	1%	3%	1%	3%
	S91	4	31%	11%	10%	0%	2%	13%	1%	32%	57%	10%	5%	5%	0%	2%	5%	1%	7%	14%
ITAB	G78	1	39%	55%	0%	0%	0%	0%	6%	0%	0%									
	G85	2	2%	64%	0%	0%	0%	3%	17%	14%	17%	2%	2%	0%	0%	0%	3%	5%	2%	5%
	S118	2	89%	5%	0%	0%	2%	0%	5%	0%	2%	2%	1%	0%	0%	2%	0%	2%	0%	2%
	S127	3	54%	41%	0%	0%	0%	0%	5%	0%	0%	7%	6%	0%	0%	0%	0%	1%	0%	0%
	S134	3	66%	22%	0%	0%	1%	0%	11%	0%	1%	6%	5%	0%	0%	1%	0%	5%	0%	1%
	S199	3	79%	19%	0%	0%	0%	0%	2%	0%	0%	5%	4%	0%	0%	0%	0%	1%	0%	0%
	S64	3	12%	57%	0%	1%	0%	2%	25%	3%	6%	2%	8%	0%	1%	0%	2%	10%	0%	2%
	S91	3	67%	31%	0%	0%	0%	0%	0%	2%	2%	5%	7%	0%	0%	0%	0%	0%	2%	2%

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Annex 3. The importance of the holobiont as potential selection target to improve resilience and elucidate the concept of microbiome mediated breeding for disease resistance in pea (*Pisum sativum* L).

Authors: Pierre Hohmann, Lukas Wille, Monika Messmer (FiBL-CH)

Summary

With the advent of modern sequencing tools and bioinformatics methodologies it becomes more and more feasible to explore not only the plant genome but also the interaction of the plant with the associated microbiome community in the roots and rhizosphere. Breeding for the holobiont is based on the concept that the performance of a plant is not only determined by plant genes but also by the genes of the whole microbial community. The hypothesis is that plants that can attract a balanced microbial community in the soil will have a higher resilience against various stresses. FiBL (together with other T3.3.2 partners) explored the importance of the holobiont as potential selection target to improve resilience and elucidate the concept of microbiome mediated breeding for disease resistance in pea (*Pisum sativum* L). The main focus was to improve plant health by elucidating plant-microbe interaction using the case study of pea and root rot caused by a complex of soil-borne pathogens.

For current report (deliverable D3.7), we present the main results of the ongoing activities in pea conducted by FiBL-CH and breeder GZPK, AREI and UBIOS and new knowledge obtained in this cutting-edge research field. The review "Insights to plant-microbe interactions provide opportunities to improve resistance breeding against root diseases in grain legumes" published in Plant, Cell & Environment (Wille et al. 2019a) summarizes (a) the current knowledge of resistance against soil-borne pathogens in grain legumes, (b) evidence for genetic variation for rhizosphere- related traits, (c) the role of root exudation in microbe-mediated disease resistance and elaborates (d) how these traits can be incorporated in resistance breeding programs.

A high-throughput screening tool was developed that successfully differentiates susceptible and tolerant pea lines against a root pathogen complex in 3 weeks under controlled conditions. 216 gene bank accessions and breeding lines were screened with this tool based on naturally infested soil. In parallel, the next-generation sequencing (NGS) pipeline including complex biometric analysis of the fungal microbiome based on the ITS regions has been established and optimized. Highest differentiation of operational taxonomic units (OTUs) was found in the pea roots compared to the rhizosphere or bulk soil. The first analysis revealed quite a large number of potential pathogenic and beneficial taxa. The most contrasting pea lines with respect to root rot tolerance were selected and tested in the screening tool using different soil from fields in Germany with history of soil fatigue. After phenotypic data confirmed previous results, DNA was extracted from the root samples and sent for microbiome sequencing. Key microbial taxa with potential beneficial or pathogenic effect were quantified using qPCR methods and related to the different resistance parameters defined in the screening tool. *Aphanomyces euteiches*, *F. solani* and *F. oxysporum* are the most abundant pathogens across the three infected soils from Kirchlindach CH, Puch DE and Neu-Eichenberg, DE. Significant genotype effect as well as significant soil x genotype interaction on microbial community composition was detected. The selected pea lines were multiplied in several steps to conduct multi-year and multi-location field trials. Results of the phenotypic root rot assessment in the field trials in Switzerland, France and Latvia could verify the resistance level of the selected pea lines selected with the screening tool. These results indicate the usefulness of the screening tool based on naturally infested field involving the entire native soil microbiome as a key element for resistance breeding. A manuscript titled "Heritable variation in pea for resistance against a root rot complex and its characterization by

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amplicon sequencing" has been published in the journal Frontiers in Plant Science (Wille et al. 2020) and another manuscript titled "Untangling the pea root rot complex reveals microbial markers for plant health" is currently under review of the same journal.

The data on microbiome data of pea roots from field trials revealed that disease pressure and sampling time are main drivers of microbiome community diversity. OTU richness was higher in sick soil than in healthy soil and generally higher at early sampling time. Within the sites, sampling time had a big effect on both alpha and beta diversity. Different pea genotypes had only a minor effect on alpha and beta diversity in healthy soil. The results of alpha- and beta-diversity of the controlled condition and field trials indicates that diseased roots still harbour a complex fungal community with certain taxa being replaced. In the meantime, a larger set of roots of SNP-genotyped pea genotypes were sent for metabarcoding analyses in order to identify QTLs and candidate genes that are associated with the recruitment of beneficial microbiomes (LIVESEED follow-up). In addition, seeds harvested from field-characterized pea genotypes were harvested for seed vitality (see WP report) and seed microbiome analyses. Due the Covid, the seed microbiome analyses are delayed. Samples are currently being sequenced and analyses and reporting (in form of publication) will occur after the end of the LIVESEED project.

Finally, the concept of the holobiont as selection target was discussed with different actors during the international workshop on Implementing Plant-Microbe Interactions in Plant Breeding in December 2019 organized in collaboration with EUCARPIA and ECO-PB. A perspective article titled "miCROPe 2019 – Emerging research priorities towards microbe-assisted crop production" combines key messages from the symposium and the satellite workshop with the author's views and thoughts on emerging research priorities and challenges and has been published in the journal FEMS Microbiology Ecology.

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1. Introduction

In recent years, plant-associated microbes have received considerable attention in research for their ability to improve crop productivity and yield stability. Benefits include improved nutrient uptake and resistance against biotic and abiotic stresses. Influences of crop management, soil parameters and climatic effects are well documented. Knowledge on plant genetic determinants for beneficial interactions with individual microbes and entire communities is growing rapidly. Several reports indicate that not only the host species but also the host genotype play a significant role in driving microbial community composition and activity, selecting for and against particular members of the microbial community. However, to what extent genetic factors are responsible for shaping beneficial plant microbiomes is still poorly understood. Today, many plant scientists recognize plants as a holobiont formed by the plant and its associated microbes (Vandenkoornhuyse et al., 2015). With the holobiont concept at hand, fundamental functions such as nutrient acquisition and response to abiotic and biotic stresses are not a solely steered by the plant, but by its interaction with the associated microbiome (Tkacz & Poole, 2015, Coleman-Derr & Tringe, 2014).

FiBL (with other T3.3.2 partners) explored the importance of the holobiont as potential selection target to improve resilience and elucidate the concept of microbiome-mediated breeding for disease resistance in pea (*Pisum sativum* L.). The main focus of this task is to improve plant health by elucidating plant-microbe interactions using the case study of pea and root rot caused by a complex of soil-borne pathogens.

2. Activities

2.1. Microbiome-mediated disease resistance in pea

FiBL-CH conducted first a review on plant-microbe interactions to improve resistance breeding against root diseases in grain legumes (Wille et al. 2019a). Pea production is severely challenged by various soil-borne pathogens that cause severe root-rot diseases also known as soil fatigue or legume yield depression syndrome. Despite considerable progress in resistance breeding against individual pathogens, current pea varieties lack resistance against multiple interacting pathogens. The overall goal of this study is to elucidate the interaction of pea genotypes and associated soil microbiome community and their impact on plant health. In 2017 a screening tool was developed that allowed to test over 200 pea accessions for tolerance against soil fatigue based on field soil under controlled conditions. The soil was shown to be naturally infested with a complex of soil-borne pathogens causing root rot in pea and other legumes. The most contrasting pea genotypes were selected in order to validate the screening tool using two other soils with knows symptoms of soil fatigue. The same lines were multiplied in 2018 and 2019 as there were only few seed available from genebanks. After the first multiplication step FiBL-CH provided seed of 8 genotypes to LIVESEED partners in different geographic regions: UBIOS in France and AREI in Latvia in 2019 in order to validate the differentiation between susceptible and resistant pea lines in different pedo-climatic conditions (as different communities of pathogenic and beneficial microbes might be present across Europe). In addition, we want to explore the influence of the region of seed multiplication on pea health and pea root microbiome via seed microbiome (T2.3.3). Pea genotypes showing contrasting resistance levels are evaluated in infected and healthy soils and assessed for differences in the root microbial community in order to link disease resistance with plant genotype-dependent microbial composition (Figure 2). Resistance level of the selected genotypes are verified in multi-location field trials in 2019 and 2020 in CH (FiBL-CH, GZPK), FR (UBIOS), LV (AREI; Figure 1). Root samples are collected from each site in 2019, DNA extracted and sent for Next Generation Sequencing-based DNA analysis (NGS) to

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subcontractor in Canada. Analysis still pending due to COVID-19 lock down. Assessment of resistance level and sampling of pea roots for microbiome analysis of second year trials are ongoing (end of May – June 2020) in running replicated field trials in all three countries. As the harvest in 2019 was very low in all three countries, a further multiplication is conducted in 2020 to allow for future experiments.

Results have been presented at several conferences (Wille et al. 2019b, c) and a scientific publication on the development of the soil-based screening tool has been submitted in March 2020 (Wille et al. submitted). Another manuscript on composition of key microbial taxa of the pea root rot complex is determined by soil x genotype interactions in almost ready for submission. On 6th December 2020 FiBL-CH organized a LIVESEED – EUCARPIA – ECO-PB workshop "Implementing Plant-Microbe Interactions in Plant Breeding" attached to the International miCROPe conference in Austria to exchange information with other researchers and disseminate first results of LIVESEED. Main work will be the analysis of 2020 field data and microbiome analysis testing the influence of plant genotype, environment, and seed source (seed microbiome) on root microbiome and the correlation to plant health.

2.1.1. Link to other tasks

In addition to the root microbiome, the influence of the environment on the submission of the plant-associated microbial composition via seeds (seed microbiome) will be tested. Therefore, seeds of the same pea cultivars propagated in different countries will be tested for seed vitality by WR to link results with Task 2.3.3. on seed health strategies (Figure 1).

Seed of the same genotypes have already been tested for Alternative oxidase (AOX) activity proposed as functional marker for resilience (Task 3.3.1). Preliminary tests with inhibition of AOX enzymes by UEV and INRAe showed significant difference between the most tolerant and the most susceptible genotypes in relative germination rates in 2019 (Deliverable 3.2). These results will be validated and further explored in 2020 by UEV (Task 3.3.1, Figure 1).

Holistic breeding strategies for resilience will be developed integrating the host plant and its microbiome as contribution to Task 3.1. on developing novel breeding concepts and modern strategies coordinated by DBH.

Resistant pea genotypes can be used as components in mixtures and for further breeding for improved diversity (mixed cropping) by AREI linked to Task 3.2.1.

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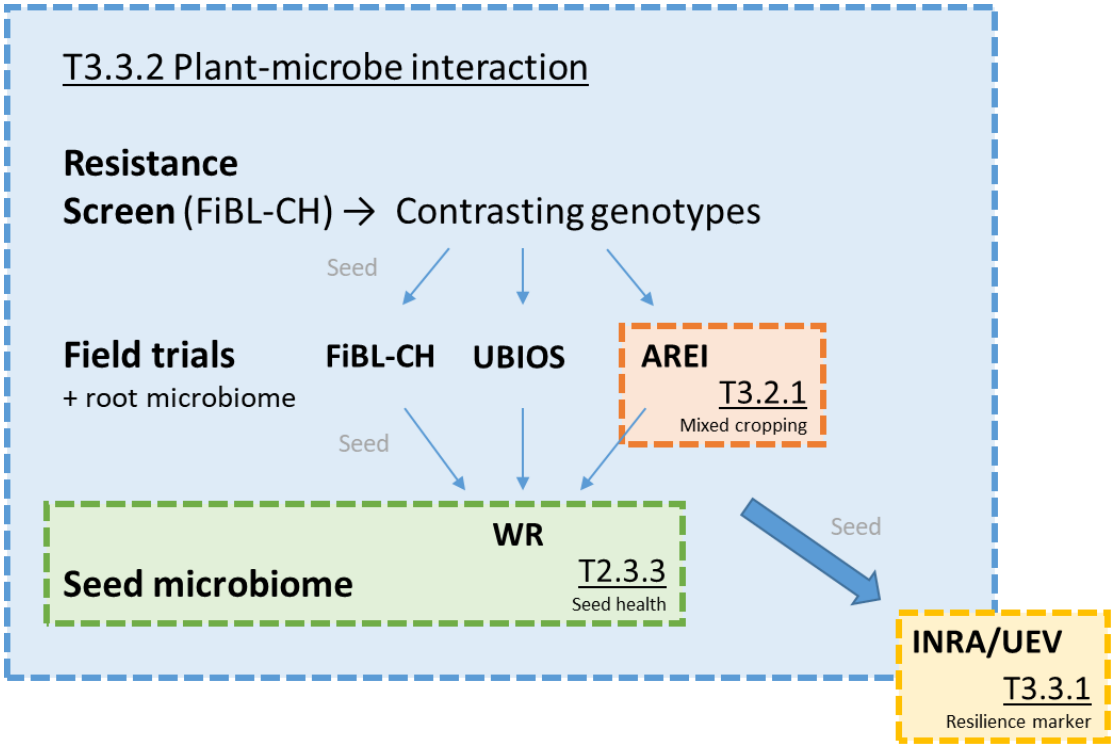

Figure 1. Schematic overview of the work on the pea root microbiome and its relation to other tasks.

