Assessment Methods for Biostimulants/Biofertilizers

Achievements and challenges

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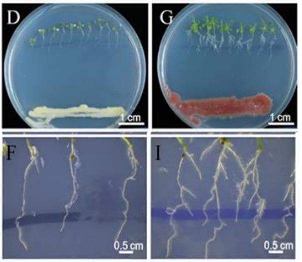
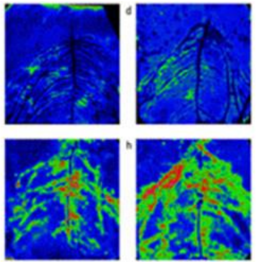
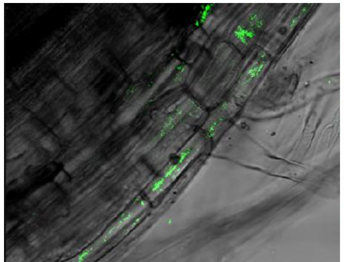
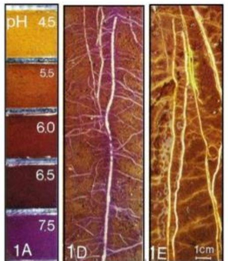
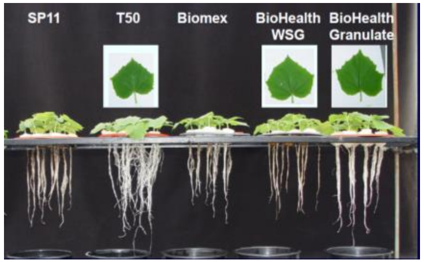




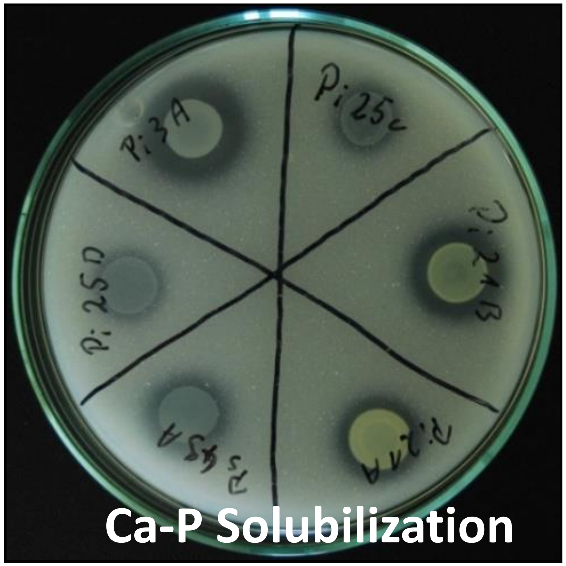


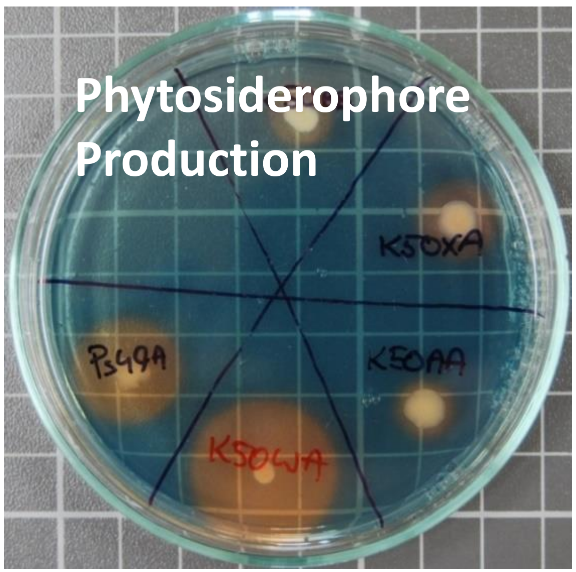


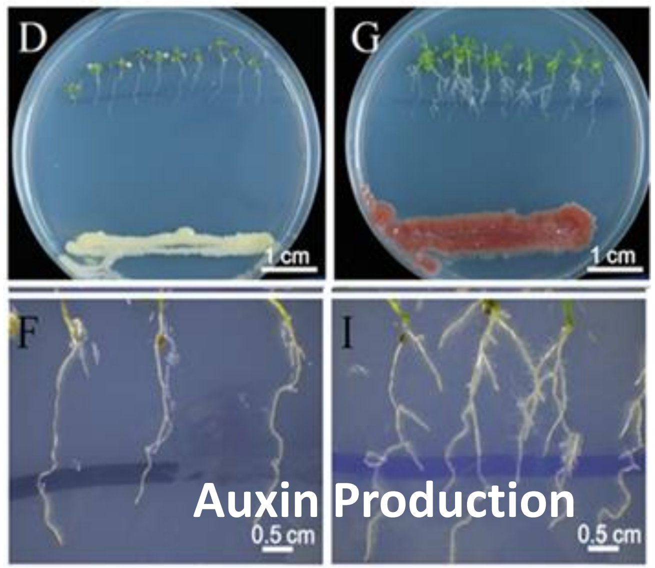


- **A wide range methodological approaches for efficacy assessment of biostiomulants has been developed particularly during the last two decades**

Examples:

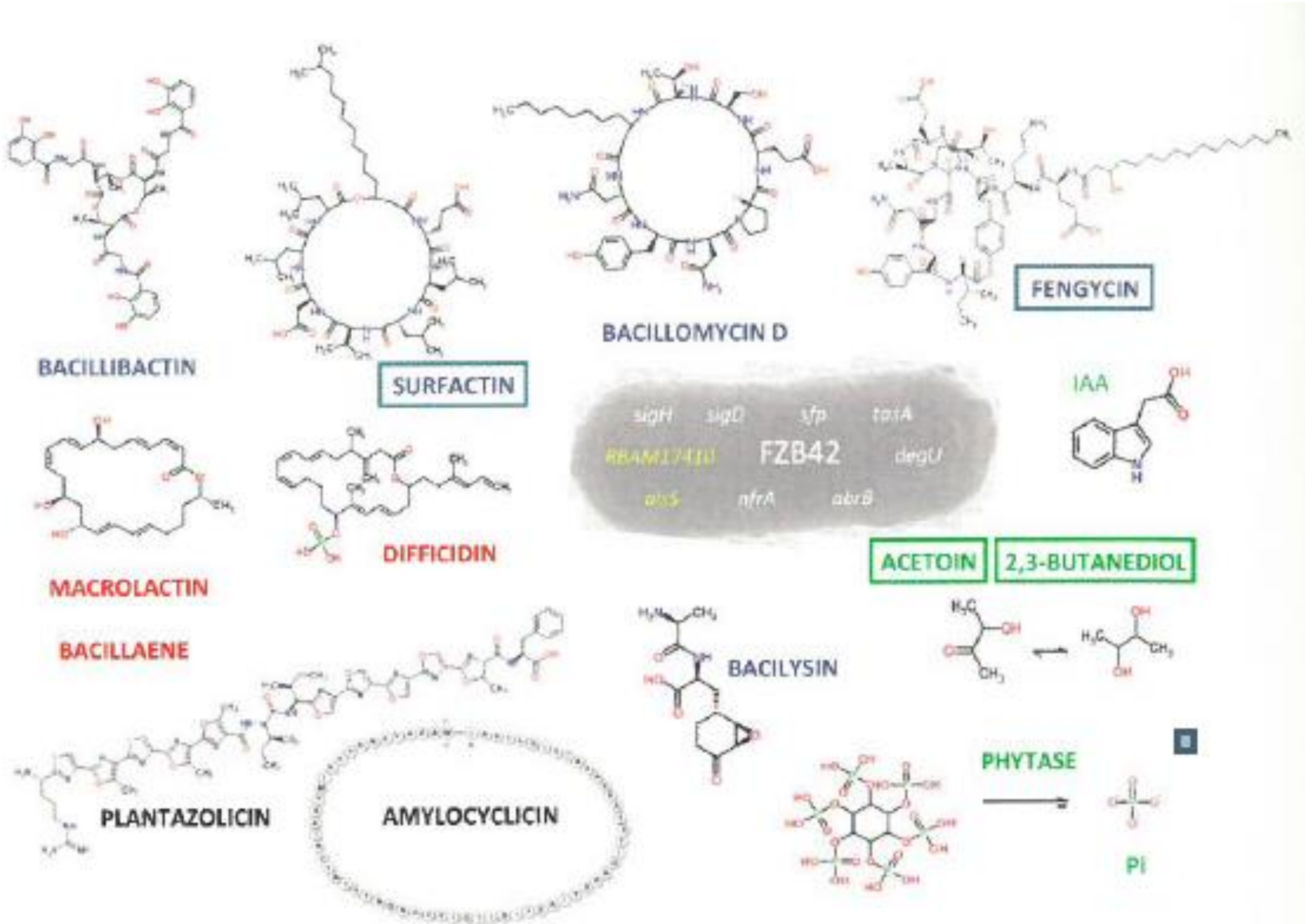






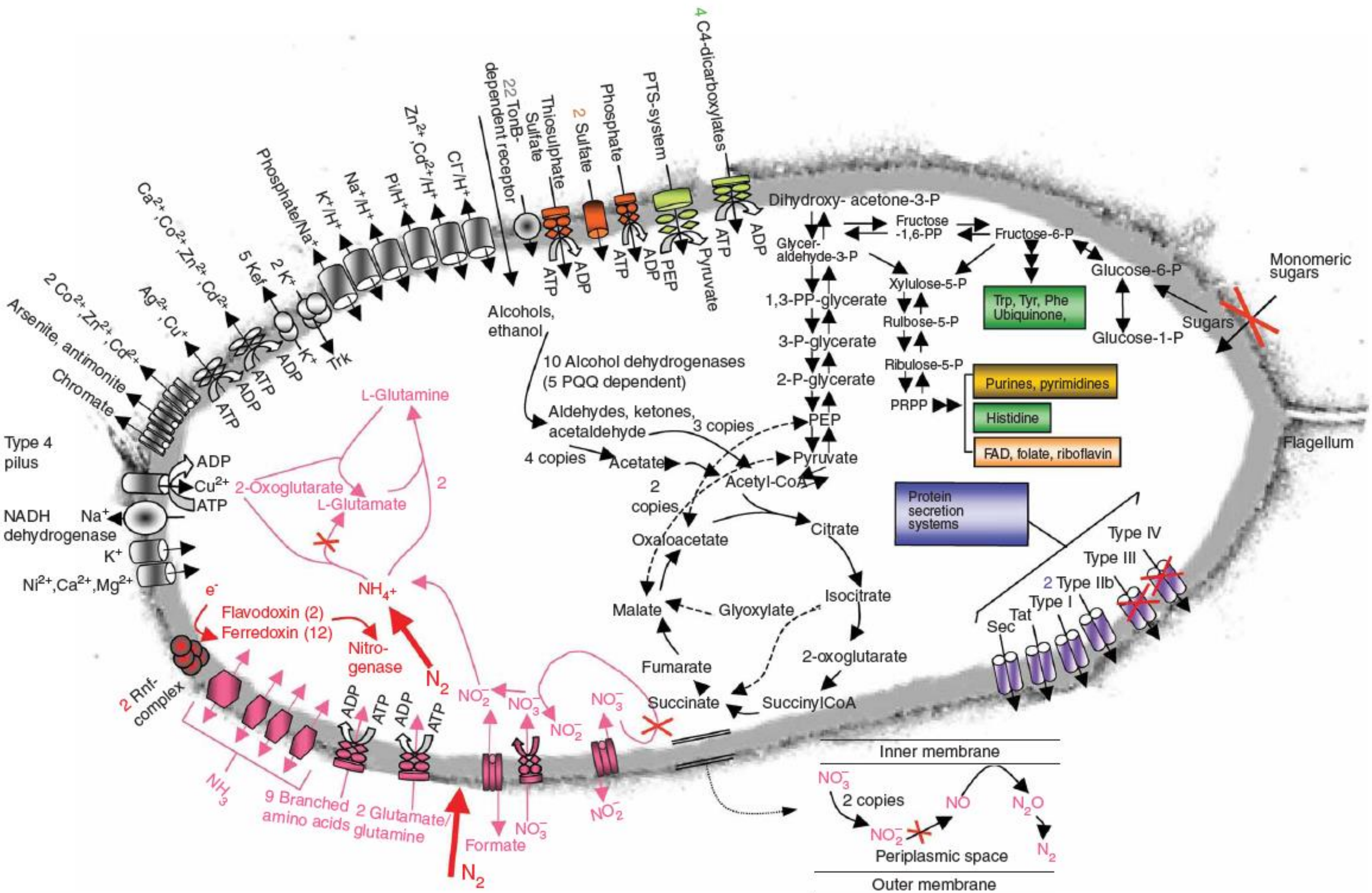
Testing metabolic properties of microbial inoculants

