Xi Cycle

School of Soil Biodiversity and Bioindication

Department of Agricultural Sciences, University of Naples Federico II, Portici Naples

4-7 June 2019

'Biodiversity and Bioindicators in Monitoring and Management of Contaminated Soils

Traceability and monitoring of target microorganisms in soil

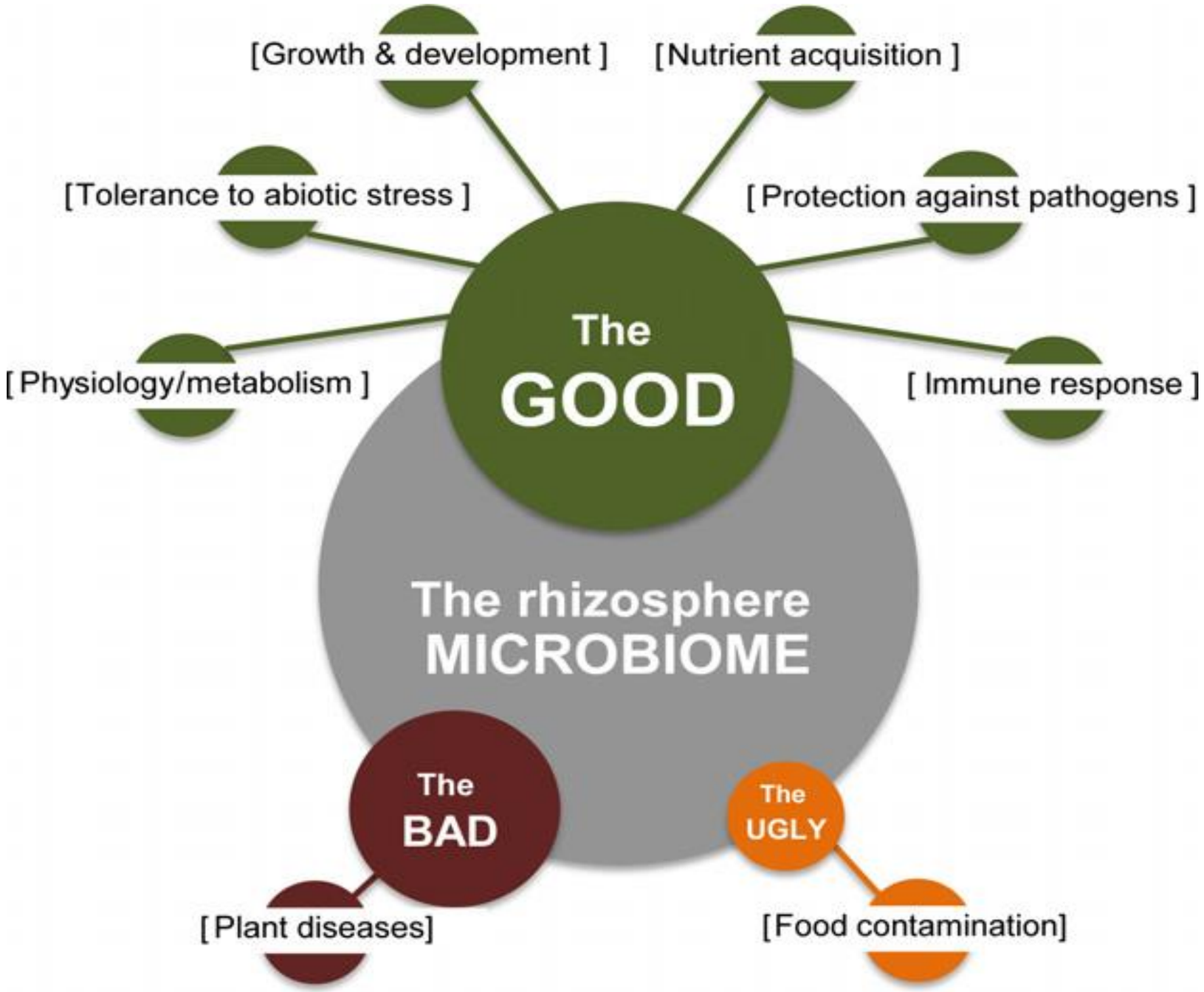
CREA – Centro di Ricerca Agricoltura e Ambiente

Loredana Canfora, Researcher

Via della Navicella, 2- 00184 Roma

loredana.canfora@crea.gov.it

In the soil there is a complex web of microorganisms respresenting the major source of biodiversity (maybe 98-99%)



It has long been recognized that soil biological diversity is the foundation of the complex ecological processes and for the maintenance of ecosystem services.