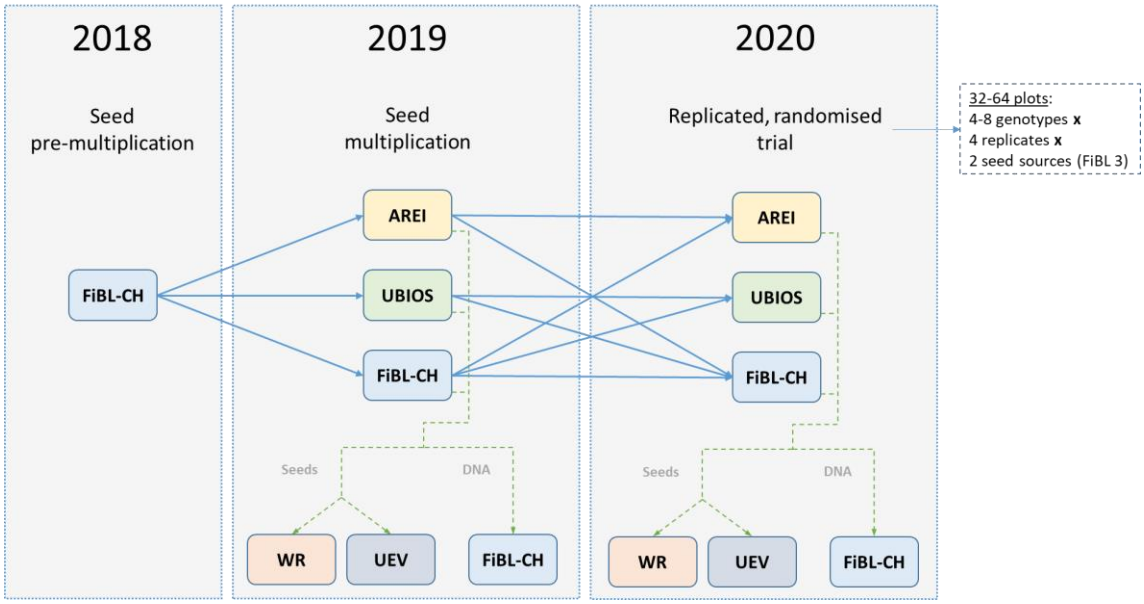

Figure 2. Schematic overview of the work on the pea root microbiome by year.

3. Main achievements

3.1. Review on Insights to plant–microbe interactions

Abstract: Root and foot diseases severely impede grain legume cultivation worldwide. Breeding lines with resistance against individual pathogens exist, but these resistances are often overcome by the interaction of multiple pathogens in field situations. Novel tools allow to decipher plant–microbiome

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interactions in unprecedented detail and provide insights into resistance mechanisms that consider both simultaneous attacks of various pathogens and the interplay with beneficial microbes. Although it has become clear that plant-associated microbes play a key role in plant health, a systematic picture of how and to what extent plants can shape their own detrimental or beneficial microbiome remains to be drawn. There is increasing evidence for the existence of genetic variation in the regulation of plant–microbe interactions that can be exploited by plant breeders. We propose to consider the entire plant holobiont in resistance breeding strategies in order to unravel hidden parts of complex defence mechanisms. This review summarizes (a) the current knowledge of resistance against soil-borne pathogens in grain legumes, (b) evidence for genetic variation for rhizosphere- related traits, (c) the role of root exudation in microbe-mediated disease resistance and elaborates (d) how these traits can be incorporated in resistance breeding programmes (see full review Wille et al. 2019a).

3.2. Development of a high-throughput screening tool for pea

A high-throughput screening tool was developed that successfully differentiates susceptible and tolerant pea lines against a root pathogen complex applying various resistance parameters. The tool was used for the screening of 261 pea accessions from the USDA genebank and Swiss breeding material under controlled conditions and evaluated after 3 weeks.

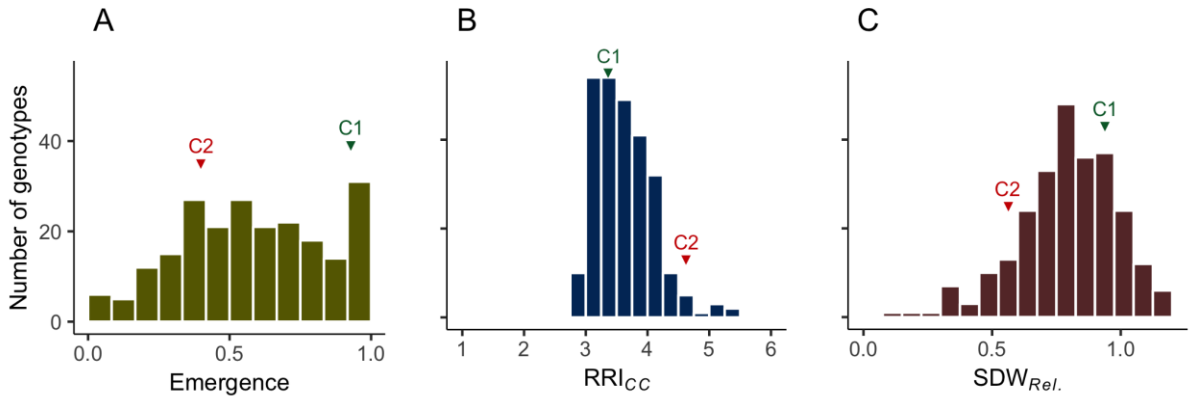

Figure 3 Frequency distributions of the estimated means of (A) plant emergence rate (Emergence), (B) root rot index (RRI$_{CC}$; levels 1-6) and (C) relative shoot dry weight (SDW$_{Rel.}$) assessed on 261 pea lines after 14 days (Emergence) or 21 days (RRI$_{CC}$ and SDW$_{Rel.}$) under controlled conditions on infested soil. The means of reference cultivars C1 ('EFB.33'; tolerant) and C2 ('Respect'; susceptible) are indicated in green and red, respectively.

Figure 4 shows the distribution of the pea accessions for emergence (0.0 no germination; 1.0 all seed germinated), root rot index (RRI$_{CC}$=1 no symptoms on the pea root; RRI$_{CC}$=6 severe symptoms of root rot), and relative shoot dry weight (SDW$_{Rel}$ = ratio between shoot biomass of pea in infected soil and shoot dry mass in sterilized soil; e.g., SDW$_{Rel}$ 0.5 = 50% reduced biomass in infected soil, SDW$_{Rel}$ 1.0 = no difference = tolerant plant). C1 indicates the value of the resistant and C2 of the susceptible checks. Along significant genotypic differences, moderate to high heritabilities could be revealed for root rot resistance and growth performance traits. Relating different resistance traits allowed to distinguish between highly susceptible, tolerant, and resistant lines and between resistance at early plant development stage. These results indicate the replicability and usefullness of a naturally infested field soil-based screening tool that involves the entire native soil microbiome as a key element for resistance breeding. A manuscript titled “Heritable variation in pea for resistance against a root rot complex and its characterisation by amplicon sequencing” is currently under review at the journal Frontiers in Plant Science (Wille et al. 2020 submitted).

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3.3. Validation of screening tool for pea using soil of different origin

A follow up experiment was conducted to test if the results of the screening tool if soil from different locations with no symptoms of soil fatigue were used in the screening tool under controlled conditions. Most contrasting pea lines were tested in three different soil with high natural infestation (original soil from Kirchlindach CH, Puch DE, Neu-Eichenberg DE) and one soil with no indication of soil fatigue (Feldbach, CH). The separation of resistant and susceptible lines with respect to relative shoot dry weight (SDW_{Rel}) could be confirmed for the three sick soil as shown in Figure 5. Results of the healthy soil from Feldbach showed no reduction of shoot biomass in native soil compared to X-ray irradiated soil, therefore no differentiation between the genotypes were observed.

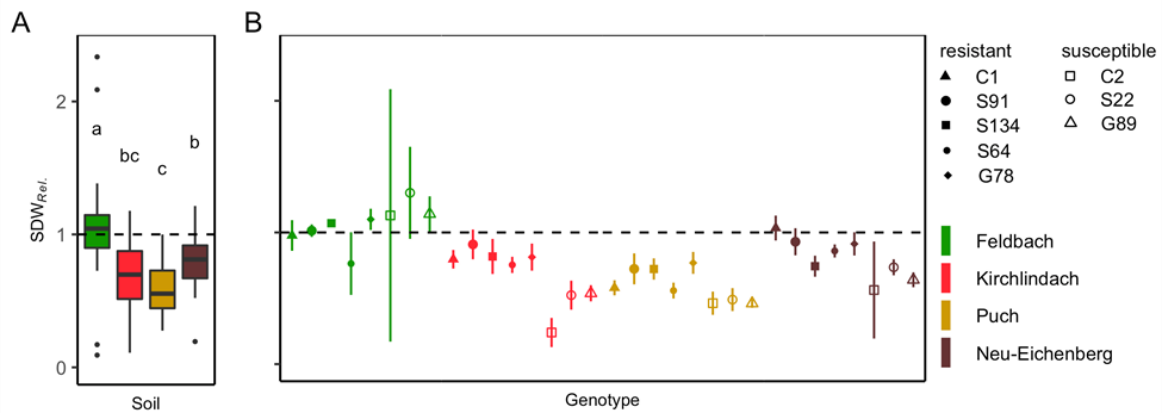

Figure 5 Relative shoot dry weight ($SDW_{Rel.}$) of eight pea lines grown for 29 days under controlled conditions on four soils (Feldbach = healthy control). A) Boxplots for each soil over all genotypes and replicates (n = 32). Soil means followed by a common letter are not significantly different (p<.05, Tukey HSD). B) Mean $SDW_{Rel.}$ for eight pea lines (symbols) in each soil (colour): Solid symbols represent pea lines categorised as resistant; open symbols represent susceptible pea lines. Bars represent the standard error of the means.

3.4. Field validation of the controlled conditions screening tool for pea

In order to validate the relevance of high-throughput screening tool conducted under controlled conditions with on-farm conditions multi-year and multi-location field trials were conducted with a selection of most contrasting pea lines. They were multiplied and assessed for symptoms of root rot disease in field trials with different disease pressure in Switzerland (FiBL), Latvia (AREI) and France (UBIOS). In Switzerland, the evaluation was conducted on field sites with moderate (EG) and high root-rot potential (EK). It appears that correlation between the root rot index of screening tool under controlled conditions (RRI_{CC}) and field root rot indices (RRI_{Field}) improved with increasing disease pressure in the field (Figure 6). Thus, highest rank correlation (rs) was found in Switzerland on the soil with known soil fatigue (EK2018/EK2020, Kirchlindach, rs=0.73/0.76, p=0.03/<0.001), and lowest in France (UBIOS, rs=0.36, p=0.39). This is an important prove of concept that the developed resistance screening tool against complex pathogens under controlled conditions is relevant for actual plant health in the field under different pedo-climatic conditions.

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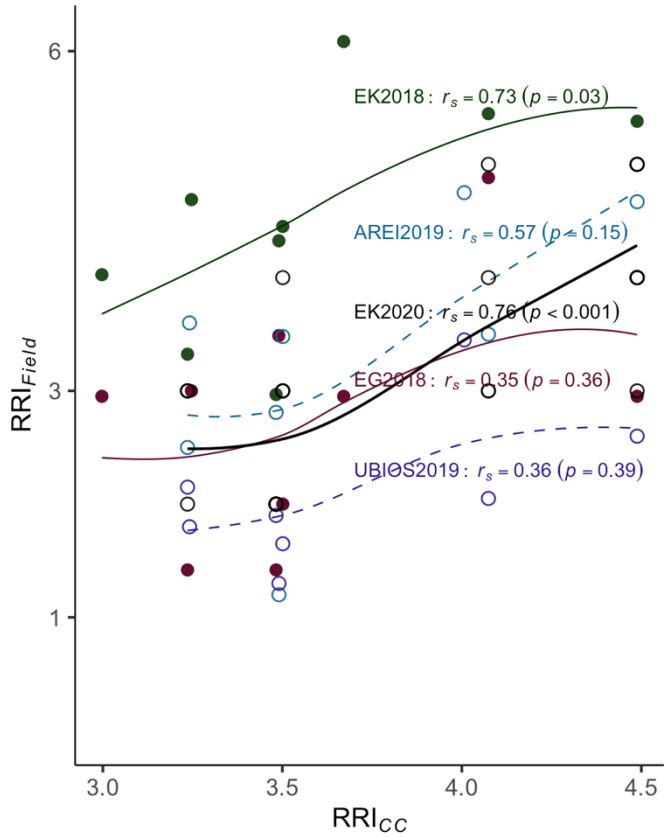

Figure 6. Correlation between root rot indices assessed under controlled conditions on infested soil 21 days after sowing (RRI_{CC}) and root rot assessed in on-farm experiments (RRI_{Field}). The pea lines with contrasting resistance phenotypes were evaluated on field sites with high (EK FiBL-CH), moderate (AREI, EG FiBL-CH) and low (UBIOS) pea root rot complex over three years (2018, 2019, 2020). Rank correlation coefficients (Spearman's rho), associated p-values and locally estimated scatterplot smoothing (LOESS) curves are shown for each site.

3.5. Characterisation of the microbial root community of diseased pea

A next-generation sequencing (NGS) pipeline for microbiome analysis has been established optimising the choice of DNA extraction protocol, primer pair, plant compartment and data processing and analysis procedures. First sequencing of the ITS1 region from total DNA extracted from bulk soil, rhizosphere soil and diseased pea roots of the screening tool under controlled conditions revealed a total of 1,190,412 high-quality sequences with a median of 55,670 sequences per sample. The microbiome sequence data were processed according Bodenhausen et al. (2019) and quality filtered sequences were clustered into operational taxonomic units (UPARSE – OTUs) with ≥ 97% sequence similarity. OTUs were assigned to taxonomic groups based on existing database for the fungal community. Taxonomic information of unassigned sequences (below family rank) were further explored using BLAST analysis of the Nucleotide collection database. BLAST taxonomic information was considered at query cover >92% and sequence identity of 100%. The most abundant OTUs and their taxonomic assignments are listed in Table 1. In total diseased pea roots display a diverse fungal community that includes known pea pathogens such Fusarium oxysporum, F. solani, Didymella sp. and Rhizoctonia solani and also antagonists such as Clonostachys rosea and several mycorrhizal species (Table 1). The bulk and rhizosphere soil did not reveal this degree of pathogen co-occurrence among the most abundant taxa therefore we concentrate for further analysis on the root microbiome. A manuscript titled "*Heritable variation in pea for resistance against a root rot complex and its*

characterisation by amplicon sequencing" is currently under review at the journal *Frontiers in Plant Science (Wille et al. 2020 submitted)*.

Table 1. Taxonomic information and mean relative abundance of the 20 most abundant Operational Taxonomic Units (OTUs) and further selected OTUs in bulk soil (n = 4), rhizosphere soil (n = 8), and root (n = 8) of 21 day-old pea grown in infested soil under controlled conditions. Bold values highlight significantly enriched OTUs compared with bulk soil (FDR-adjusted p<0.05).