Secretory Metabolite Profiling in *Bacillus amyloliquefaciens* FZB42



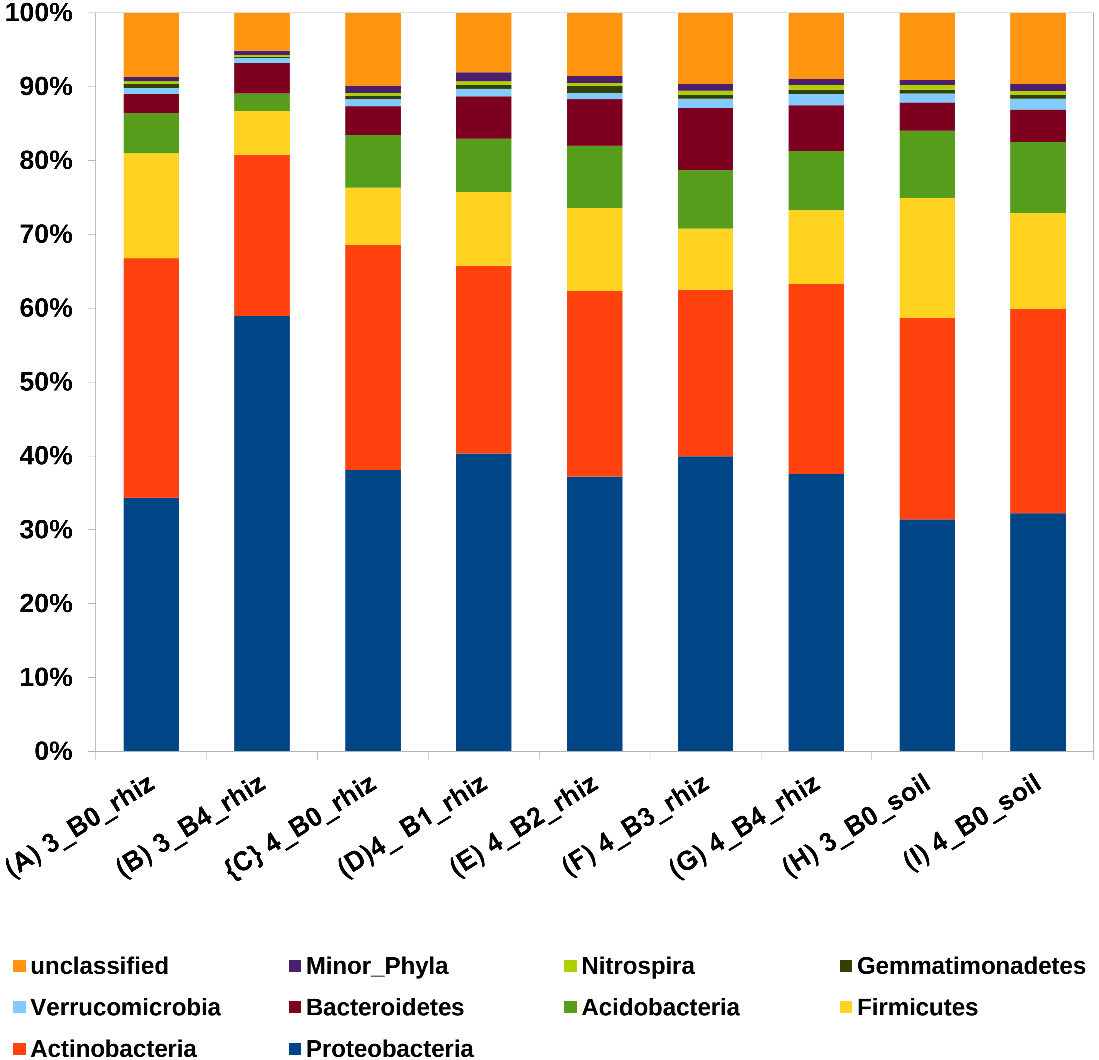
Borriss, 2015: Principles of Plant Microbe Interactions. pp.379-389 Springer

Genome sequencing to demonstrate
the genetic potential to interact with the environment