Different kinds of soil microorganisms colonizing the rhizosophere of plant tissues and promoting PGPM, can be utilized for the production of MICROBIAL-BASED FERTILIZERS

….exploiting microbial-based fertilizers can be traced back to ancient times

- The request by the market of **high quality horticultural production** is fostering the overuse of the different technical means, in particular chemical fertilizers and plant protection products as well as water for irrigation purposes, which has a negative impact on the soil environment.
- Furthermore, specialized productions are limiting rotations or other agronomical practices (such as diversification), known to improve the soil characteristics.
- There is thus the **need to improve the soil management practices** in crops systems to improve soil fertility and soil resilience against pathogens and pests, and at the same time limiting the negative environmental impact of these intensive crops.

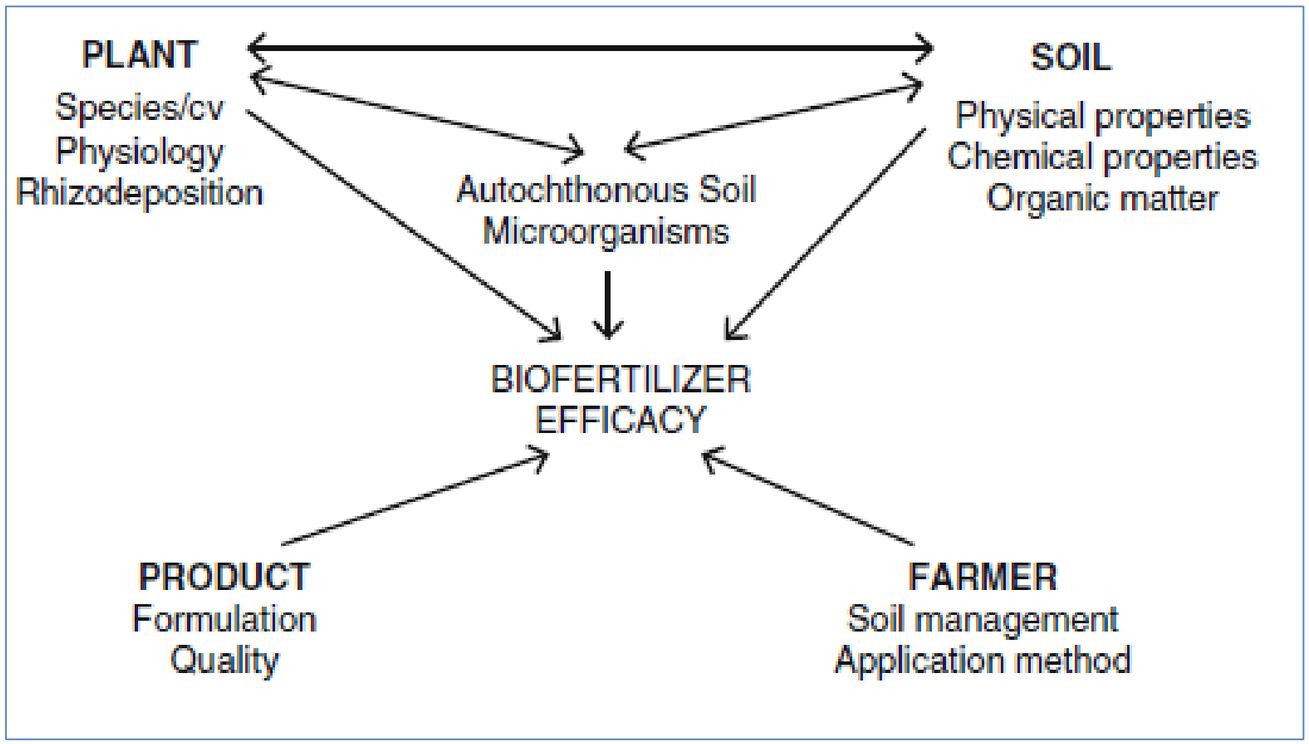
- The traceability, environmental fate, impact and efficiency-related measurements of bioinocula in soil, water and crops is the foundation for the safe use of microorganisms in agriculture;