Taxon	OTU	Relative abundance [‰]		
	ID	bulk soil	rhizosphere	root
Top 20 taxa in the root				
Ilyonectria	OTU4	1.79	**9.06**	**241.62**
Fusarium oxysporum	OTU10	8.97	14.22	**95.25**
Polyphilus	OTU3	0.10	**0.66**	**60.60**
Olpidium brassicae	OTU5	0.32	2.19	**48.62**
Clonostachys rosea	OTU11	0.73	**7.07**	**33.46**
Fusarium solani	OTU396	1.23	2.89	**27.69**
Fungi	OTU31	0.00	**1.29**	**9.81**
Fusarium solani	OTU34	0.72	1.55	**8.39**
Tetracladium	OTU19	8.91	4.45	7.21
Cylindrocarpon	OTU25	2.74	4.25	6.84
Microdochium bolleyi	OTU38	0.80	0.22	**6.46**
Rhizoctonia solani	OTU111	0.00	**0.81**	**5.87**
Sordariomycetes	OTU29	1.87	0.32	5.81
Dendryphion nanum	OTU46	0.98	1.11	**5.24**
Didymella	OTU7	1.09	2.16	4.95
Stephanosporaceae	OTU13	0.01	0.52	**3.96**
Fusarium	OTU20	2.03	1.81	3.54
Plectosphaerella	OTU114	1.10	0.37	3.37
Exophiala equina	OUT1671	8.55	6.18	3.19
Orbiliaceae	OTU100	0.01	**2.21**	**3.13**
		Further known pea pathogens		
Fusarium solani	OTU185	0.02	0.30	0.68
Fusarium solani	OTU122	0.07	0.01	0.67
Fusarium	OTU80	1.52	2.21	0.36
Didymellaceae	OTU33	11.01	5.22	0.08
		Further putative beneficials		
Corprinellus	OTU28	0.00	**0.97**	**2.38**
Arthrobotrys oligospora	OTU37	0.15	3.15	1.29
Funneliformis mosseae	OTU355	0.18	0.13	0.98
Entrophspora	OTU489	0.20	0.01	0.55
Funneliformis mosseae	OTU470	0.23	0.01	0.52
Funneliformis mosseae	OTU408	0.17	0.11	0.19
Diversispora	OTU780	0.05	0.05	0.15
Diversispora	OTU1390	0.11	0.08	0.11
Arthrobotrys musiformis	OTU222	0.01	0.65	0.09

[a] red = putative pea pathogen, orange = putative plantathogen, green = putative plant beneficial

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In the first field trials in 2018 of pea genotypes with contrasting resistance we wanted to explore how the microbiome community of pea roots is affected by soil, plant genotype and sampling time by comparing alpha (local, within treatment) and beta (the differentiation between environment or treatments) diversity and identify fungal taxa that respond to these effects. This shall serve as basis to relate microbial community to plant health. Alpha diversity can be characterized by the number of different OTUs (richness), relative OTU abundance (Shannon diversity), and equal abundancy of OTUs (Pielou's evenness).

Preliminary results revealed that field site and sampling time are main drivers of microbiome community diversity. As expected, the field site (heavy infested sick soil vs. healthy soil) had a big effect on the OTU diversity (Figure 7, Table 2). OTU richness was higher in sick soil than in healthy soil and generally higher at early sampling time (Figure 7). Within the sites, sampling time had a big effect on both alpha and beta diversity. The alpha diversity seems more pronounced when plants are grown in the sick soil compared to healthy soil. Different genotypes had only a minor effect on alpha and beta diversity in healthy soil, but not in heavily infested sick soil (Table 2).

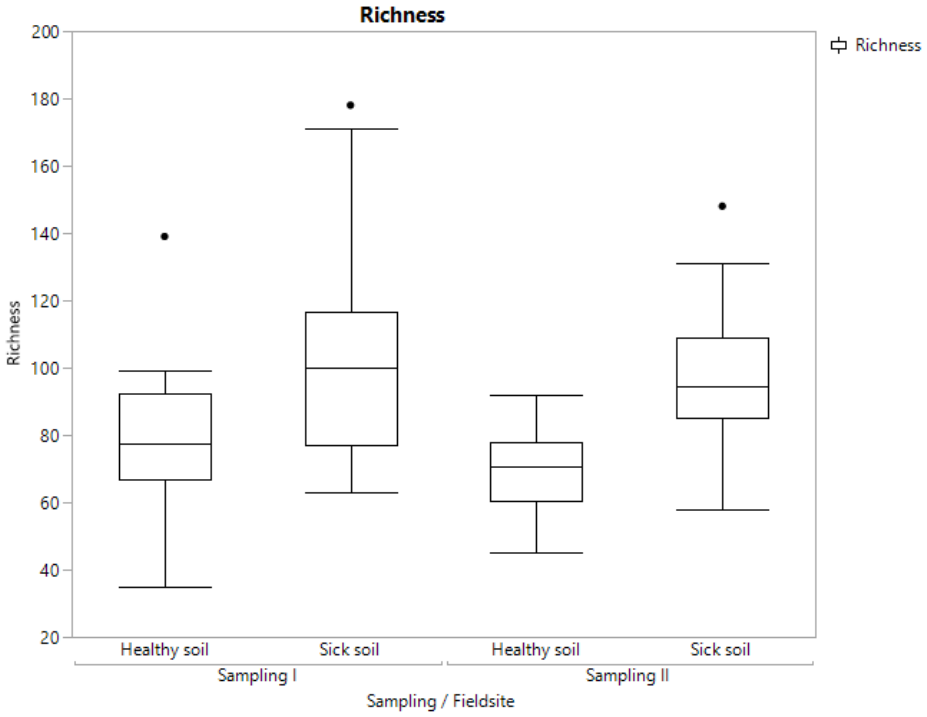

Figure 7. OTU richness of fungal community of pea roots sampled in field trials in 2018 on soils with different natural infestation of soil fatigue (Healthy vs. Sick soil) sampled at two different times (Sampling I vs. Sampling 2)

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Table 2. P-values of (PERM)ANOVA analyses of fungal root beta- and alpha-diversity (richness, evenness and Shannon diversity) of operational taxonomic units for sampling time, pea genotype and their interaction for pea lines grown in either a heavily infested (sick) or a low infestation (healthy) soil.

	Beta diversity		Alpha diversity					
	Soil		Richness		Evenness		Shannon diversity	
	Sick	Healthy	Sick	Healthy	Sick	Healthy	Sick	Healthy
Sampling time	**0.0001**	**0.0001**	0.45	0.32	**<0.0001**	**0.011**	**<0.0001**	**0.04**
Genotype	0.36	**0.045**	0.87	0.27	0.57	0.12	0.42	**0.053**
Time x Genotype	0.47	0.36	0.59	0.80	0.65	**0.067**	0.61	0.11
p-values that show a significant effect (p<0.05) or are tending towards significance (0.05<p<0.1) are highlighted in bold								

The follow-up experiment assessed the microbiome structure of 8 pea genotypes (resistant: S91, S134, G78, C1, S64; susceptible: G89, S22, C2) grown in four different agricultural soils under controlled conditions (with the 'Feldbach' soil being the only healthy one). The fungal microbiome showed a significant effect of the factor soil and pea genotype on the root fungal diversity and composition (Figures 8-10). While alpha diversity was not affected by genotype resistance level, the community composition (beta-diversity) significantly differed between susceptible and resistant genotypes (Figure 10).

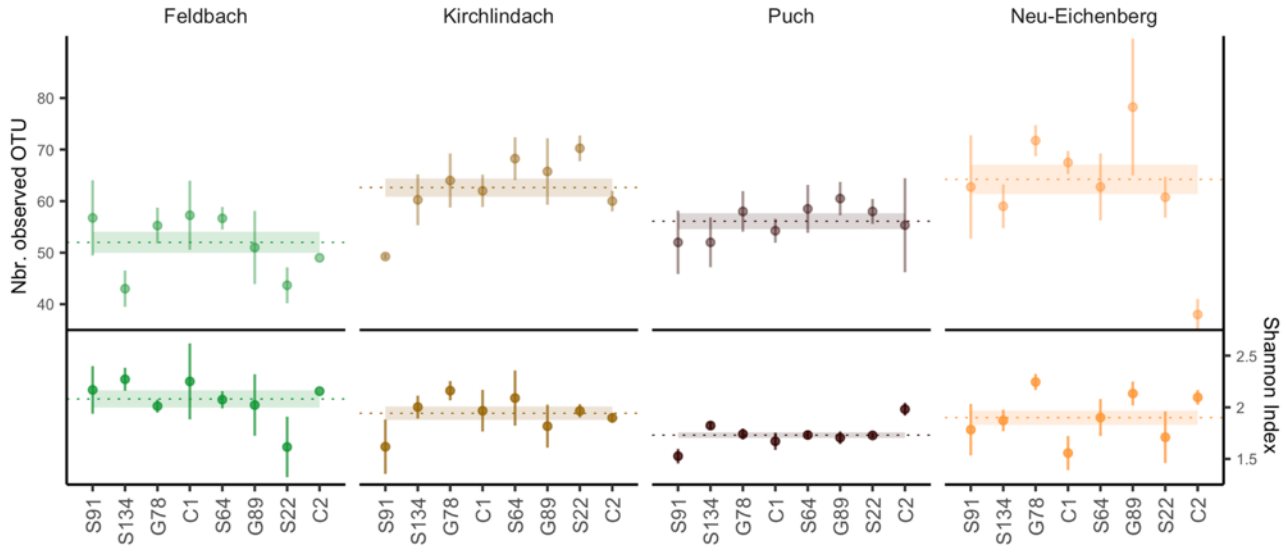

Figure 8. Fungal alpha diversity in diseased roots of eight pea genotypes grown in four soils with different disease pressure. The upper part of the figure shows the mean value over four replicates and standard errors of the number of sequenced fungal taxa (OTU), the lower part the Shannon diversity. The dashed line and the coloured bar show the mean value and standard error over all samples of a soil.

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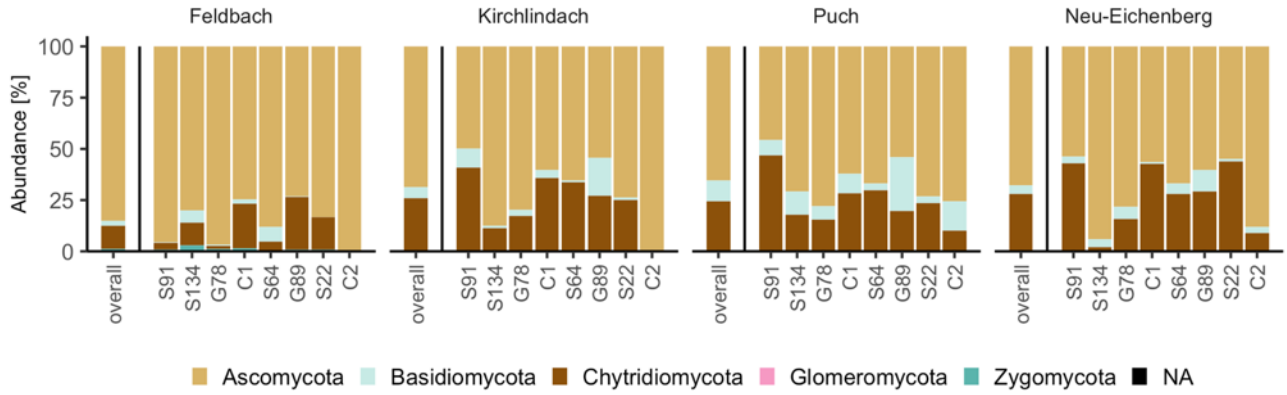

Figure 9. Relative abundances of the fungal taxa sequenced in pea roots at the phylum level. The mean values over four replicates for each pea genotype and for each soil (overall) are shown.

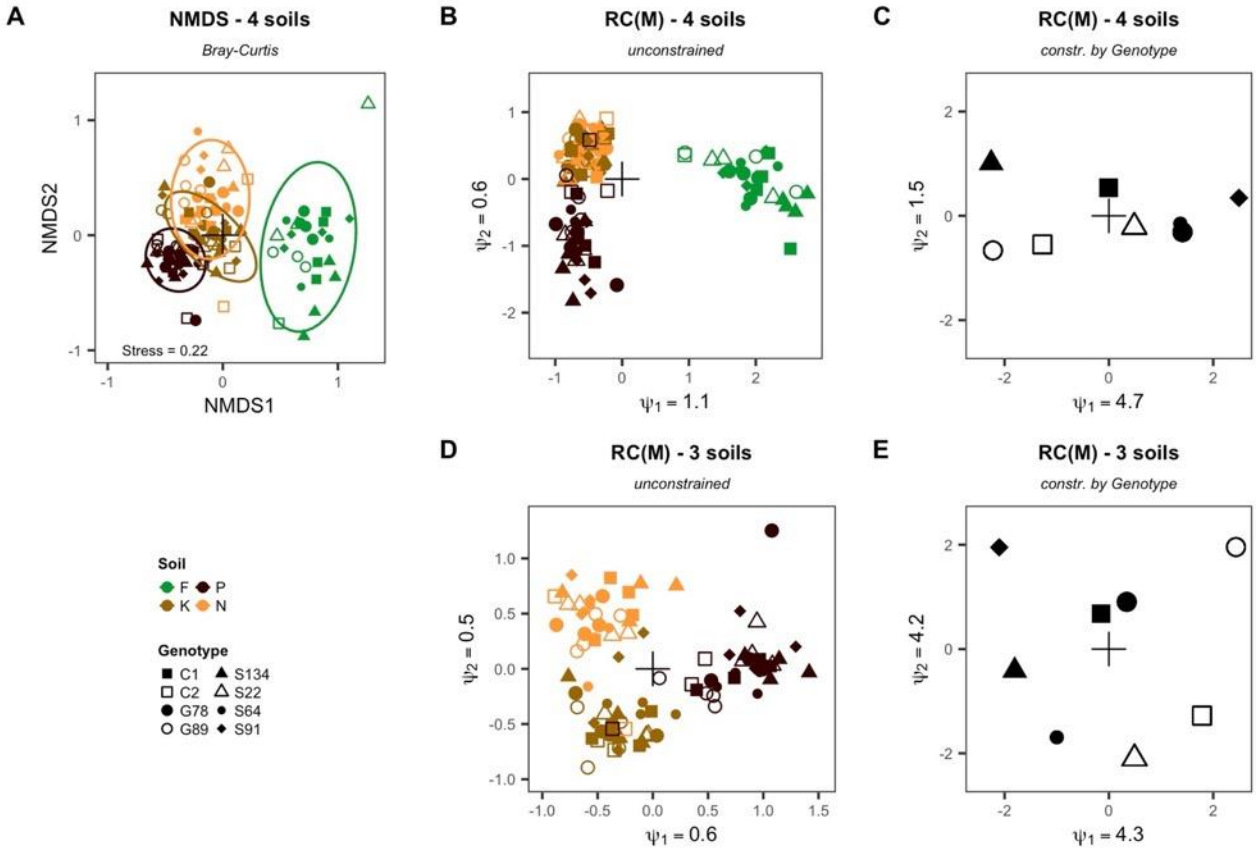

Figure 10. Effect of soil and pea genotype on the root fungal community in diseased pea roots. (A) Non-metric multidimensional scaling based on the Bray-Curtis distances between samples. B) - E) Row-Column-Interaction-Model based ordinations on all four soils (B and C) or only the three soils with increased disease pressure (D and E). Panels C and E show the models contrained by genotype.