Krause et al. 2006, Nat Biotech 24: 1385

Metagenomics to study interactions with the soil microbiome

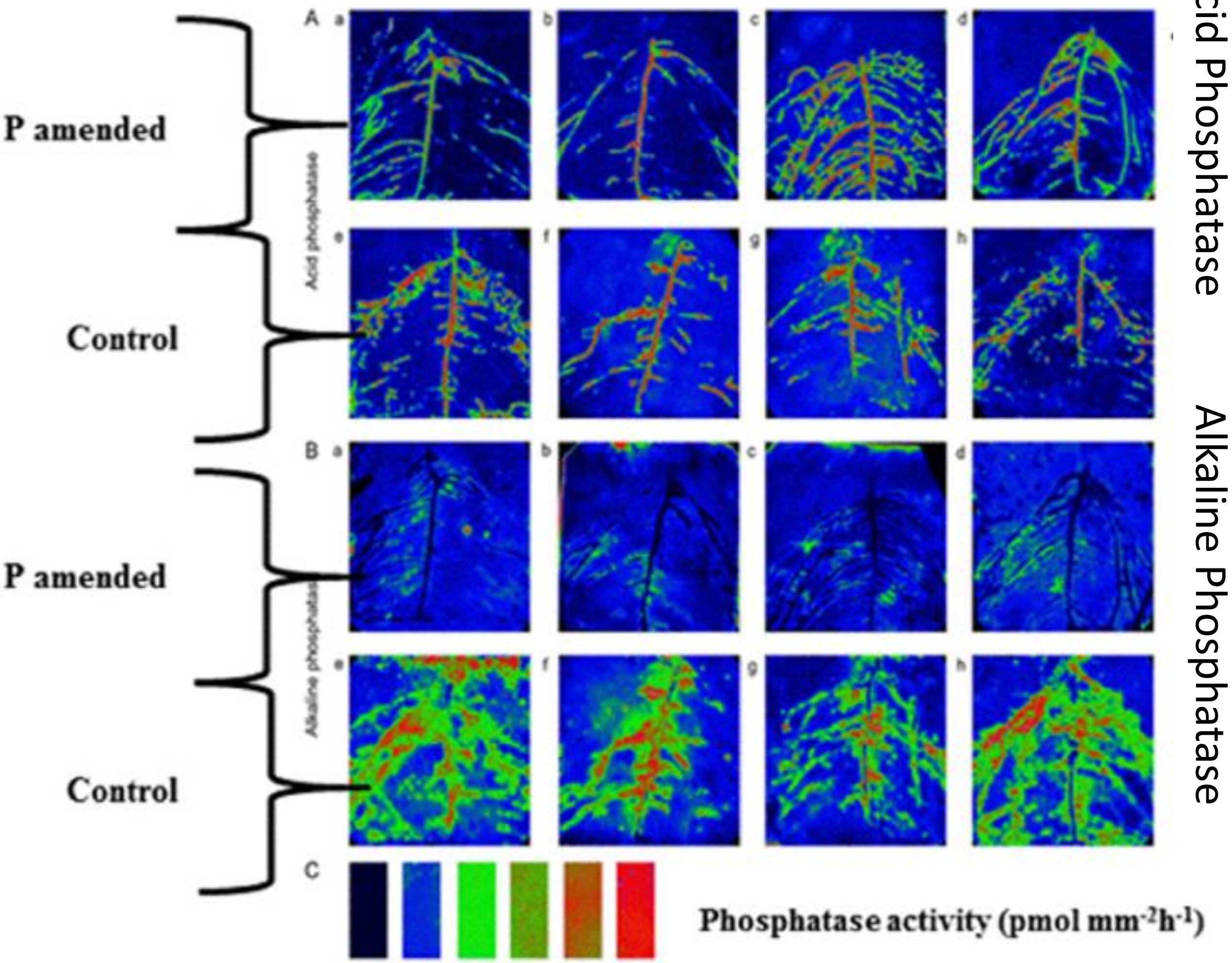


- Amplicon pyrosequencing of 16S rRNA genes obtained from rhizosphere and soil DNA from tomato revealed that ***Proteobacteria***, ***Actinobacteria***, ***Firmicutes*** and ***Acidobacteria*** were the dominant phyla in all treatments.

Eltlbany 2015

Functional characterization of plant PGPR interactions In the Rhizosphere

Zymographic detection of rhizosphere phosphatases by use of fluorogenic substrates and image analysis



Marker enzymes for nutrient in the rhizosphere

Expression of functional genes

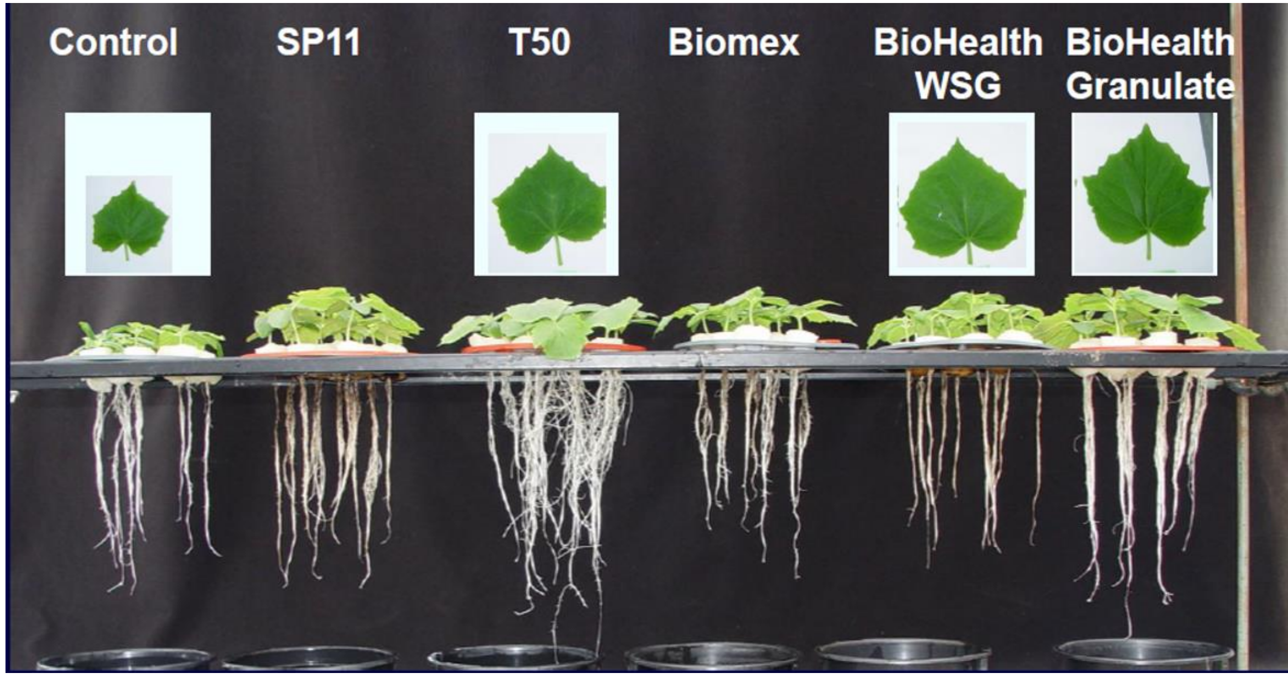
Gianfreda 2015
J. Soil Sci Plant Nutr 15: 1

Rhizosphere tracing of inoculants

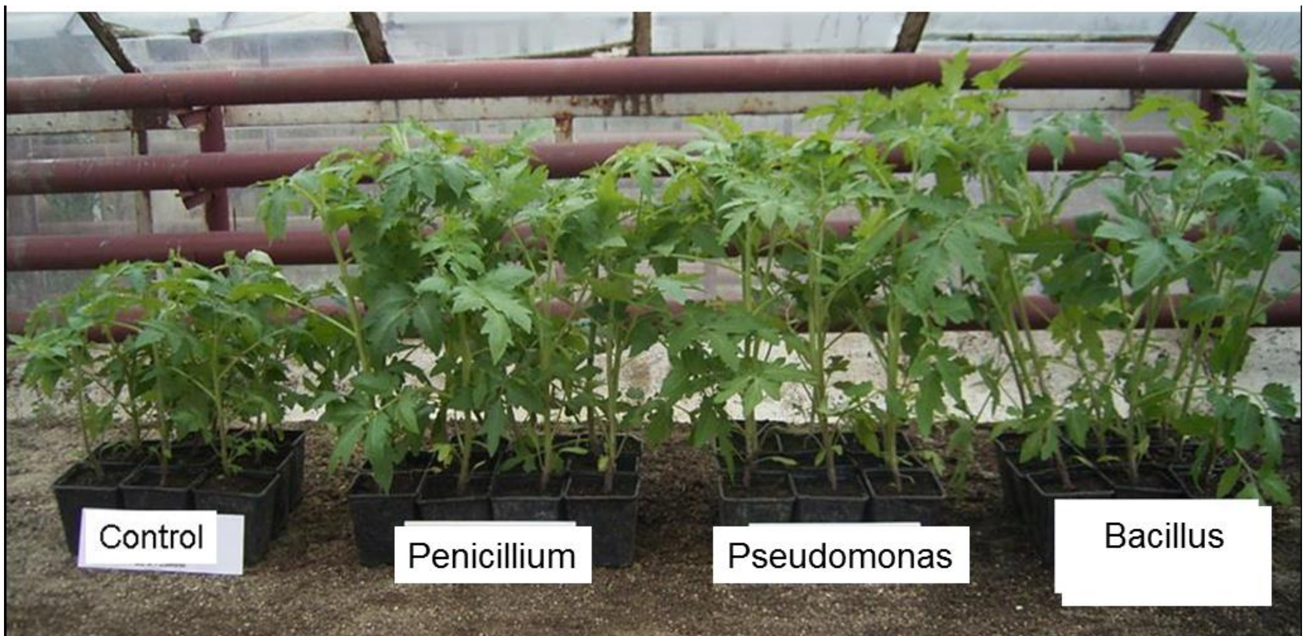
- **Transgenic reporter strains expressing fluorescence labelling** (GFP, RFP etc)
- **Rifampicin-resistant selectants for tracing under field conditions**
- **Tracing via species/strain-specific primers**
- **FISH techniques**