….Therefore there are two kinds of problems not necessarily connected to each other:

i) **the monitoring of introduced species in soil addressing their persistence and their fate that indirectly provide important insights into soil microbial ecology overall effects;**

ii) **the monitoring impact of these introduced species on native soil microbial communities that directly provide insights on what extent introduced species impact on local communities.**

The monitoring of the fungi and bacteria introduced in soil, however, is not an easy task, due to **the complex web of microorganisms** already present in the soil, and their **patchy distribution across space and time**.



Major factors affecting the efficacy of biofertilizers in improving crop nutrition, growth and yield

(Malusa' E, Pinzari F and Canfora L. 'Efficacy of Biofertilizers: Challenges to improve crop production. Springer, *2016-Microbial Inoculants in Sustainable agricultural Productivity)*

an overview of methods for tracing microbial inoculants in soil

& monitoring target microbial species

Methods	Scale	Advantages	Limitations	Applicability to monitor introduce species in soil
PCR-Universal primers based	whole community	parallel analysis of high sample number	spatial and macro scale information depending on sampling accurancy	**Not**
PCR-trait based	Selected groups/domains	targeted gene amplicfication	applicability depending on degree of knowledge of target species	**yes**
Amplicon sequencing	Selected microorganisms	targeted gene detection	qualitative information; accurancy depending on taxonimic assignments against reference databases	**yes**
Real-time PCR (qPCR)	whole community; selected organisms; selected groups/domanis	targeted gene amplification and quantification	applicability depending on specific and discriminant primer pair design	**yes**

Paper in progress

by Early detection approaches:

- ✓ Culture-dependent tools, such as direct microscopic examination, plate profiling, FISH

to effectively characterize the soil microbial assemblages in space and time, evaluate their functional and trophic interactions

- ✓ Culture-independent tools: DNA and RNA- based analyses, PCR-based techniques

The field of DNA-based species identification transitioned to metabarcoding communities, where a plethora of methods have been developed to enable **inventories of microbial species composition** and a **good understanding of dynamics and processes of biodiversity**

However high degree of resolution is fundamental to monitor bacteria and fungi in soil!!!!

- **to evaluate the success or failure of inoculation,**
- **to trace and discriminate the 'introduced DNA' in a mixture of genomes from thousands of different organisms**

One of the critical issues is the lack of knowledge for some species of discriminant genetically traits.

How? And what we need?

1. **Species-specific markers that focus on genetic attributes that make microorganisms unique;**
2. **Species-specific markers discriminate target specie of interest from native strains;**
3. **A tool that allow to detect and quantify introduced species in soil at very low density**

Real-time PCR (qPCR) is a quantitative technique in which detection chemistries such as SYBRgreen and TaqMan are used to quantify genera or domain-specific genetic biomarkers.

an overview of methods for tracing microbial inoculants in soil

& monitoring target microbial species

Methods	Scale	Advantages	Limitations
T-RFLP	whole community; selected groups/domanis	Discriminant bands could show a putative species, characteristic of a certain soil/treatment; PCR products can be purified and used to identify microorganisms through several tools such as for example the enzymatic digestion coupled with the separation of single amplicons by sequencer, the sequence analysis of excised bands, and the construction of clone libraries, facilitating a more reliable phylogenetic identification of microorganisms.	Many problems come from PCR step and primers biases: length and sequence polymorphism, choice of primers, primer specificity and degree of mismatch, great variability of extracted eDNA
DGGE	whole community; selected groups/domanis	The bands reflect microbial diversity in the sample and their relative intensity reflects abundance.	Patterns with low discriminitary power.
Metagenomics	whole community	untargeted gene screening; assessment of diversity and functional potential	limited number of replicates; computational power required

Paper in progress

To decipher how the inoculative process may affect soil ecosystem as well as soil key ecosystem service remains the major challenge!!