Further, the root microbiome structure of adult pea plant at time of flowering was investigated in a multi-site field experiment over two years. Altough year, location and pea genotype showed an effect

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on taxa richness and Shannon diversity, no clear link was found between alpha diversity and pea resistance level (Figure 11, 12).

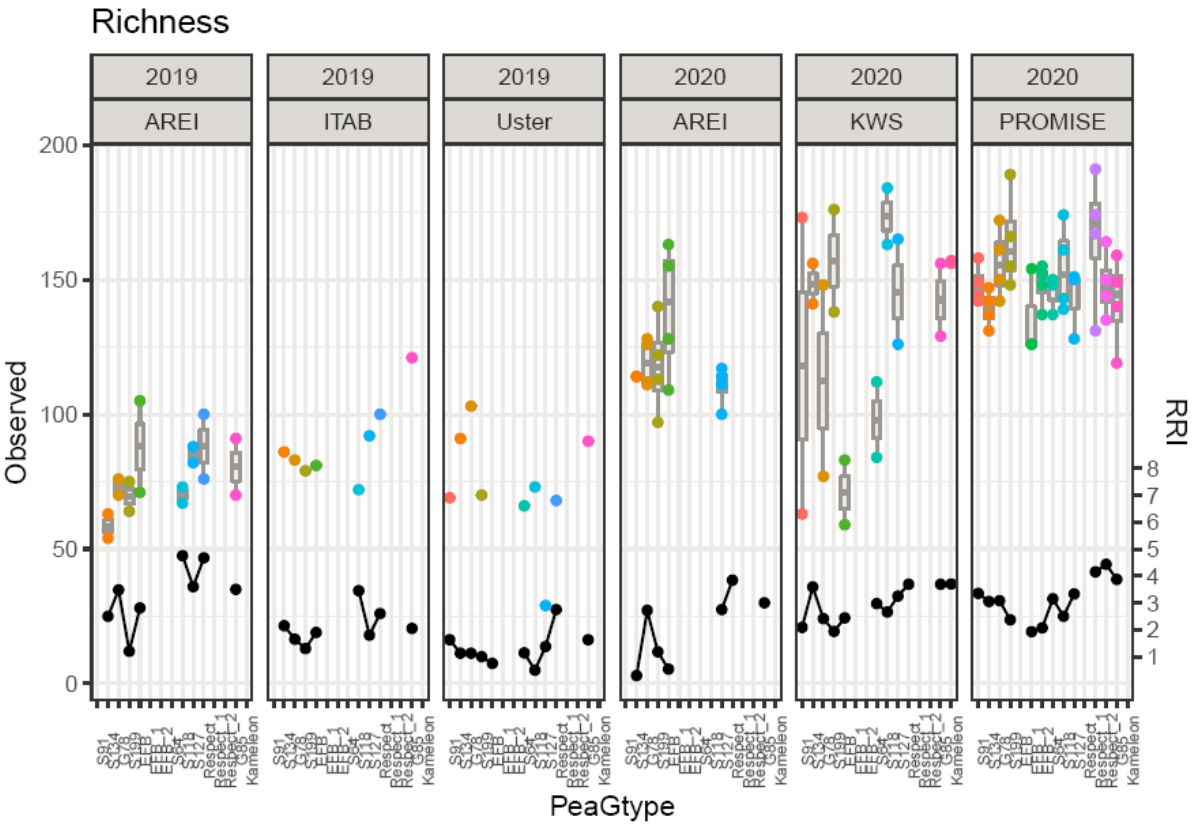

Figure 11. Fungal taxa richness in roots of different pea genotypes (PeaGtype) grown in different field trials in 2019 and 2020. Genotypes are ordered based on their tolerance identified in a previous screening assay. The coloured dots refer to richness, the black dots refer to the root rot index (RRI) assessed in the field.

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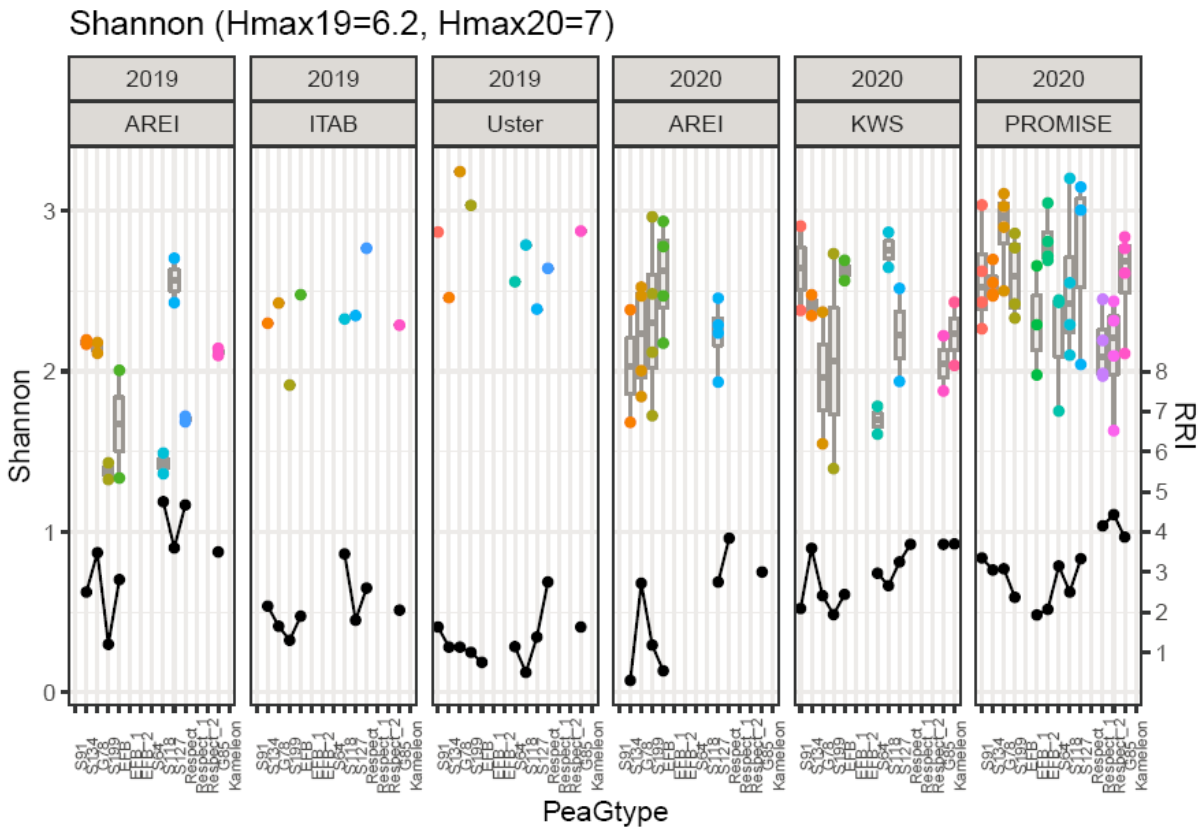

Figure 12. Fungal Shannon diversity in roots of different pea genotypes (PeaGtype) grown in different field trials in 2019 and 2020. Genotypes are ordered based on their tolerance identified in a previous screening assay. The coloured dots refer to richness, the black dots refer to the root rot index (RRI) assessed in the field.

With regard to beta diversity, pea genotype show an overall significant effect on community composition ($p<0.001$) grouping resistant and susceptible genotypes as shown representatively for the two environments Promise 2020 (*Figure 13A*) and AREI 2020 (*Figure 13B*).

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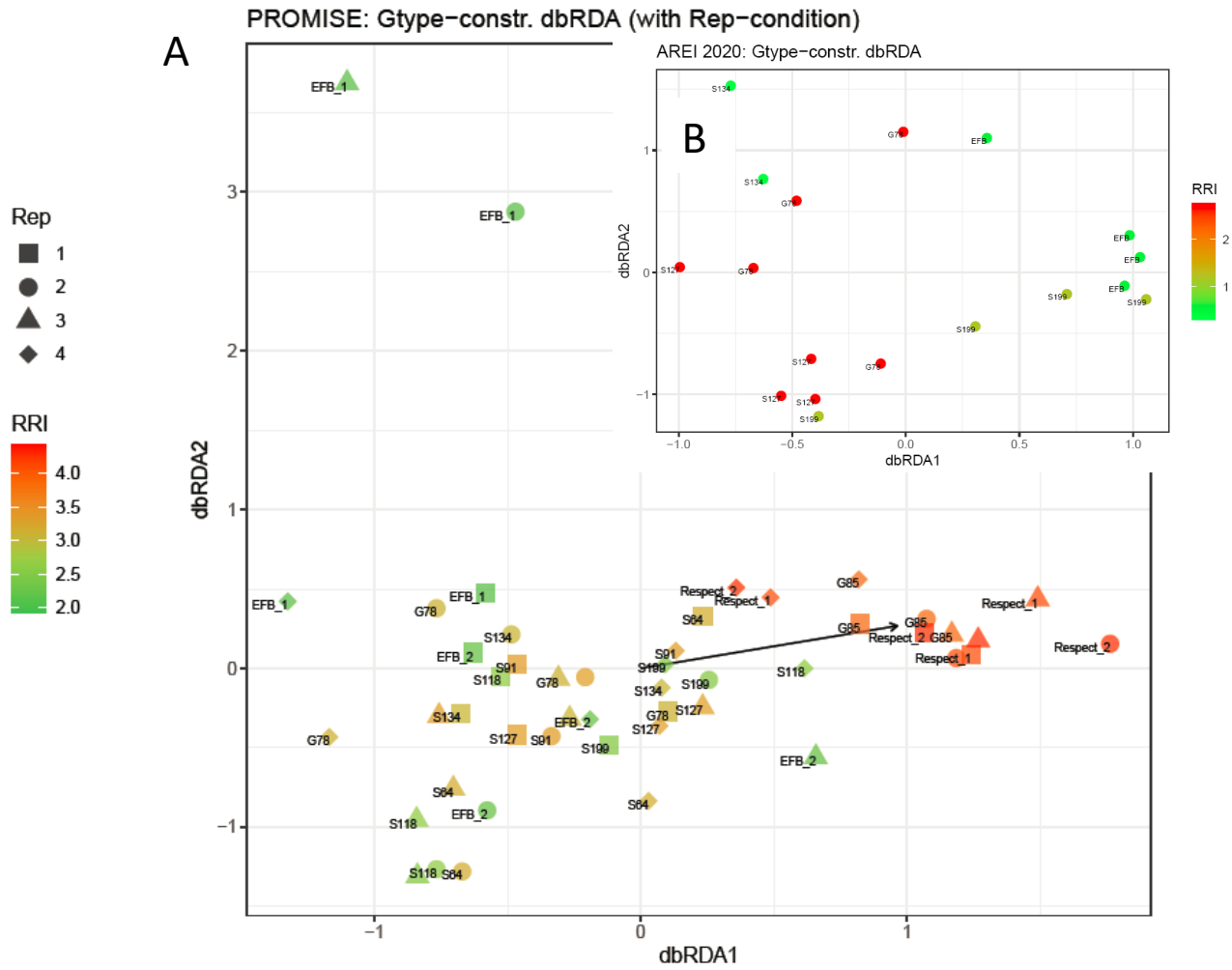

Figure 13 Distance-based redundancy analysis of root fungal community composition of different pea genotypes grown in environment Promise 2020 (A) and AREI 2020 (B). Colour coding indicates the root rot index (RRI) assessed in the field.

The results of alpha- and beta-diversity of the controlled condition and field trials indicates that diseased roots still harbour a complex fungal community with certain taxa being replaced. In Chapter 3.6, we will use quantitative real-time PCR to have a closer look at potential pea pathogens to provide further insights into these replacements.

In the meantime, a larger set of roots of SNP-genotyped pea genotypes were sent for metabarcoding analyses in order to identify QTLs and candidate genes that are associated with the recruitment of beneficial microbiomes. In addition, seeds harvested from field-charaterised pea genotypes were harvested for seed vitality (see WR report) and seed microbiome analyses. Due the Covid, the seed microbiome analyses are delayed. Samples are currently being sequenced and analyses and reporting (in form of publication) will occur after the end of the LIVESEED project.

3.6. Key microbial taxa in diseased pea roots of screening tool

A quantitative real-time PCR-based methodology has been developed to target specific pathogenic and beneficial microbial species involved in root rot of pea in different soils. Molecular quantification of pea roots harvested in screening under controlled conditions show a diverse microbial complex in diseased roots with *Aphanomyces euteiches*, *F. solani* and *F. oxysporum* being the most abundant pathogens for the three infected soil in Kirchlindach CH, Puch DE and Neu-Eichenberg, DE (Figure 14). Permutational MANOVA revealed a significant genotype effect on microbial community composition.

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More specifically, resistant pea genotypes showed significantly lower *F. solani* and *A. euteiches*, and higher arbuscular mycorrhizal fungi abundance in the roots. However, significant soil x genotype interaction could also be observed. These results demonstrate the value of plant-microbe interactions for pea resistance breeding programs. A manuscript titled "*Composition of key microbial taxa of the pea root rot complex is determined by soil x genotype interactions*" is in progress for submission to the *ISME* journal.

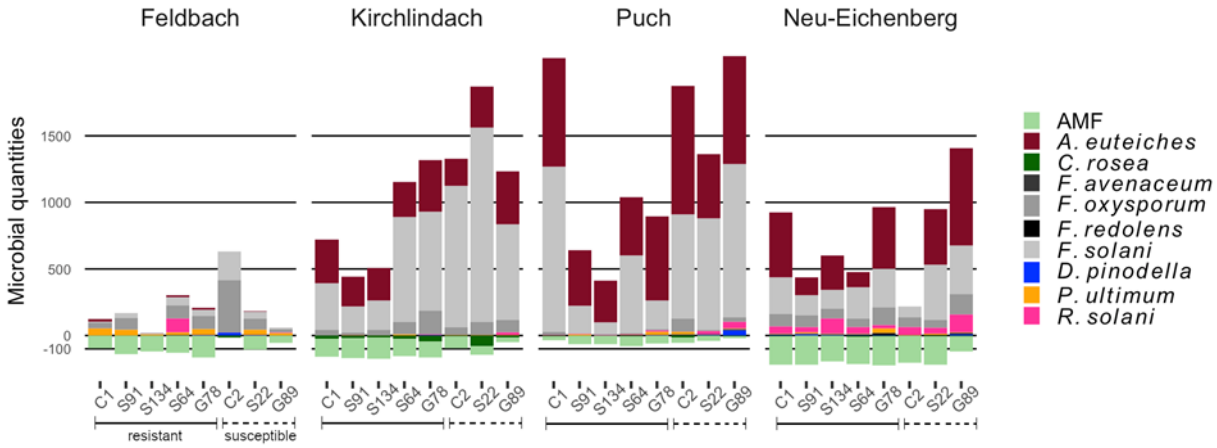

Figure 14. Composition of ten microbial taxa in pea roots of pot trials using different soils. Microbes were quantified by qPCR in roots of eight different pea lines (either resistant or susceptible to root rot) grown on four soils (Feldbach = healthy control): Mean quantification (in pg rct-1, or copies rct-1 for AMF) of the ten microbial taxa, with pathogens extending above of the 0-scale bar, beneficial taxa below (AMF quantifications were square root transformed for the presentation).