Eltlbany 2015

Screening for growth responses under controlled conditions



.Akter et al, 2013
J. Plant Nutr. 36: 1439



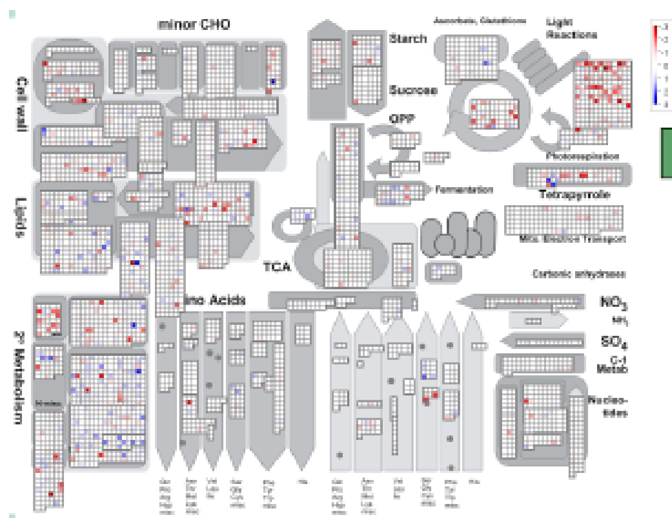
Greenhouse testing in soil culture

Posta et al. 2014

Root transcriptome changes in soil-grown maize plants In response to inoculation with microbial biostimulants

Whole transcriptome analysis

MapMan metabolism overview for whole transcriptome



Up-regulated BINs

- **Protein degradation** (Ubiquitin-mediated)
- **Hormones** (**ethylene** biosynthesis and signalling, cytokinin degradation)
- **Transcriptional regulation** (TFs): AP2/EREBP, WRKY, NAC, MYC
- **Phenylpropanoid/Flavonoid metabolism**
- **Biotic stress**: PR genes, SA (only FZB42)

Down-regulated BINs

- **Protein synthesis**
- **DNA synthesis/ chromatin structure/ histones**
- **RNA processing**
- **Cell organisation, cell division**

→ **No stimulation of auxin-related genes**

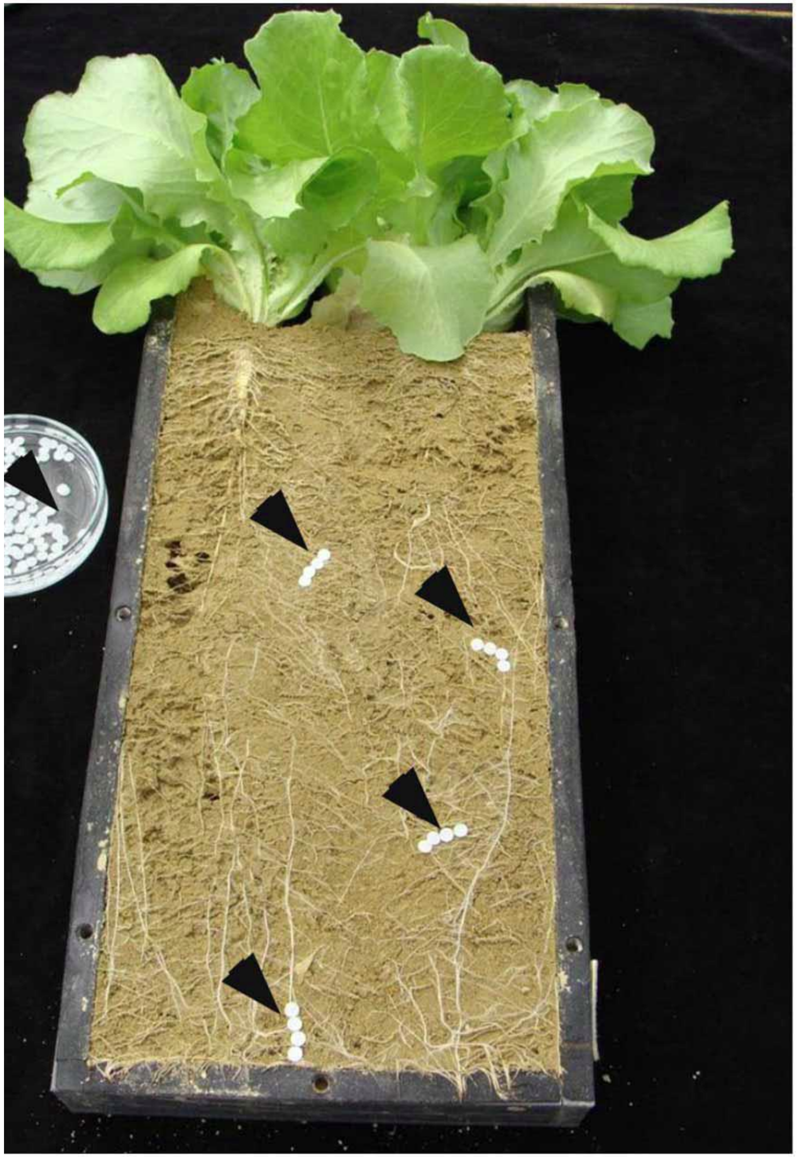
→ **Stress-related responses** (e.g. TFs, Ethylene, ubiquitin-mediated protein degradation) (Flick & Kaiser, 2012)