- T-RFLP, DGGE, AFLP, RAPD and similar have been extensively used to study the complex microbial communities in diverse soil ecosystem and environment, proving to be unable to discriminate target single species within communities of species coming from different genera
- however suitable to evaluate the impact of local communities structure and diversity after the introduction of inoculative species;
- In the last decade metagenomic extends DNA-based identification to communities of multispecies using a massive sequencing of environmental DNA (eDNA)

- Several markers do not overlap with the standardized barcode used to build international collection of sequences derived from taxonomically identification, generating an increasing gap between taxonomical, morphological identification and DNA-based identification.
- A robust taxonomic inference can help us calibrate microbial diversity estimates and prevent erroneous interpretations.

… if we start by a good knowledge based on morphological, physiological, or functional traits, we will able to generate specie-specific barcode……
Unfortunately, taxonomic identification is scarce for many groups of microorganisms!!!

A case study

Pest and damage

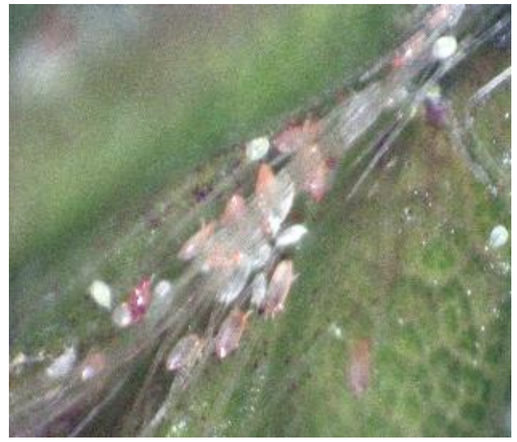
the application of biological control agents (BCA) and biofertilizers

Melolontha melolontha

In brief: we have tested

Beauveria bassiana (biopesticide Naturalis) to control *Phytonemus pallidus* on strawberry,

Łabanowska B.H., Tartanus M., Gruchała M., Masny A., 2015 . Efficacy of *Beauveria bassiana* and Abamectin in the control of strawberry mite - *Phytonemus pallidus* (Banks) (Acari: Tarsonemidae) and susceptibility of some cultivars to pest infestation. Journal of Berry Research 5 (2015) 1-7.



B. bassiana and *Heterorhabditis megidis* (as Larvanem) against *Otiorhynchus ovatus* and *O. sulcatus* on strawberry

Tkaczuk C., Łabanowska B. H., Augustyniuk-Kram A. 2005. The potential of entomopathogenic fungi and nemathodes against strawberry root weevil *Otiorhynchus ovatus* L. (Coleoptera, Curculionidae). Insect Pathogens and Insect Parasitic Nematodes IOBC/wprs Bulletin Vol. 28(3): 173-177.

Beauveria brongniartii (biopesticide Melocont) against ‘white grubs’ larvae (*Melolontha melolontha*)