3.7. Workshop on Breeding for Plant-Microbe Interactions

The workshop "*2nd EUCARPIA Workshop on Implementing Plant-Microbe Interactions in Plant Breeding*", co-organized by LIVESEED and ECO-PB in December 2019 in Tulln, Austria, as a satellite workshop of the miCROPe 2019 symposium. The booklet is publicly available at the Organic Eprints repository (Hohmann et al. 2019 https://orgprints.org/36920/). A perspective article titled "*miCROPe 2019 – Emerging research priorities towards microbe-assisted crop production*" combines key messages from the symposium and the satellite workshop with the author's views and thoughts on emerging research priorities and challenges and is currently in progress for submission to the journal *FEMS Microbiology Ecology*.

The workshop aimed to strengthen a network among plant breeders and scientist of different disciplines and to explore the integration of knowledge of plant-microbe interactions in plant breeding. In recent years, plant-associated microbes have received considerable attention in research for their ability to improve crop productivity and yield stability. Benefits include improved nutrient uptake and resistance against biotic and abiotic stresses. Influences of crop management, soil parameters and climatic effects are well documented. Knowledge on plant genetic determinants for beneficial interactions with individual microbes and entire communities is growing. Several reports indicate that not only the host species but also the host genotype play a significant role in driving microbial community composition and activity, with the host plant selecting for and against particular members of the microbial community. However, to what extent genetic factors are responsible for shaping beneficial plant microbiomes is still poorly understood. Similarly, seed or plant microbiome manipulation via the introduction of biologicals offers great promise, but still suffers from variable outcomes due to insufficient knowledge of the factors involved for a successful integration. In

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conclusion, there are still many uncertainties on how to implement this knowledge into plant breeding and seed multiplication programmes. In 2015 the EUCARPIA Working Group on Plant-Microbe Interactions of the Section Organic and Low-Input Agriculture had their kick-off workshop. The present workshop was a follow up supported by research results of LIVESEED in order to explore the potential and limitations of implementing the growing knowledge on plant-microbe interactions in plant breeding in order to improve stress resistance, plant nutrition, plant health and general adaptability, and links between upstream disciplines and breeding.

At miCROPe and the satellite workshop, plant genetics of and breeding for beneficial plant microbiome interactions were highlighted as underutilised and promising approaches to improve crop resilience and yield stability. There have been fruitful discussions on the benefit of targeted microbiome-based genotype selections. While Jos Raaijmakers argued that beneficial plant-associated microbiomes were indirectly co-selected throughout the history of breeding, Richard Jefferson concluded that plant genome-focussed breeding has neglected agile trait contributions from the microbiome. The group work session of the EUCARPIA workshop led to fruitful discussions on opportunities and challenges of implementing plant-microbe interactions in plant breeding (see Figure 17 for a summary of group work outputs).

Figure 17. Word cloud of written highlights after the group work session on opportunities and challenges of implementing plant-microbe interactions in plant breeding.

Opportunities were particularly seen in the area of yield stability (increased resilience for challenging conditions) and productivity (maintaining yield while reducing fossil-based inputs). Emphasised tools and applications were high-throughput microbiome-based phenotyping, machine learning and modelling approaches, novel seed treatments and the focus on endophytes, plant genetic markers, gene editing, and monitoring and decision tools for agricultural practice and crop/genotype selection in general. The need to work closely with farmers and to link controlled experiments with field conditions was highlighted. Identified challenges involved the development of standards for omics protocols, understanding of microbiome functions (notably, beyond description), registration of microbial products and still unresolved problems with their performance variability. In conclusion, it emerges from the various reports that the variation between plant genotypes in regard to the

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interaction with beneficial microorganisms or more generally with soil microbiota indicates that plant breeding for improved interactions is a research priority. This has so far been hardly addressed by the industry, primarily because there are no or only few screening tools available for selecting appropriate plant genotypes, which, in turn, is due to a limited understanding of genotype-microbiome interactions. To further elucidate the potential, the variability of soil microbiomes has to be considered (e.g., between different environments) and we have to better understand the complexity of plant responses. Eventually, screening systems have to be developed to promote breeding programmes that allow high-throughput selection of plant genotypes that enable beneficial microbe interactions.

This workshop fostered the dialogue and collaboration between the different actors in order to develop advanced breeding strategies for the future.

References

Bodenhausen N, Somerville V, Desirò A, Walser J-C, Borghi L, van der Heijden MGA, Schlaeppi K (2019) Petunia- and arabidopsis-specific root microbiota responses to phosphate supplementation. Phytobiomes Journal 3(2): 112-124.

Coleman-Derr, D. & Tringe, S.G. (2014). Building the crops of tomorrow: advantages of symbiont-based approaches to improving abiotic stress tolerance. Frontiers in microbiology 5, 283.

Hohmann P., Trognitz, F., Messmer, M.M. (Editors) (2019) 2nd EUCARPIA workshop booklet. . Proceedings zu: 2nd EUCARPIA Workshop on Implementing Plant-Microbe Interactions in Plant Breeding, Tulln, Vienna, 6 Dec 2019 https://orgprints.org/36920/

Hohmann, P., Schlaeppi, K., and Sessitsch, A. (2020). miCROPe 2019 - emerging research priorities towards microbe-assisted crop production. FEMS Microbiol Ecol 96, 1-7.

Tkacz, A. & Poole, P. (2015). Role of root microbiota in plant productivity. Journal of experimental botany 66(8), 2167-2175.

Vandenkoornhuyse P., Quaiser A., Duhamel M., Le Van A., Dufresne A. (2015) The importance of the microbiome of the plant holobiont. New Phytologist 206(4): 1196-1206.

Wille, L., Messmer, M.M., Bodenhausen, N., Studer, B., and Hohmann, P. (2020). Heritable Variation in Pea for Resistance Against a Root Rot Complex and Its Characterization by Amplicon Sequencing. Frontiers in Plant Science 11, 1-15.

Wille, L., Messmer, M. M., Studer, B., Hohmann, P. (2019a) Insights to plant-microbe interactions provide opportunities to improve resistance breeding against root diseases in grain legumes. Plant, Cell & Environment 42: 20-40. https://zenodo.org/record/2547601#.XtuPFsBCSuU

Wille, L., Studer, B., Messmer, M. M. and Hohmann, P. (2019b) Screening pea for resistance against a root rot complex on naturally infested field soil. 2nd EUCARPIA Workshop on Implementing Plant-Microbe Interactions in Plant Breeding, Tulln (AT), 6.12.2019

Wille, L., Studer, B., Messmer, M. M. and Hohmann, P. (2019c) Resistant and susceptible pea lines harbour different root-rot pathogens and antagonistic fungi. Poster flash talk: MiCROPe, Vienna (AT), 2.-5.12.2019

Wille, L., Messmer, M.M., Studer, B., and Hohmann, P. (2019). Insights to plant-microbe interactions provide opportunities to improve resistance breeding against root diseases in grain legumes. Plant, Cell & Environment 42, 20-40.

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Annex 4. Effect of farming systems on maize rhizosphere (task 3.2.1)

Authors: Dr. Pedro Mendes Moreira (IPC), Fitolab (Portugal)

Abstract

Maize is one of the most important crops worldwide and is the number one arable crop in Portugal. A transition from the conventional farming system to organic agriculture requires optimization of cultivars and management, being the interaction plant-soil rhizosphere microbiota pivotal.

The strategy followed in collecting samples for microbiome analysis, was based on collecting a total of 450 samples in the field at flowering (50 accessions x 3 repetitions x 3 samples per plot). The samples were frozen and kept until field data collection and analysis.

The selection of the samples for microbioma were based on the following criteria: to verify the performance of the varieties and their behavior throughout selection, as well as their response to different environments. To select varieties that are more contrasting in organic and conventional farming and whose ranking in AB and conventional are the most convergent or divergent.

Rhizosphere soil samples of 16 open-pollinated maize populations and CCPs cultivated under conventional, organic farming and/or agroecological systems depending on the population were taken during flowering and analysed by NGS. Phenological data was collected from the replicated field trial. Due to COVID-19 data are delayed, so as the data analyses. We intend to publish as we did, with the same team for the present data.

https://www.frontiersin.org/articles/10.3389/fmicb.2021.636009/full

1. Introduction

Maize (*Zea mays*) is one of the most important cereal crops in human and animal diets worldwide. Together with rice and wheat, it provides at least 30% of the food calories to more than 4.5 billion people in 94 developing countries (Shiferaw *et al.,* 2011). Aside from providing nutrients for humans and animals, maize serves as a basic raw material to produce starch, oil, protein, alcoholic beverages, food sweeteners and fuel (Wu and Guclu, 2013).

According to FAOSTAT, from 2012 to 2017, the average world production per year of corn was around 1.036.263.896 t. In Portugal, maize is the most important arable crop, occupying an area of approximately 150.000 hectares (ANPROMIS, 2019), producing an annual average of 826.417 Mg from 2012 to 2017 (FAOSTAT, 2019).

Due to new challenges related to the expansion of the human and animal populations allied with the global climatic changes all opportunities for sustainably increasing the yield are relevant and should be developed. For example, the improvement on both agronomic practices and breeding related with microorganism interactions (e.g. control of root diseases) and organic management are appellative proposals since the rhizosphere microbial communities are critically important for soil nitrogen cycling and plant productivity (Schmidt *et al.*, 2016; Emmett *et al.,* 2017; Wille *et al.,*2018).

In this context, alternatives that promote more resilient farming systems, increasing the economic activity of rural areas, as well as preventing the significant loss of biodiversity in these areas, would contribute to more sustainable agriculture. In line with this thought, the traditional varieties are extremely important and should not be neglected(Altieri, 2004) since they present a genetic reservoir

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that can be used to select for advantages associated with the adaptability and resilience of cultivars to low input and a permanently changing environment. One of the characteristics of interest of these varieties is the possibility to maintain genetic diversity through open pollination. New genetic combinations may present new features or capabilities that will allow the plant population to respond more resilient towards pests, diseases or even the most adverse weather conditions (Garcia-Tejero *et al.,* 2015). Therefore, traditional varieties can adapt themselves to the environment, having greater adaptability to external factors increasing the crop population fitness (Altieri and Merrick, 1986; Lane and Jarvis 2007).

Although traditional Portuguese varieties do not have high yields, they are still cultivated due to their high yield stability even under unfavourable conditions like drought (Garcia-Tejero *et al*., 2015; Leitão *et al*., 2019). It is important to note that these varieties also play an important role in the country's rural economy, especially in the Central and Northern regions of Portugal, as their market value for bread making has increased thanks to their health benefits. This practice is also seen as a viable way to preserve the biodiversity of threatened farming systems (Vaz Patto *et al.,*2013).

Since 2002, the knowledge about microbiota and rhizosphere has increased exponentially, however, we are just beginning to understand the mechanisms of the plant-microorganism interactions (Vandenkoornhuyse *et al.*, 2015; Compant *et al*, 2019). According to Philippot *et al.,* 2013, the rhizosphere is a hotspot of plant-microbe interactions with a profound influence on plant productivity and all its functions are extremely important in terms of nutrition, health, and plant quality. Indeed, the rhizosphere is a critical interface that supports the exchange of resources between plants and their related soil environment. It is already known that several plants produced components interact with the rhizosphere microbiota, thus forming a dynamic structure in which microbial diversity can be modified with soil composition, plant species, different genotypes within the same cultivar and the development stage of the organism (Turner *et al*., 2013; Kandel *et al*., 2017; Chaparro *et al*., 2014). Moreover, the microbiota can help plants survive climate changes; modify the tolerance to abiotic and biotic stresses; affect the plant-pathogen interactions and change the nutrient content inside the plant (Chadha *et al*.,2015). In addition to all these factors, management agricultural practices, the addition of fertilizers, presence of pathogens, or extreme climatic conditions cause important effects on the microbial diversity composition (Andreote *et al.,* 2017). All these factors are highly relevant to improve the vigour, growth, and plant's health (Muller *et al*., 2016).

In this context, this study aimed to unravel the effect of genotype and farming system on structural diversity and putative functions of the microbial communities in the rhizosphere of 16 open-pollinated maize populations and CCPs to verify the performance of the varieties and their behavior throughout selection, as well as their response to different environments. In addition, the varieties were selected according to their contrasting yield in organic and conventional farming and whose ranking in AB and conventional are the most convergent or divergent.

With this approach groups of microorganisms with the potential to modulate the soil quality and fertility were identified and linked to specific conditions, thus potentially contributing to the increasing crop tolerance for stress conditions and to minimize the use of synthetic fertilizers and pesticides.

2. Material and methods

2.1 Germplasm

In 2020 two trials were performed; Organic vs Conventional: in two locations distance 1.3 km and intended to compare two agriculture systems - low input organic agriculture versus conventional agriculture (50 accessions); On-farm PPB trial: in two farmer's locations in Sousa Valley (10 accessions).

The germplasm used in 2020 trials (Table 1) included:

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- 41 open-pollinated populations (OPPs) from Azores (Portugal), used by the farmers for food (e.g. maize bread and polenta) and feed; 36 OPPs were collected in a collection mission on both Terceira and S. Miguel Islands, between 1979 and 1982 (Bettencourt and Gusmão, 1982) and kept in cold storage in Banco Português de Germoplasma Vegetal (BPGV) in Braga (Portugal) and multiplied in 2018 and 2019 in the low-input organic, at Escola Superior Agrária de Coimbra (ESAC); the remaining 5 Azorean OPPs were donated by farmers and collected by Duarte Pintado e Emanuel Ferreira in 2018 - BSM17, BT17, BT18, VT17, MT17.

-8 accessions were derived from 4 OPPs obtained from the participatory plant breeding program ("VASO") in Sousa Valley that has been active in the northern part of Portugal since 1984:

'Pigarro' is a white flint type FAO 300 cycle, with high kernel-row numbers (18 to 28), selected from a traditional Portuguese landrace (3 accessions: Pg C0S0 19 (Lousada 2019) - subjected to stratified mass selection on-farm in Lousada, Portugal in 2019; Pg C0S0 18 (Caldeirão 2018) and Pg C0S0 19 (Caldeirão 2019) - subjected to breeder's stratified mass selection in ESAC in 2018 and 2019).