Weber 2016

GC-MS root exudate profiling of lettuce in different soils

Chemical group	Compound	Loess Loam	Alluvial Loam	Dilluvial Sand
Amino acids and amines	Alanine	+	+	++
	beta-Alanine	+	-	+
	Aspartate	+	+	+
	Glutamate	-	-	+
	Glutamine	+	+	+
	Glycine	+++	++	++
	Leucine	++	+	+
	Isoleucine	+	+	+
	Proline	+	+	+
	4-Hydroxyproline	+	+	+
	Pyroglutamate	++	+	+
	Serine	++	++	+
	Threonine	++	+	+
	Valine	++	+	++
	beta-Aminobutyric acid	+	+	+
	4-Aminobutyric acid	+	-	+
	Putrescine	+	+	+
Sugars and sugar alcohols	Glucose	+++	+	++
	Fructose	+++	+	++
	Mannose	+	-	-
	Maltose	+++	+	+++
	Trehalose	+++	+	+++
	Sucrose	+++	++	++
	Glycerol	+++	+++	+++
	Inositol	+++	+	+
Organic acids	Malate	+	+	+
	Fumarate	+	+	+
	Succinate	++	+++	++
	Lauric acid	++	+	+
	Benzoic acid	++	++	++
Others	Urea	+++	++	++
	Phosphate	+	+	+
	Ornithine	+	+	+

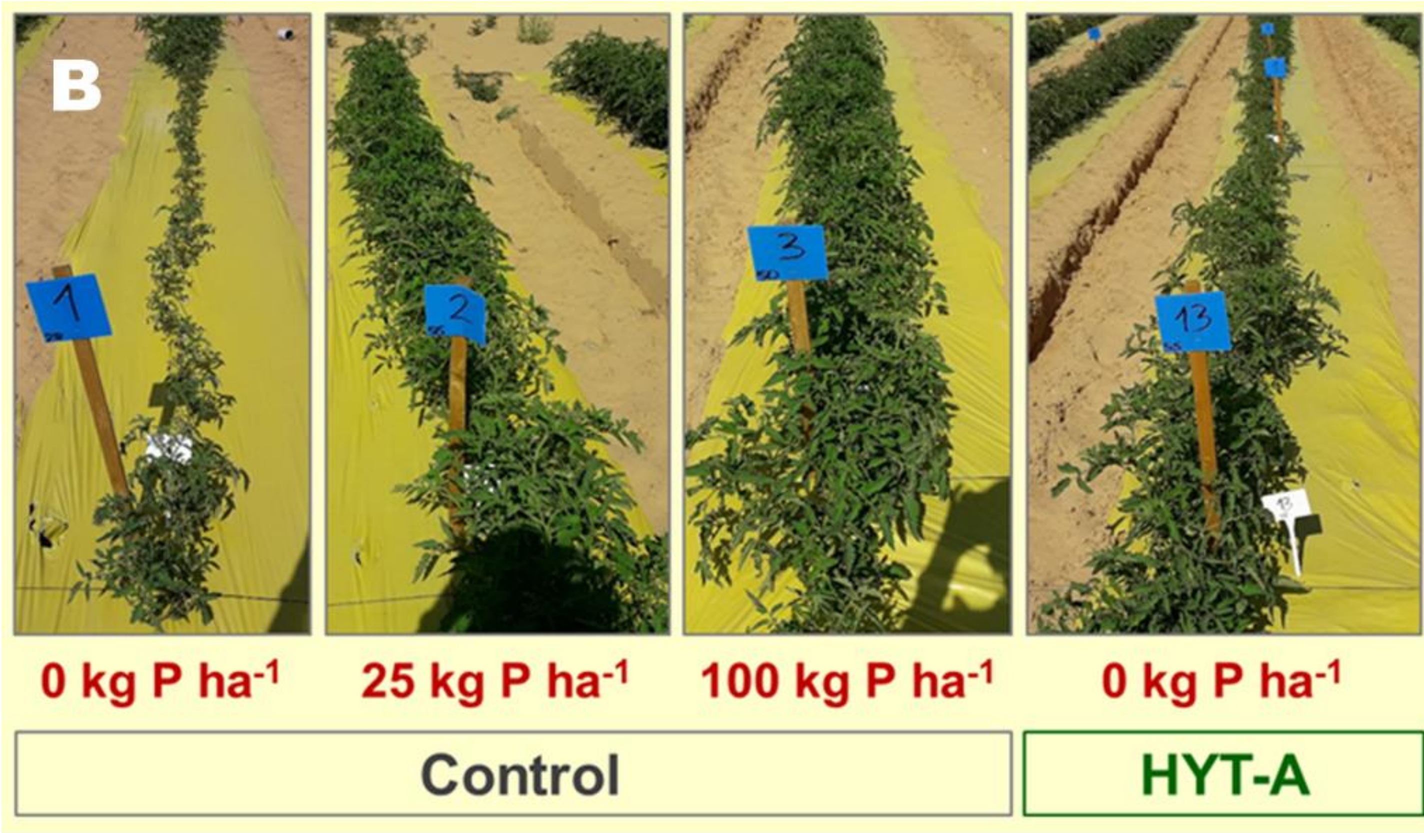
Differences in profiles of exudate samples (rhizosphere soil solution) are rather quantitative than qualitative.



Neumann et al., 2014 Front Microbiol. 2014; 5: 2

Field testing: field inoculation with the microbial consortium product HYT-A in drip-irrigated tomato production(Negev Israel)