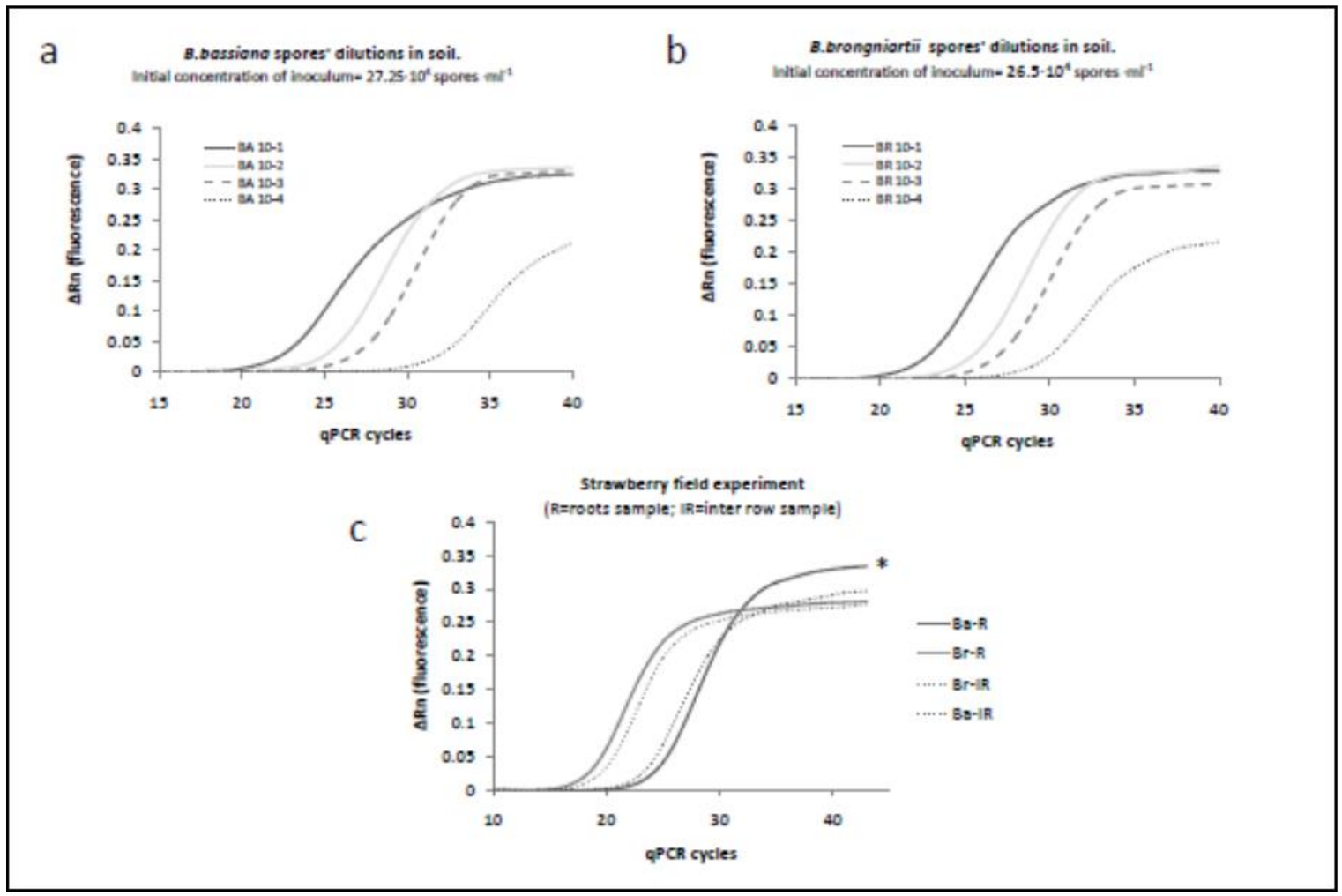
Łabanowska B. H., Bednarek H. 2011. Efficacy of *Beauveria brogniartii* as Melocont in the control of the European cockchafer (*Melolontha melolontha*). IOBC/wprs Bulletin, Vol. 66, 2011: 179-182.