'Verdeal´ is a white flint type FAO 300 cycle, selected from a traditional Portuguese landrace (3 accessions: VA C0S0 17 (Sequeiro Lousada 2017), VA C0S0 19 (Regadio Lousada 2019), VA C0S0 19 (Sequeiro Lousada 2019) - subjected to stratified mass selection on-farm in Lousada, Portugal in different irrigation systems in 2017 and 2019).

'Fandango' is a yellow dent type FAO 600 cycle synthetic composite, selected from the intercrossing of 77 yellow, elite inbred lines (dent and flint; 20% Portuguese and 80% American germplasm), with big kernel-row number and large ear size (1 accession: FN-2014 (Lousada 2019) - subjected to stratified mass selection on-farm in Lousada, Portugal in 2019).

'Amiúdo' is a yellow flint early population (FAO 200) adapted to poor soils (1 accession: Am C397 (Lousada 2019) - subjected to stratified mass selection on-farm in Lousada, Portugal in 2019).

-3 Composite Cross Populations (CCP) obtained using a polycross method based on 'Nutica' experience (Mendes-Moreira et al., 2009): 'SinPre' (Sintético Precoce) obtained through the crossing of 12 maize populations (10 Portuguese landraces and 2 American populations) subjected to on-farm stratified mass selection under PPB ("VASO") and multiplied in 2019 at ESAC. 'BulkAzores1' and 'BulkAzores2' were obtained in 2018-19 through the crossing of 40 Azorean OPPs and subjected to mass selection at ESAC and on-farm in 2019.

Seeds were dried and kept in cold storage (4 ◦C) until its use.

The strategy followed in collecting samples for microbiome analysis, was based on collecting a total of 450 samples in the field at flowering (50 accessions x 3 repetitions x 3 samples per plot). The samples were frozen and kept until its use.

The selection of the samples for microbioma were based on the following criteria: to verify the performance of the varieties and their behavior throughout selection, as well as their response to different environments. To select varieties that are more contrasting in organic and conventional farming and whose ranking in AB and conventional are the most convergent or divergent. After the selection of the samples, they were sent for microbiome analises.

The selected samples intended to respond to the following hypothesis (Table 2).

1H0 - Are there an impact of the genotype on the rhizosphere microbiota? (From de 50 entries that we had we select 14, some that were contrasting for yield and yield stability both for organic and conventional)

2H0- Is there an impact of the location and genotype on the maize rhizosphere microbiota? 8 varieties in 3 regions;

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3H0 - Are there a selection of the maize rhizosphere microbiota when the same population was selected in two locations; Are there a selection of the maize rhizosphere microbiota for the two mass selections across 4 environments (Pg C0S0 19 - (185-2019 Caldeirão); Pg C0S0 19 - (Lousada 2019)).

4H0 - Are there any change in microbiota during the selection process? Are there any changes in microbiota when selection was done for stress or for well irrigated area? (VA C0S0 17 - (Sequeiro Lousada 2019); VA C0S0 19 - (Regadio Lousada 2019); VA C0S0 19 - (Sequeiro Lousada 2019))

Table 1. Populations used in the trials and chosen for microbiota studies.

#	Name (OPPs,CCPs)	Origin	Organic vs Conventional trial	On-farm PPB Trial Agroecological site	Chosen Populations microbiota
1	2444	Azores, Portugal	X		
2	2445	Azores, Portugal			
3	2446	Azores, Portugal			
4	2448	Azores, Portugal	X		
5	2449	Azores, Portugal	X		
6	2488	Azores, Portugal	X		
7	2489	Azores, Portugal	X		
8	2492	Azores, Portugal			
9	2493	Azores, Portugal	X		
10	2494	Azores, Portugal	X		
11	2496	Azores, Portugal	X		
12	2497	Azores, Portugal			
13	2498	Azores, Portugal	X		
14	2499	Azores, Portugal	X		X
15	2500	Azores, Portugal			
16	2501	Azores, Portugal	X		X
17	2502	Azores, Portugal	X		
18	2503	Azores, Portugal			
19	2504	Azores, Portugal	X		
20	2505	Azores, Portugal	X		
21	2506	Azores, Portugal			
22	2507	Azores, Portugal	X		
23	2508	Azores, Portugal	X		
24	2509	Azores, Portugal	X		
25	2510	Azores, Portugal	X		
26	2511	Azores, Portugal			
27	2512	Azores, Portugal			

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28	2513	Azores, Portugal	X		
29	2514	Azores, Portugal	X		
30	2515	Azores, Portugal	X		
31	2516	Azores, Portugal	X		X
32	2517	Azores, Portugal	X		
33	2518	Azores, Portugal	X		
34	2519	Azores, Portugal	X		
35	2520	Azores, Portugal			
36	2521	Azores, Portugal			
37	2522	Azores, Portugal	X		
38	2524	Azores, Portugal	X		
39	2525	Azores, Portugal	X		
40	2526	Azores, Portugal	X		
41	2527	Azores, Portugal	X		
42	2528	Azores, Portugal	X		
43	2529	Azores, Portugal	X		
44	2530	Azores, Portugal	X		
45	2531	Azores, Portugal	X		X
46	Amc397	Vale do Sousa Portugal	X	x	X
47	Fn 2014	Vale do Sousa Portugal	X	x	X
48	Pg C0S0 18	Vale do Sousa Portugal	X	x	X
49	Pg C0S0 19 (Caldeirão 2019)	Vale do Sousa Portugal	X	x	X
50	Pg C0S0 19 - (Lousada 2019)	Vale do Sousa Portugal	X	x	X
51	SinPre – CCP	Vale do Sousa Portugal	X	x	X
52	VA C0S0 17 - (Sequeiro Lousada 2017)	Vale do Sousa Portugal	X	x	X
53	VA C0S0 19 - (Regadio Lousada 2019)	Vale do Sousa Portugal	X	x	X
54	VA C0S0 19 - (Sequeiro Lousada 2019)	Vale do Sousa Portugal	X	x	X
55	VT17 - (Caldeirão 2018)	Azores, Portugal	X		
56	MT17 - (Caldeirão 2018)	Azores, Portugal	X		
57	MONJ-3 - (Caldeirão 2018)	Azores, Portugal	X		
58	MONJ-2 - (Caldeirão 2018)	Azores, Portugal	X		
59	BT18 - (37-2019 Caldeirão 2019)	Azores, Portugal	X		
60	BT17 - (Caldeirão 2018)	Azores, Portugal	X		X

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61	BSM17 - (Caldeirão 2018)	Azores, Portugal	X		
62	Bulk-Azores1 - (Caldeirão 2018) CCP	Coimbra, Portugal	X		
63	Bulk-Azores 2 CCP	Coimbra, Portugal	X	x	X

Table 2. Germplasm used by the hypothesis

Hypothesis	1H0	2H0	3H0	4H0
Bulk soil				
Bulk-Azores 2 - (110-2019 Caldeirão)	1	1		
Pg C0S0 18 - (Caldeirão 2018)		1		
Pg C0S0 19 - (185-2019 Caldeirão)	1	1	1	
Pg C0S0 19 - (Lousada 2019)	1	1	1	
SinPre - (68-2019 Caldeirão 2019)	1	1		
VA C0S0 17 - (Sequeiro Lousada 2019)	1	1		1
VA C0S0 19 - (Regadio Lousada 2019)	1	1		1
VA C0S0 19 - (Sequeiro Lousada 2019)	1	1		1
Fn- 2014 - (Caldeirão 2018)	1			
Amc397 - (Caldeirão 2018)	1			
2499 - (9-2019 Caldeirão 2019)	1			
2501 - (11-2019 Caldeirão 2019)	1			
2516 - (23-2019 Caldeirão 2019)	1			
2531 - (34-2019 Caldeirão 2019)	1			
BT17 - (Caldeirão 2018)	1			

2.2 Field characterization and agronomical practices

Field trials were established at:

-2 locations in Coimbra, Portugal that belong to Coimbra College of Agriculture (ESAC) ("Caldeirão" in Low-input Organic 40°130 0.22”N, 8°260 47.69” W and "Vagem grande" in Conventional 40°13'16.2"N 8°28'29.3"W) distanced 1,3 km

-2 locations distanced 15 km in Sousa Valley region, (Macieira de Lixa 41°34'51 N, -8°16'92”W and Lousada 41°29'41 N, -8°26'72”W - agroecological sites). ESAC locations have alluvial soils with a medium field texture however they can be differentiated between low-input organic and conventional in the organic matter (1.9% versus 1.6%), pH (7.5 versus 5.9).

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The maize seed used was multiplied in the low-input organic location (ESAC) and in a low input conventional farm (PPB) in Lousada, Portugal in 2019 and seeds were not treated. For all Locations, the preceding crop was maize.

Conventional vs Low input Organic Trial: In conventional system soil tillage started in 15-05-2020 followed by the first fertilization in 20-05-2020, the horse manure was distributed (300 kg/ha) with a manure spreader and incorporated with a rotor tiller. Sowing occurred on 04-06-2020 and was conducted with a single seed sowing machine (5 plants per m2), followed by herbicide application (609.38 g/ha terbuthylazine + 121.88 g/ha mesotrione + 1015.63 g/ha S-metolachlor). Mechanical weed control with a harrow was also used.

In organic and low input farming, soil tillage was done on 15-05-2020. Sowing occurred on 04-06-2020 and was conducted with a single seed sowing machine (5 plants per m2). No fertilizer was applied.

The 50 accessions were evaluated in a randomized complete block design with 60 000 stand, in plots of two lines with 6.4 length and 0,8 interrow distance.

On-Farm PPB trial: In Macieira de Lixa soil tillage started in 12-05-2020 followed by the first fertilization in 20-05-2020, the cow manure was distributed (3000 kg/ha aprox.) with a manure spreader and incorporated with a rotor tiller. Sowing occurred on 25-05-2020 and was conducted with a single seed sowing machine with 5 plants per m2. Mechanical weed control with a harrow was used.

In Lousada soil tillage started in 15-05-2020 followed by the first fertilization in 18-05-2020, the cow manure was distributed (3000 kg/ha aprox.) with a manure spreader and incorporated with a rotor tiller. Sowing occurred on 25-05-2020 and was conducted with a single seed sowing machine (5 plants per m2). Mechanical weed control with a harrow.

The 10 accessions were evaluated in a randomized complete block design with 60 000 stand, in plots of two lines with 6.4 length and 0,8 interrow distance.

The maize rhizosphere samples were collected during flowering (BBCH 61) in 2020.

2.3 Germplasm characterization

For 10 randomly chosen plants from each population, an adaptation of the HUNTERS descriptor was used based on the field data collected during the monitorization of the maize crop: height (H); the height of 1st ear insertion (H1E), uniformity (U), Root (R%) and Stalk lodging percentage (S%). IBMSPSS® statistics program was used for phenotyping data analyses (Mendes-Moreira et al, 2017).

2.4 Sampling of maize rhizosphere

The samples were collected at the flowering stage in the 16 maize populations plus a bulk soil of the respective environments, in four locations Within each plot, 3 plant individuals, separated by at least 5 m from each other, were selected. Entire plants were dug up with a soil monolith in the middle of them. Bulk soil was shaken off the plant roots by vigorous shaking. Plant fine roots were collected from each plant, stored in cool temperature and moved rapidly to the laboratory where rhizospheric soil samples (1–2 mm soil adhering to roots) were collected. A bulk soil was also collected per each of the four locations.

2.5 DNA extraction and sequencing

Total DNA was extract using Nucleospin Soil Kit (Macherey Nagel, Düren, Germany) with Buffer SL1 in combination with Enhancer SX, according to manufacturer's instructions. Internal Transcribed Spacer 2 region amplicon libraries and Illumina 16S rRNA gene were generated and sequenced at Genoinseq (Portugal). The DNA was amplified for the hypervariable regions with specific primers and further reamplified in a limited-cycle PCR reaction to add sequencing adapters and dual indexes. First PCR

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reactions were performed using a pool of forward primers: ITS3NGS1_F-5'-CATCGATGAAGAACGCAG-3', ITS3NGS2_F-5'-CAACGATGAAGAACGCAG-3', ITS3NGS3_F-5'-CACCGATGAAGAACGCAG-3', ITS3NGS4_F-5'-CATCGATGAAGAACGTAG-3', ITS3NGS5_F-5'-CATCGATGAAGAACGTGG-3', ITS3NGS10_F-5'-CATCGATGAAGAACGCTG-3' and reverse primer ITS4NGS001_R-5'-TCCTSCGCTTATTGATATGC-3' for fungi (Tedersoo*et al.,* 2014) and forward primer Bakt_341F-5'-CCTACGGGNGGCWGCAG-3' and reverse primer Bakt_805R-5'-GACTACHVGGGTATCTAATCC-3' for bacteria (Herlemann*et al.,* 2011; Klindworth *et al.,* 2013). The second PCR reaction added indexes and sequencing adapters to both ends of the amplified target region according to the manufacturer's recommendations (Illumina, 2013). Negative PCR controls were included for all amplification procedures. PCR products were then one-step purified and normalized using SequalPrep 96-well plate kit (ThermoFisher Scientific, Waltham, USA) (Comeau *et al.,* 2017), pooled and pair-end sequenced in the Illumina MiSeq® sequencer with the V3 chemistry, according to manufacturer's instructions (Illumina, San Diego, CA, USA) at Genoinseq (Cantanhede, Portugal).

2.6 *In silico* functional analysis

Prediction of functional bacterial and fungal diversity within 16SrRNA and ITS2 sequence libraries were performed using PICRUSt (Langille *et al.,* 2013) and FUNGuild (Nguyen *et al.,* 2015), respectively. PICRUSt predicts the potential metagenomic gene content of a 16S amplicon library, based on genomic information of bacteria represented within the greengenes 16S database. To perform the process within the PICRUSt program, samples derived from the QIIME2 process, before taxonomic assignment were selected and grouped into 97% OTU's against the Greengenes database v.13.8. The NSTI (Nearest Sequence Taxon Index) within the PICRUSt pipeline was also calculated as quality control to validate the accuracy of the predicted functional annotations. FUNGuildassigns trophic modes to fungal taxa based on comparison to a curated database of fungal lifestyles(*sensu*Tedersoo*et al.,* 2014): pathotrophos, symbiotroph and saprotroph. Trophic mode refers to the mechanisms through which organisms obtain resources, providing putative information on the ecology of such organisms (Nguyen *et al.,* 2015). Functional assignments through FUNGuild are based on taxonomy and are possible only if taxa have been classified at the genus level or if taxa belong to a fungal group with exclusive lifestyle. Input data for FUNGuild was the OTU´s table.