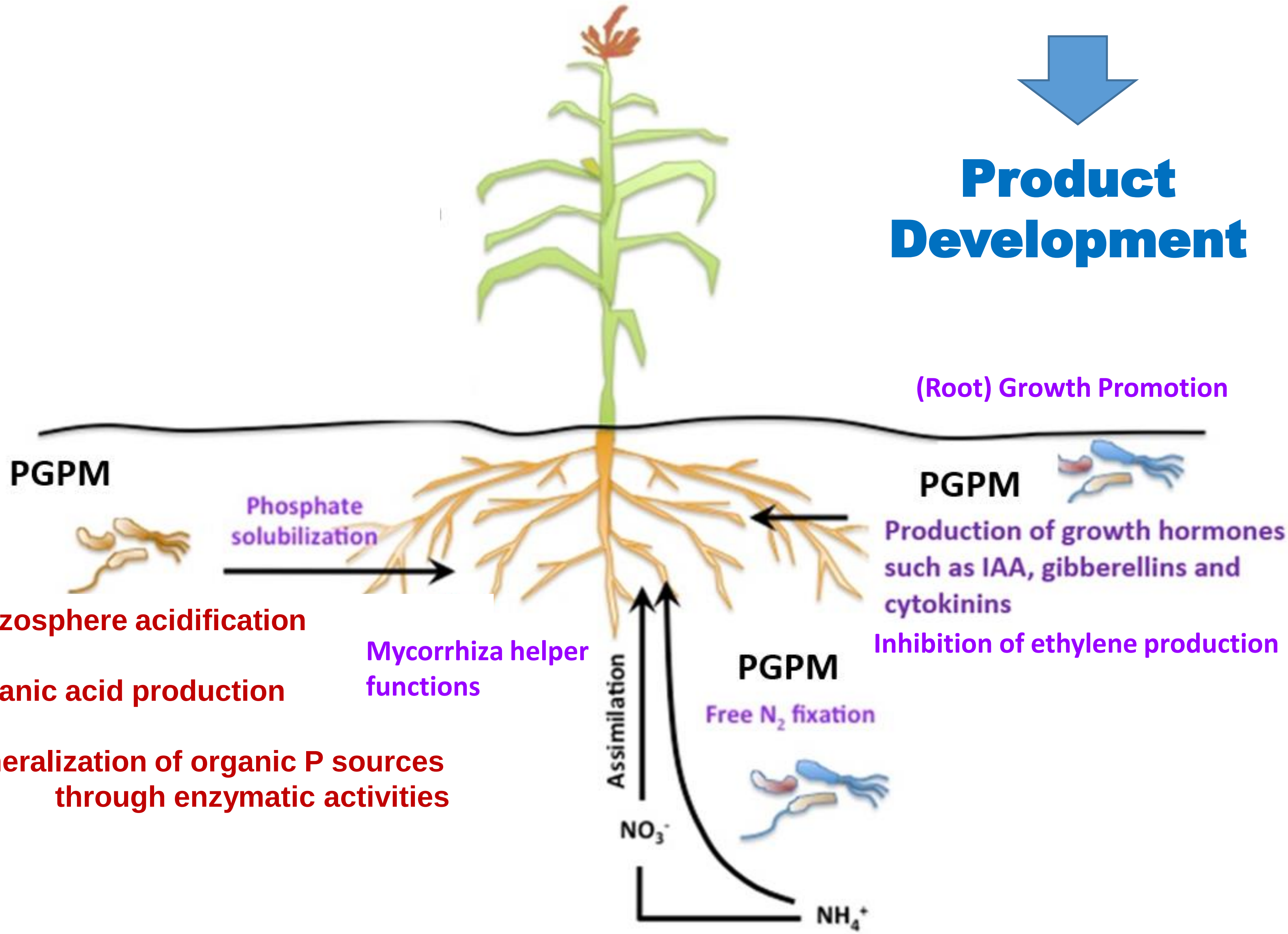
Shoot FW [kg/plant]	0.30c	0.42 bc	0.62 ab	0.64 ab
Yield [kg/m²]	1.44 c	3.61 b	5.67 a	3.32 bc



Bar Tal et al. 2017

Conceptual models for plant-biostimulant interactions as a basis for product development

Product Development



- Rhizosphere acidification
- organic acid production
- Mineralization of organic P sources through enzymatic activities

<u>Example:</u>

Consortium products for improved nutrient acquisition and stress resistance of crops

- **Associative or symbiontic N_2 fixation (BNF) promoted by inoculation with selected strains of BNF inoculants** (Azotobacter, Azosporillum, Clostridium, Rhizobium etc)

- **Carbon supply for BNF inoculants promoted by co-noculation with selected strains of C-decomposer**s (Bacillus, Trichoderma etc)

- **Phosphate supply for BNF microbes and host plants promoted by co-inoculation with selected strains of P-solubilising microorganisms (PSMs)** (Bacillus, Pseudomonas, Acetobacter, Micrococcus, Trichoderma etc)

- **Additional protective agents and co-factors** (microbial pathogen antagonists, micronutrients, seaweed extracts , starter C, etc)

⇒
- **Improved fertilizer use efficiency**
- **Reduction of N fertilizer demand 40-80%**
- **Improved stress resistance**

Poster Bradacova et al.

(information based on patent applications and product descriptions)

But: **Inoculation with accociative N_2 fixers rarely leads to improved N acquisition and higher yields under field conditions in temperate climates**

Parameter	N-fertilization level (kg N ha^{-1}) 0	40	80	120
Number of cobs per plant				
Control	1.069a	1.181a	1.233a	1.289ab
A. brasilense Sp245	1.039a	1.138a	1.177a	1.194b
A. irakense KBC1	1.065a	1.121a	1.194a	1.310a
Fresh cob yield (kg ha^{-1})				
Control	12409a	15225a	16350a	16924a
A. brasilense Sp245	12439a	15008a	15923a	16380a
A. irakense KBC1	13500a	15158a	16421a	17719a
N content in grains (%)				
Control	1.322a	1.367a	1.445a	1.496a
A. brasilense Sp245	1.343a	1.398a	1.481a	1.486a
A. irakense KBC1	1.346a	1.399a	1.419a	1.536a

Azotobacter field inoculation experiments with maize in Belgium

Similar results in three EU-funded projects During 2000 – 2010 (Micro N-Fix, RHIBAC, MicroMAIZE)

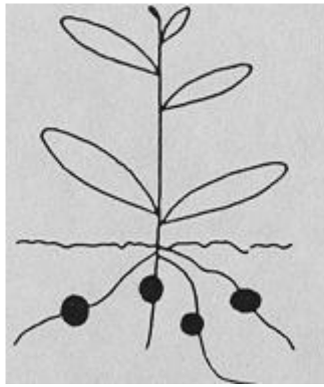
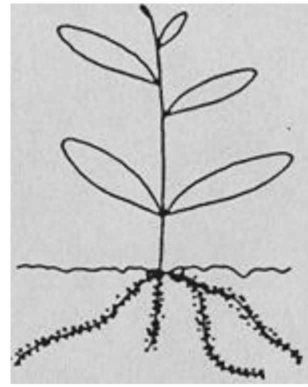
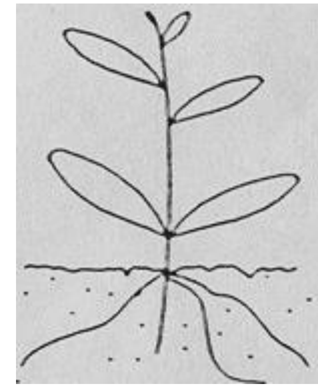
(Dobbelaere et al. 2001, Austr. J. Plant Physiol. 28: 871-879)

Inoculation with associative N_2 fixers more frequently leads to improved growth and higher yields in sub-/tropical climates

Field inoculation of cereals with Azospirillum in Mexico

Crop	No. of evaluated sites	Evaluated area (ha)	Positive effects (%)	Range of grain yield increase (%)	Average increase (%)
Maize: no N-fertilization or less than 90 kg N ha^{-1}	62	248	95	11–98	33
Maize: fertilized in the range of 100 to 150 kg N ha^{-1}	69	276	62	6–49	12
Sorghum: no N-fertilization or less than 90 kg N ha^{-1}	12	48	62	11–73	36
Sorghum: fertilized in the range of 100 to 150 kg N ha^{-1}	8	32	63	6–17	10
Wheat: no N-fertilization or less than 90 kg N ha^{-1}	7	35	83	10–58	22
Barley: no N-fertilization or less than 110 kg N ha^{-1}	13	39	86	17–65	42