- The effect of the treatments was assessed by checking the **changes in soil microorganisms population and metabolism**: T-RFLP & qPCR analysis of soil bacterial and fungal communities;

- A culture independent method (SSR markers) based on qPCR was developed for the detection and quantification of BCA inoculants in soil, resulting in relatively low cost protocol suitable for the **detection, identification and traceability of fungal bio-inoculants in soil;**

- In vitro tests were performed using *B. bassiana* and *B. brongniartii* to evaluate the **metabolic changes and the antagonism** derived from the **co-inoculation** of the two strains. The Phenotype MicroArrayTM system was used to assess the metabolic structure, while the FF MicroPlateTM array system was used to evaluate the use of different carbon sources, so to reveal which fungus dominated;

- The **qPCR results** obtained from in vitro soil dilutions of *B. bassiana* and *B. brongniartii* spores from the qPCR suggested that the method is able to detect also a small number of gene copies in just 1 g of soil.
- The results obtained from soil samples inoculated with B. bassiana or *B. brongniartii* and taken from the strawberry plants' roots (R) and from the plants' inter rows (IR) showed the persistence of *B. bassiana* only in soil samples taken close to plants' roots (R) while in the samples taken from inter rows (IR) did not result in a sufficient amount of PCR product also after a significant number of PCR cycles. *B. brogniartii* was absent, or in undetectable amounts in both inter rows (IR) and strawberries' roots (R) samples.



SCIENTIFIC REPORTS

OPEN

Development of a method for detection and quantification of *B. brongniartii* and *B. bassiana* in soil

Received: 10 November 2015
Accepted: 25 February 2016
Published: 15 March 2016

L. Canfora[1], E. Malusà[1,2], C. Tkaczuk[3], M. Tartanus[2], B.H. Łabanowska[2] & F. Pinzari[1,4]

A culture independent method based on qPCR was developed for the detection and quantification of two fungal inoculants in soil. The aim was to adapt a genotyping approach based on SSR (Simple Sequence Repeat) marker to a discriminating tracing of two different species of bioinoculants in soil

crea

SCIENTIFIC REPORTS

OPEN

Co-inoculum of *Beauveria brongniartii* and *B. bassiana* shows *in vitro* different metabolic behaviour in comparison to single inoculums

Received: 3 July 2017
Accepted: 13 September 2017
Published online: 12 October 2017

L. Canfora[1], N. Abu-Samra[1,5], M. Tartanus[2], B. H. Łabanowska[2], A. Benedetti[1], F. Pinzari[1,3] & E. Malusà[2,4]

- ✓ Results on carbon sources use by the two fungal species, and their joint inoculums showed that actually some compounds triggered the co-inoculums while other depressed its development;

- ✓ The two species showed a very different metabolic profile by Phenotype MicroArrayTM. *B. bassiana* showed a broader metabolism than *B. brongniartii* on a range of substrates. *B. brongniartii* showed a greater specificity in substrate utilization.

- ✓ Several carbon sources (L-Asparagine, L-Aspartic Acid, L- Glutamic Acid, m- Erythritol, D-Melezitose, D-Sorbitol**) triggered** the fungal metabolism in the co-inoculum.

- ✓ SSR markers and Real Time qPCR analysis showed that different substrates promoted either the growth of one or the other species, suggesting a **form of interaction** between the two fungi, related to their different ecological niches.