2.7 Statistical and bioinformatic analysis

Raw reads were extracted from IlluminaMiSeq® System in fastq format and quality filtered with PRINSEQ version 0.20.4 to remove sequencing adapters, reads with less than 100 bases for the ITS2 region and 150 bases for the 16S rRNA gene, and trim bases with an average quality lower than Q25 in a window of 5 bases (Schmieder *et al.,* 2011). The forward and reverse reads were merged by overlapping paired end reads with Adapter Removal version 2.1.5 using default parameters (Schubert *et al.,* 2016). After sequencing the bacterial and fungal communities were analyzed using the QIIME software package. Chimeric sequences were removed using the consensus method and clustered in operational taxonomic units (OTU's) at 99% using a reference. Taxonomy was assigned to bacterial and fungal OTU's sequences using Greengenes v13.8 and UNITEv.7.2, respectively. The phylogenetic classification was performed to the genus level. The rarefaction curves obtained were saturated for each sample, demonstrating that the OTU's recovered were representative of the bacterial and fungi diversity, supporting a robust analysis.

The alfa diversity indexes Shannon index (H'), Simpson (D), and Chao1 were calculated with Phyloseq package to include in MicrobiomeAnalyst (Dhariwal*et al.*, 2017). The statistical significance of grouping based on experimental factor was estimated using T-test/ANOVA ($P < 0.05$) to determine differences in alpha diversity indexes among variables: 'SinPre' and 'Pigarro' populations and conventional and organic farming system. A non-supervised principal component analysis (PCA)was performed to compare the bacterial and fungal community structure. Statistical analyses were

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performed with analysis of variance (ANOVA) at $P < 0.05$ using R software v.4.0. Venn diagrams were generated with Venny 2.1 (Oliveros, 2015) to identify shared and unique taxa of each population according to the farming system. To identify fungal and bacterial taxa that differed in the relative abundance among population genotypes and farming systems in the rhizosphere of maize a Linear Discriminant Analysis (LDA) was performed combined with Effect Size (LEfSe) using a graphical interface in the Galaxy Version 1.0 (The Huttenhower Lab, 2018). A p-value of < 0.05 and a score ≥ 2.0 were considered significant in Kruskal–Wallis and pairwise Wilcoxon tests, respectively.

3. Results

3.1 Germplasm agronomic characterization

The averages of data collected in the field according to the HUNTERS (Mendes-Moreira *et al.*, 2017) descriptor for all populations in conventional and organic farming is detail (Table 3).

Table 3. Mean results and results of ANOVA in both organic and conventional

No.	Name	Conventional		Organic	
1	2444 - (1-2019 Caldeirão 2019)	3473,70	bc	2473,95	a
2	2448 - (Caldeirão 2018)	7578,13	abc	2321,77	a
3	2449 - (4-2019 Caldeirão 2019)	5994,03	abc	2727,59	a
4	2488 - (Caldeirão 2018)	5335,03	abc	2381,07	a
5	2489 - (Caldeirão 2018)	7410,37	abc	2236,02	a
6	2493 - (Caldeirão 2018)	5535,13	abc	3306,08	a
7	2494 - (Caldeirão 2018)	6465,53	abc	3252,70	a
8	2496 - (Caldeirão 2018)	9613,00	a	2529,74	a
9	2498 - (8-2019 Caldeirão 2019	5579,30	abc	3534,08	a
10	2499 - (9-2019 Caldeirão 2019)	7961,27	ab	4127,95	a
11	2501 - (11-2019 Caldeirão 2019	6344,97	abc	4252,30	a
12	2502 - (12-2019 Caldeirão 2019	6000,43	abc	3493,91	a
13	2504 - (14-2019 Caldeirão 2019	6750,73	abc	3114,09	a
14	2505 - (Caldeirão 2018)	5536,63	abc	2318,74	a
15	2507 - (16-2019 Caldeirão 2019	5577,30	abc	3598,94	a
16	2508 - (17-2019 Caldeirão 2019	5831,43	abc	2207,63	a
17	2509 - (Caldeirão 2018)	5354,90	abc	1959,21	a
18	2510 - (18-2019 Caldeirão 2019	9143,80	a	3332,94	a
19	2513 - (21-2019 Caldeirão 2019	5972,03	abc	2018,67	a
20	2514 - (22-2019 Caldeirão 2019	5198,97	abc	2787,86	a
21	2515 - (Caldeirão 2018)	4651,70	abc	1949,29	a
22	2516 - (23-2019 Caldeirão 2019	5675,80	abc	4235,95	a
23	2517 - (24-2019 Caldeirão 2019	6994,67	abc	3303,96	a
24	2518 - (Caldeirão 2018)	5961,70	abc	2620,23	a
25	2519 - (25-2019 Caldeirão 2019	6710,23	abc	2060,04	a
26	2522 - (28-2019 Caldeirão 2019	7749,67	abc	3090,10	a
27	2524 - (Caldeirão 2018)	6188,80	abc	2568,46	a
28	2525 - (31-2019 Caldeirão 2019	5284,60	abc	2230,07	a
29	2526 - (32-2019 Caldeirão 2019	5667,23	abc	3492,22	a

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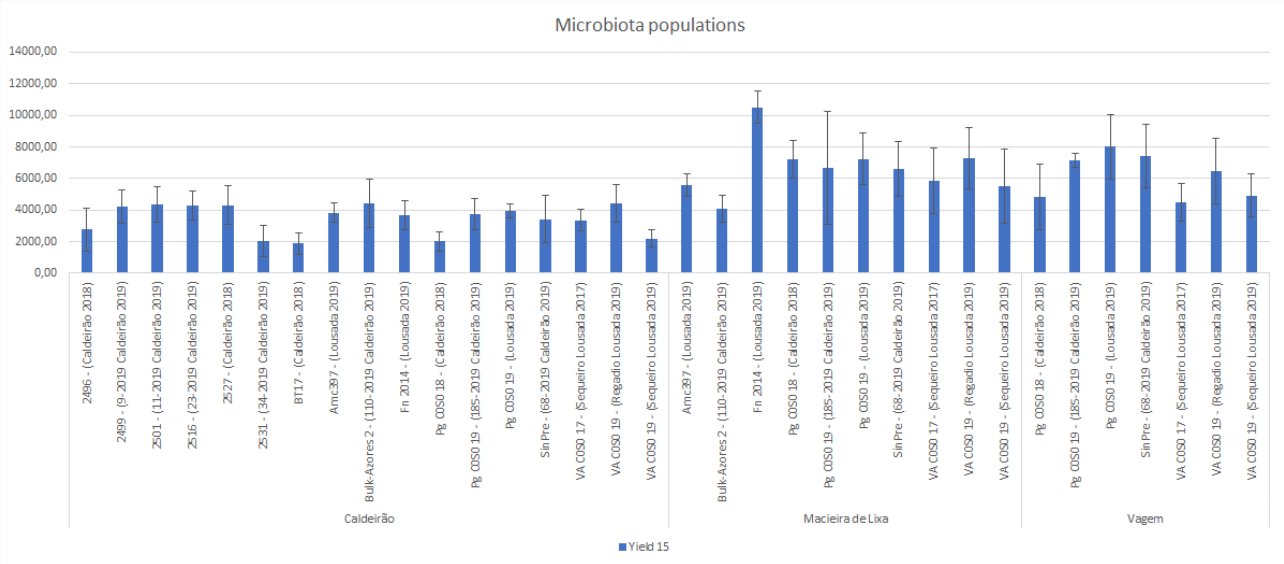
30	2527 - (Caldeirão 2018)	5575,57	abc	4195,12	a
31	2528 - (Caldeirão 2018)	7117,30	abc	2854,88	a
32	2529 - (33-2019 Caldeirão 2019	5597,87	abc	2638,94	a
33	2530 - (35-2019 Caldeirão 2019	5573,13	abc	2545,97	a
34	2531 - (34-2019 Caldeirão 2019	6519,50	abc	1886,28	a
35	BSM17 - (Caldeirão 2018)	6381,10	abc	2078,77	a
36	BT17 - (Caldeirão 2018)	4511,53	abc	1790,64	a
37	BT18 - (37-2019 Caldeirão 2019	7998,80	ab	2757,31	a
38	Bulk-Azores 2 - (110-2019 Cald	7308,47	abc	4248,98	a
39	Bulk-Azores1 - (Caldeirão 2018	7667,10	abc	3897,20	a
40	MONJ-2 - (Caldeirão 2018)	5339,33	abc	1522,41	a
41	MONJ-3 - (Caldeirão 2018)	5836,27	abc	2865,28	a
42	MT17 - (Caldeirão 2018)	6040,47	abc	2533,52	a
43	Pg C0S0 18 - (Caldeirão 2018)	4847,20	abc	1954,68	a
44	Pg C0S0 19 - (185-2019 Caldeir	7128,17	abc	3670,44	a
45	Pg C0S0 19 - (Lousada 2019)	8019,57	ab	3927,24	a
46	SinPre - (68-2019 Caldeirão 20	7448,30	abc	3205,65	a
47	VA C0S0 17 - (Sequeiro Lousada	4495,50	abc	3311,00	a
48	VA C0S0 19 - (Regadio Lousada	6450,77	abc	4313,74	a
49	VA C0S0 19 - (Sequeiro Lousada	4932,57	abc	2157,75	a
50	VT17 - (Caldeirão 2018)	2655,17	c	3131,05	a



4. Discussion

The rhizosphere is a specialized region where the interaction between plant roots and the surrounding soil associated microorganisms take place. Rhizospheric soil differs from bulk soil not only due to the direct effect of these microorganisms but because root growth modifies the composition of the soil. Roots can release rhizodeposits, a wide range of substances containing carbon (e.g., root cells, mucilage, volatiles and exudates), selecting and enhancing groups of microorganisms (Harkes *et al.,*

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2019), which in addition to modifying some soil characteristics and can play a role in the plant health status.

This work intends to characterize the structural composition of fungal and bacterial communities present in the rhizospheric soil associated with 16 maize populations cultivated in different farming systems.

References

Ahmed, M.S., Sallam, N.M., Mohamed, A.A., and Hassan, M.H. (2013). Arch. Phytopathol. 46(7), 868-881. doi: 10.1080/03235408.2012.753707.

Altieri, M. (2004). Linking ecologists and traditional farmers in the search for sustainable agriculture. Front. Ecol. Environ. 2(1): 35-42.doi.org/10.1890/1540-9295(2004)002[0035:LEATFI]2.0.CO;2.

Andreote, F., and Silva, M.C. P. (2017). Microbial communities associated with plants: learning from nature to apply it in agriculture. Curr. Opin. Microbiol. 37, 29-34 doi.org/10.1016/j.mib.2017.03.011.

ANPROMIS. (Last Access 13th August 2019). Associação Nacional de Produtores de Milho e Sorgo. http://www.anpromis.pt/o-milho.html.

Beirinckx, S., Viaene, T., Haegeman, A. Debode, J., Amery, F., Vandenabeele, S. et al. (2020). Tapping into the maize root microbiome to identify bacteria that promote growth under chilling conditions. Microbiome 8, 54 doi.org/10.1186/s40168-020-00833-w.

Berlanas, C., Berbegal, M., Elena, G., Laidani, M., Cibriain, J.F., Sagües, A., et al. (2019). The fungal and bacterial rhizosphere microbiome associated with grapevine rootstock genotypes in mature and young vineyards. Front. Microbiol. 10, 1142. doi: 10.3389/fmicb.2019.01142.

Brisson, V.L., Schmidt, J.E., Northen, T.R., Vogel, J.P., and Gaudin, A.C.M. (2019). Impacts of maize domestication and breeding on rhizosphere microbial community recruitment from a nutrient depleted agricultural soil. Sci. Rep.9, 15611. doi.org/10.1038/s41598-019-52148-y.

Byrt, C.S., Grof, C.P., and Furbank, R.T. (2011). C4 Plants as biofuel feedstocks: optimizing biomass production and feedstock quality from a lignocellulosic perspective free access. J. Integr. Plant Biol. 53, 120–135. doi: 10.1111/j.1744-7909.2010.01023.x.

Chadha, N., Mishra, M., Rajpal, K. Bajaj, R., Choudhary D.K., Varma, A. (2015). An ecological role of fungal endophytes to ameliorate plants under biotic stress. Arch Microbiol 197, 869–881. doi.org/10.1007/s00203-015-1130-3.

Chaparro, J., Badri, D., Vivanco, J. (2014). Rhizosphere microbiome assemblage is affected by plant development. The ISME Journal 8, 790–803. doi.org/10.1038/ismej.2013.196.

Cobo-Díaz, J.F., Legrand, F., Le Floch, G., Picot, A. (2019). Maize residues changes soil fungal composition and decrease soil microbial co-occurrence networks complexity.bioRxiv Doi.org/10.1101/703967.

Comeau, A.M., Douglas, G.M., and Langille, M.G.I. (2017). Microbiome Helper: a Custom and streamlined workflow for microbiome research. mSystems. 2, e00127-16. doi.org/10.1128/mSystems.00127-16.

Compant, S., Samad, A., Faist, H., and Sessitsch, A. (2019). A review on the plant microbiome: Ecology, functions, and emerging trends in microbial application. J. Advanced Res.19, 29–37. doi.org/10.1016/j.jare.2019.03.004

LIVESEED is funded by the European Union's Horizon 2020 under grant agreement No 727230 and by the Swiss State Secretariat for Education, Research and Innovation (SERI) under contract number 17.00090.

Dhariwal, A., Chong, J., Habib, S., King, I. L., Agellon, L. B., and Xia, J. (2017). MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. Nucleic Acids Res. 45, W180–W188. doi: 10.1093/nar/gkx295.

Desirò, A., Salvioli, A., Ngonkeu, E., Mondo, S.J., Epis, S., Faccio, A. et al. (2014) Detection of a novel intracellular microbiome hosted in arbuscular mycorrhizal fungi. ISME J. 8**,** 257–270. doi.org/10.1038/ismej.2013.151.

Duniere, L., Xu, S., Long, J., Elekwachi, C., Wang, Y., Turkington, K., et al. (2017). Bacterial and fungal core microbiomes associated with small grain silages during ensiling and aerobic spoilage. BMC Microbiol. 17, 50. doi: 10.1186/s12866-017-0947-0.

Emmett, B. D., Youngblut, N. D., Buckley, D. H., and Drinkwater, L. E. (2017). Plant phylogeny and life history shape rhizosphere bacterial microbiome of summer annuals in an agricultural field. Front. Microbiol. 8, 2414. doi.org/10.3389/fmicb.2017.02414.

FAOSTAT. Agriculture Organization of the United Nations (Last Access 13th August 2020). http://www.fao.org/faostat/en/#data/QC.

Figueroa-López, A.M., Cordero-Ramírez, J.D., Martínez-Álvarez, J.C. et al. 2016. Rhizospheric bacteria of maize with potential for biocontrol of Fusarium verticillioides. SpringerPlus 5, 330 doi.org/10.1186/s40064-016-1780-x.