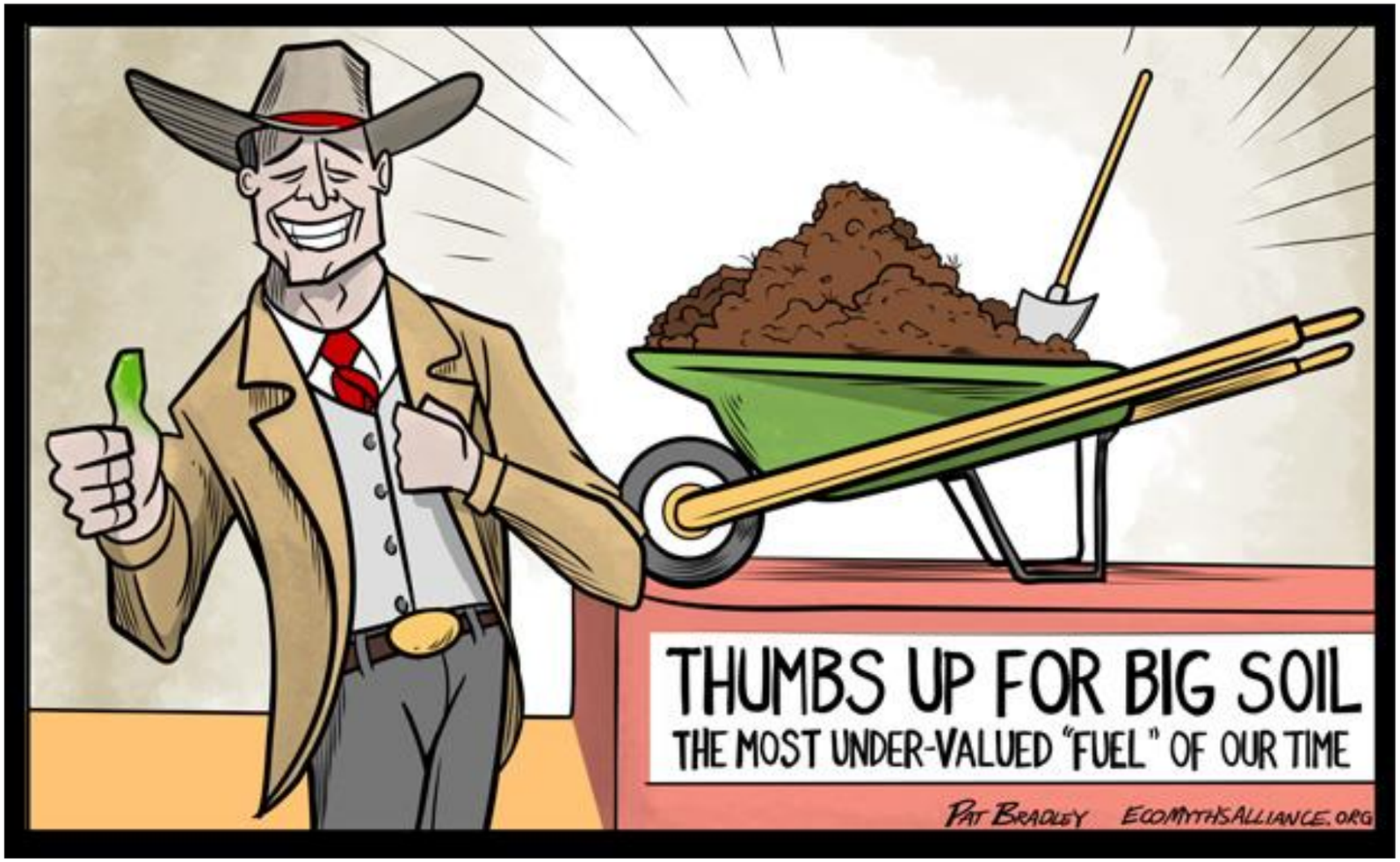
- ✓ **The methodological approach that combines Phenotype MicroArrayTM and SSR genotyping appeared useful to assess the performance and potential competition of co-inoculated entomopathogenic fungi.**

- The formulation of inoculums with the combination of more than one species of biocontrol fungi implicates possible interactions, either synergic or inhibitory, between the strains/species that can affect the production phase and the biocontrol activity.

- The two Beauveria species, when tested alone, showed different behaviour in carbon source use. *B. bassiana* showed a higher metabolism than *B. brongniartii* on a wide range of substrates, paralleled by higher biomass production. The comparable metabolic and growth patterns of the co-inoculum to those of B. bassiana single inoculum suggests that this species would dominate in the co-inoculum.

- The results suggest the hypothesis that the two fungi have a little niche overlap and therefore are different enough not to enter in a real competition when co-inoculated but, at the same time, at the presence of specific stimuli (i.e. competition for specific carbon sources) they can react with a higher respiration and biomass production that could possibly be accompanied by a higher virulence (yet to be verified).

new possibilities inspire application to soil where tools like the 'lab-on-chip' (Aleklett et al., 2018)



In the next weks, follow the starting Project H2020

EXCALIBUR

Exploiting the multifunctional potential of belowground biodiversity in horticultural farming