Galazka, A., and Grzadziel. (2018). Fungal genetics and functional diversity of microbial communities in the soil under long-term monoculture of maize using different cultivation techniques. Front. Microbiol. 9, 76. doi=10.3389/fmicb.2018.00076.

Garcia-Tejero, I., Costa, J., Lima, R., Zuazo, V., Chaves, M., and Vaz-Patto, M. (2015). Thermal imaging to phenotype traditional maize land races for drought tolerance. Comunicata Scientiae 6, 34-343. doi.org/10.14295/cs.v6i3.960.

Ginnan, N. A., Dang, T., Bodaghi, S., Ruegger, P. M., Peacock, B. B., McCollum, G., England, G., et al. (2018). Bacterial and fungal next generation sequencing datasets and metadata from citrus infected with '*Candidatus* Liberibacter asiaticus'. Phytobiomes J. 2(2), 64-70.

Granzow, S., Kaiser, K., Wemheuer, B., Pfeiffer, B., Daniel, R., Vidal, S., et al. (2017). The effects of cropping regimes on fungal and bacterial communities of wheat and faba bean in a greenhouse pot experiment differ between plant species and compartment. Front. Microbiol. 8, 902. doi: 10.3389/fmicb.2017.00902.

Harkes, P., Afnan, K.A., Suleiman; van den Elsen., Sven, J.J., de Haan, J.J., Holterman, M., et al. (2019). Conventional and organic soil management as divergent drivers of resident and active fractions of major soil food web constituents. Sci. Rep. 9, 13521. doi.org/10.1038/s41598-019-49854-y.

Herlemann, D. P., Labrenz, M., Jürgens, K., Bertilsson, S., Waniek, J. J., Andersson, A. F. (2011). Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. The ISME Journal, 5 (10), 1571–1579. doi.org/10.1038/ismej.2011.41.

Hohmann, P.; Messmer, M.M. (2017). Breeding for mycorrhizal symbiosis: focus on disease resistance. Euphytica 213, 113 (2017). https://doi.org/10.1007/s10681-017-1900-x

ILLUMINA. 16s-metagenomic-library-prep-guide-15044223-b. (s.f.). (Last Access 10th August 2020) https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf.

Ismail, Y., McCormick, S., and Hijri, M. (2013). The arbuscular mycorrhizal fungus, *Glomus irregulare*, controls the mycotoxin production of *Fusarium sambucinum* in the pathogenesis of potato. FEMS Microbiol. Letters. 348(1), 46-51. https://doi.org/10.1111/1574-6968.12236.

LIVESEED is funded by the European Union's Horizon 2020 under grant agreement No 727230 and by the Swiss State Secretariat for Education, Research and Innovation (SERI) under contract number 17.00090.

Kandel, S., Joubert, P., Doty, S. (2017). Bacterial Endophyte Colonization and Distribution within Plants. Microorganisms 5, 4. doi: 10.3390/microorganisms5040077.

Kane, S. R., Chakicherla, A. Y., Chain, P. S. G., Schmidt, R., Shin, M. W., Legler, T. C., et al. (2007). Whole-genome analysis of the methyl tert-butyl ether-degrading beta-proteobacterium *Methylibiumpetroleiphilum* PM1. J. Bacteriol.189(5), 1931. doi.org/10.1128/JB.01259-06.

Klaubauf, S., Inselsbacher, E., Zechmeister-Boltenstern, S., Wanek, W., Gottsberger, R., Strauss, J., et al. (2010). Molecular diversity of fungal communities in agricultural soils from Lower Austria. Fungal Divers. 44(1), 65-75.doi.org/10.1007/s13225-010-0053-1.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., et al. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res.41(1), 1-11. doi.org/10.1093/nar/gks808.

Lagos, M.L., Maruyama, F., Nannipieri, P., Mora, M.L., Ogram, A., Jorquera, M.A. (2015). Current overview on the study of bacteria in the rhizosphere by modern molecular techniques: a mini-review. J. Soil Sci. Plant Nut. 15 (2), 504-523.doi.org/10.4067/S0718-95162015005000042

Langille, M. G. I., Zaneveld, J., Caporaso, J. G., McDonald, D., Knights, D., Reyes, J. et al. (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat. Biotechnol. 31(9), 814–821. doi.org/10.1038/nbt.2676.

Leitão, S., Ferreira, E., Bicho, M., Alves, M., Pintado, D., Santos, D., Vaz-Patto, M. (2019). Maize open-pollinated populations physiological improvement: validating tools for drought response Sustainability. 11(21). doi.org/10.3390/su11216081.

Levasseur, A., Piumi, F., Coutinho, P., Rancurel, C., Asther, M., Delattre, M. et al. (2008). FOLy: An integrated database for the classification and functional annotation of fungal oxidoreductases potentially involved in the degradation of lignin and related aromatic compounds. Fungal Genetics and Biology, Vol. 45, 5, 638-645. doi: 10.1016/j.fgb.2008.01.004.

Li, G., Yang, S., Li, M., Qiao, Y., and Wang, J. (2009). Functional analysis of an *Aspergillus ficuum* phytase gene in *Saccharomyces cerevisiae* and its root-specific, secretory expression in transgenic soybean plants. Biotechnol. Lett. Vol. 31 (8), 1297-1303. doi.org/10.1007/s10529-009-9992-6.

Lin, Z., Zhen, Z., Ren, L., Yang, J., Lou, C., Zhong, L., et al. (2018). Effects of two ecological earthworm species on atrazine degradation performance and bacterial community structure in red soil. Chemosphere. 196, 467-475.

McMullen, M., Bergstrom, G., De Wolf, E., Dill-Macky, R., Hershman, D., Shaner, G., et al. (2012). A unified effort to fight an enemy of wheat and barley: Fusarium Head Blight. PlantDis. 96(12), 1712-1728. doi.org/10.1094/PDIS-03-12-0291-FE-.

Mendes-Moreira, P.,Satovic, Z., Mendes-Moreira, J., Santos, J.P.P., Santos, J.P.N.S., Pêgo, S., Vaz Patto, M.C. (2017),.Maizeparticipatorybreeding in Portugal. Comparison of farmer's and breeder's on-farm selection. Plant Breeding. doi:10.1111/pbr.12551

Mendes-Moreira P, VazPatto MC, Mota M, Mendes-Moreira J; Santos JPP, Santos JPNS, Andrade E, Hallauer A, Pêgo S (2009) 'Fandango': long term adaptation of exotic germplasm to a Portuguese on-farm-conservation and breeding project. Maydica 54: 269-285. ISSN: 0025-6153

Medina, J., and López, N. (2007). Resistance to *Puccinia polysora* in maize accessions. Plant Dis. 91 (11), 1489-1495. doi.org/10.1094/PDIS-91-11-1489.

Miransari, M., Bahrami, H., Rejali, F., and Malakouti, M. (2009). Effects of soil compaction and arbuscular mycorrhiza on corn (*Zea mays* L.) nutrient uptake. Soil Tillage Res. 103,(2) 282-290. doi.org/10.1016/j.still.2008.10.015.

LIVESEED is funded by the European Union's Horizon 2020 under grant agreement No 727230 and by the Swiss State Secretariat for Education, Research and Innovation (SERI) under contract number 17.00090.

Mu, Y., Pan, Y., Shi, W., Liu, L., Jiang, Z., Luo, X., et al. (2016). *Luteimonasarsenicasp*. nov., an arsenic-tolerant bacterium isolated from arsenic-contaminated soil. Int. J. Syst. Evol. Micr. (66), 6. doi.org/10.1099/ijsem.0.001024.

Muller, D., Vogel, C., Bai, Y., and Vorholt, J. (2016). The plant microbiota: systems-level insights and perspectives. Annu. Rev. Genet. 50:1, 211-234. doi.org/10.1146/annurev-genet-120215-034952.

Nguyen, N.H., Smith, D., Peay, K.G., and Kennedy, P.G. (2015). Parsing ecological signal from noise in next generation amplicon sequencing. New. Phytol. 205, 1389-1393. doi.org/10.1111/nph.12923.

Oehl, F., and Sieverding, E. (2004). *Pacispora*, a new vesicular arbuscular mycorrhizal fungal genus in the *Glomeromycetes*. J. Appl. Bot. 78, 78-82.

Oliveros, J.C. (2007-2015) Venny. An interactive tool for comparing lists with Venn's diagrams. Available online at: https://bioinfogp.cnb.csic.es/tools/venny/index.html.

Pereira, P., Ibáñez, F., Rosenblueth, M., Etcheverry, M., and Martínez-Romero, E. (2011). Analysis of the bacterial diversity associated with the roots of Maize (*Zea mays* L.) through culture-dependent and culture-independent methods. ISRN Ecol.2011, 1-10. doi.org/10.5402/2011/938546.

Philippot, L., Raaijmakers, J., Lemanceau, P., and van der Putten, W. (2013). Going back to the roots: the microbial ecology of the rhizosphere. Nat. Rev. Microbiol. 11, 789–799. doi.org/10.1038/nrmicro3109

Salgado, J. D., Madden, L. V., and Paul, P. A. (2015). Quantifying the effects of *Fusarium* head blight on grain yield and test weight in soft red winter wheat. Phytopathol. 105, 295–306. doi: 10.1094/PHYTO-08-14-0215-R.

Schmieder, R., and Edwards, R. (2011). Quality control and preprocessing of metagenomic datasets. Bioinformatics. 27(6), 863–864. doi.org/10.1093/bioinformatics/btr026.

Schmidt, J., Bowles, T., and Gaudin, A. (2016). Using ancient traits to convert soil health into crop yield: impact of selection on maize root and rhizosphere function. Front. Plant Sci. 7.373.https://doi.org/10.3389/fpls.2016.00373

Schubert, M., Lindgreen, S. and Orlando, L. (2016). Adapter Removal v2: rapid adapter trimming, identification, and read merging. BMC Res. Notes. 9, 88. doi.org/10.1186/s13104-016-1900-2.

Shiferaw, B., Prasanna, B., Hellin, J., and Bãnzinger, M. (2011). Crops that feed the world. Past successes and future challenges to the role played by maize in global food security. Springer Netherlands, Vol. 3, 307. doi.org/10.1007/s12571-011-0140-5.

Smith, S.E., Smith, F.A., Jakobsen, I. (2003). Mycorrhizal fungi can dominate phosphate supply to plants irrespective of growth responses. Plant Physiology 133: 16–20. doi: 10.1104/pp.103.024380.

Stopnisek, N., Zühlke, D., Carlier, A., Barberán, A., Fierer, N., Becher, D., et al. (2016). Molecular mechanisms underlying the close association between soil B*urkholderia* and fungi. ISME J. 10, 253-264. doi.org/10.1038/ismej.2015.73.

Sun, M., Xiao, T., Ning, Z., Xiao, E., and Sun, W. (2015). Microbial community analysis in rice paddy soils irrigated by acid mine drainage contaminated water. Appl. Microbiol. Biotechnol. 99(6), 2911-2922.doi.org/10.1007/s00253-014-6194-5.

Tedersoo, L., Bahram, M., Polme, S., Koljalg, U., Yorou, N. S., Wijesundera, R., et al. (2014). Global diversity and geography of soil fungi. Science, 346(6213), 1256688–1256688. doi.org/10.1126/science.1256688.

The Huttenhower Lab (2018). Department of Biostatistics. Boston, MA: Harvard T.H.

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Trappe, J.M. (1987) Phylogenetic and ecologic aspects of mycotrophy in the angiospems from an evolutionary viewpoint. In: Safir GR (ed) Ecophysiology of VA mycorrhizal plants. CRC, Boca Raton, Fla., pp 5–25.

Turner, T., Ramakrishnan, K., Walshaw, J., Heavens, M., Swarbreck, D., Osbourn, A., et al. (2013). Comparative metatranscriptomics reveals kingdom level changes in the rhizosphere microbiome of plants. The ISME Journal volume 7, 2248–2258. doi.org/10.1038/ismej.2013.119.

Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., and Dufresne, A. (2015). The importance of the microbiome of the plant holobiont. New Phytol. 206, 1196-1206. doi.org/10.1111/nph.13312.

Vaz Patto, M. C., Mendes-Moreira, P. M., Alves, M. L., Mecha, E., Brites, C., do Rosário Bronze, M., & Pego, S. (2013). Participatory Plant Quality Breeding: An Ancient Art Revisited by Knowledge Sharing. The Portuguese Experience., Plant Breeding from Laboratories to Fields, Prof. Sven Bode Andersen (Ed.), ISBN: 978-953-51-1090-3, InTech, Available from: http://www.intechopen.com/books/plant-breeding-from-laboratories-to-fields/participatory-plant-quality-breeding-an-ancient-art-revisited-by-knowledge-sharing-the-portuguese-ex.

Walbot, V., and Skibbe, D. (2009). Maize host requirements for *Ustilago maydis* tumor induction. Sex. Plant Reprod. 23, 1–13. doi.org/10.1007/s00497-009-0109-0

Wille, L., Messmer, M.B.S., and Hohmann, P. (2018). Insights to plant-microbe interactions provide opportunities to improve resistance breeding against root diseases in grain legumes. Plant Cell Environ. 42 (1), 20-40. doi.org/10.1111/pce.13214.

Wolf, J., Espadas-Moreno, J., Luque-Garcia, J., & Casadevall, A. (2014). Interaction of Cryptococcus neoformans Extracellular Vesicles with the Cell Wall. Eukaryot Cell, 13 (12), 1484–1493. doi: 10.1128/EC.00111-14.

Wu, F., and Guclu, H. (2013). Global maize trade and food security: implications from a social network model. Risk Anal. 33(12), 2168–2178. doi.org/10.1111/risa.12064.

Kong, X., Han, Z., Tai, X., Jin, D., Ai, S., Zheng, X., et al. Maize (*Zea mays* L. Sp.) varieties significantly influence bacterial and fungal community in bulk soil, rhizosphere soil and phyllosphere, FEMS Microbiol. Ecol. 96 (3), fiaa020. doi.org/10.1093/femsec/fiaa020.

Yang, Y., Wang, N., Guo, X., Zhang, Y., Te, B. (2017). Comparative analysis of bacterial community structure in the rhizosphere of maize by high-throughput pyrosequencing. PLoS ONE 12(5), e0178425.doi.org/10.1371/journal.pone.0178425.

Zhang, H., Wu, X., Li, G., and Qin, P. (2011). Interactions between arbuscular mycorrhizal fungi and phosphate-solubilizing fungus (*Mortierella* sp.) and their effects on *Kostelelzkya virginica* growth and enzyme activities of rhizosphere and bulk soils at different salinities. Biol. Fert. Soils. 47, 543. /doi.org/10.1007/s00374-011-0563-3.

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