N2 fixation potential in different BNF systems

	symbiosis	associations	free living	
system of N_2 fixation ($N_2 \rightarrow NH_3$) and microorganisms involved	 (e.g. *Rhizobium*, *Actinomycetes*)	 (e.g. *Azospirillum*, *Azotobacter*)	 (e.g. *Azotobacter*, *Klebsiella*, *Rhodospirillum*)	
energy source (organic carbon)	sucrose metabolites (from the host plant)	root exudates from host plant	heterotroph. plant residues	autotroph. photosynt.
estimates of amounts fixed (kg N/ha*a)	Legumes: 50-400 Nodulated non-egumes: 20-300	30-40 (tropical C4 grasses) 10-30 (temperate climates)	<5 (temperate climates)	10-80

(adapted from Marschner, 1995 and Roper and Gupta, 2016 Open Agr. J. 10: 7-27)

Comparison of symbiotic and free living N_2 fixation

Symbiotic N_2 fixation:

about 7g carbohydrates /g reactive N

= 1 t carbohydrates /150 kg N

- direct and preferential access to host carbohydrates,
- low competition due to endophytic colonization;
- N release by microsymbionts

N_2 fixation by free living C-heterotrophic microorganisms:

= 1 t carbohydrates (crop residues) /1.5 kg N

- Substrate (C) limitation
- Competition with other C-heterotropghic microorgamnisms
- N release mainly related with turnover of microbial biomass

Aspects to be considered for efficiency assessment of associative BNF inoculants

- Benefits **largely determined by site-specific conditions**
  - Tropical climates with rapid and intense C turnover,
  - C_4 grasses with high C-input as root exudates and crop residues and preferential endophytic associations

- Global change: Increased temperatures ? Increased rhizosphere C alloation ?
- Conservation agriculture – reduced tillage, C accumulation in top soil ?

- More widespread PGPR effects by additional modes of action (root growth promotion, stress priming)

- Low input vs standard input systems

Wheat Production	Grain yield [t ha^{-1}]	N demand [kg ha^{-1}]	N_2 fixation [t ha^{-1}]	BNF share of N demand [%]
Germany	6-8 t/ha	160	10-30	6-19
Australia	1-2 t/ha	40	10-30	25-75

(adapted from Roper and Gupta, 2016 Open Agr. J. 10: 7-27)

Example: Phosphate Solubilizing Microorganisms (PSMs)

Proradix

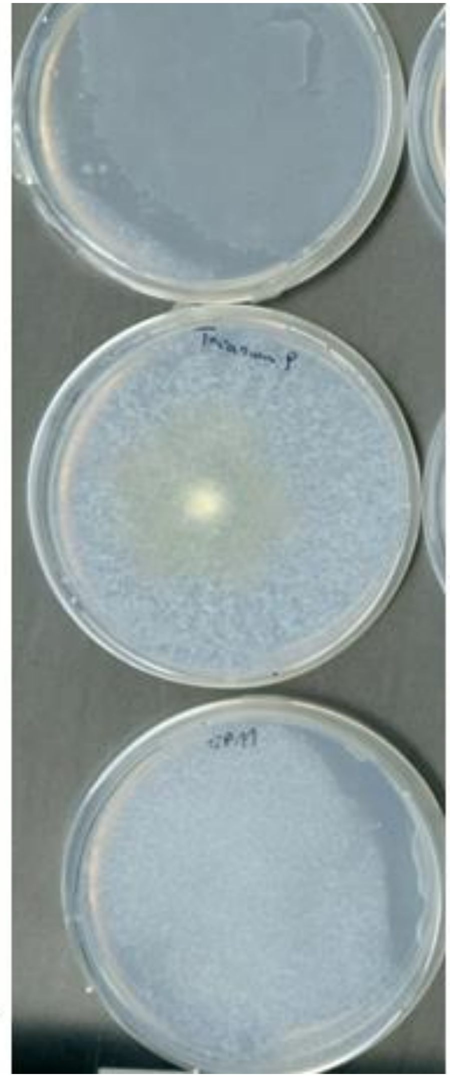
(***Pseudomonas DMSZ 13134***)

Paenibacillus m.

Biol. Fertilizer OC

(***Penicillium sp.***)

Kuhlmann & Nkebiwe, 2014



Rhizovital

(***Bacillus amylo-liquefaciens***)

Trianum P

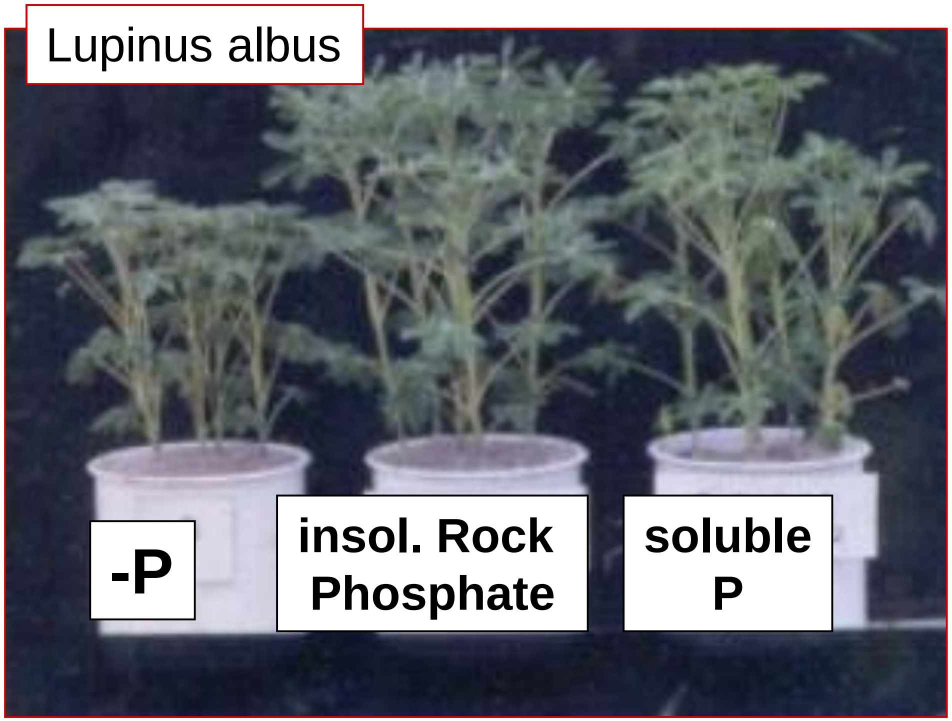
(***Trichiderma Harzianum T22***)

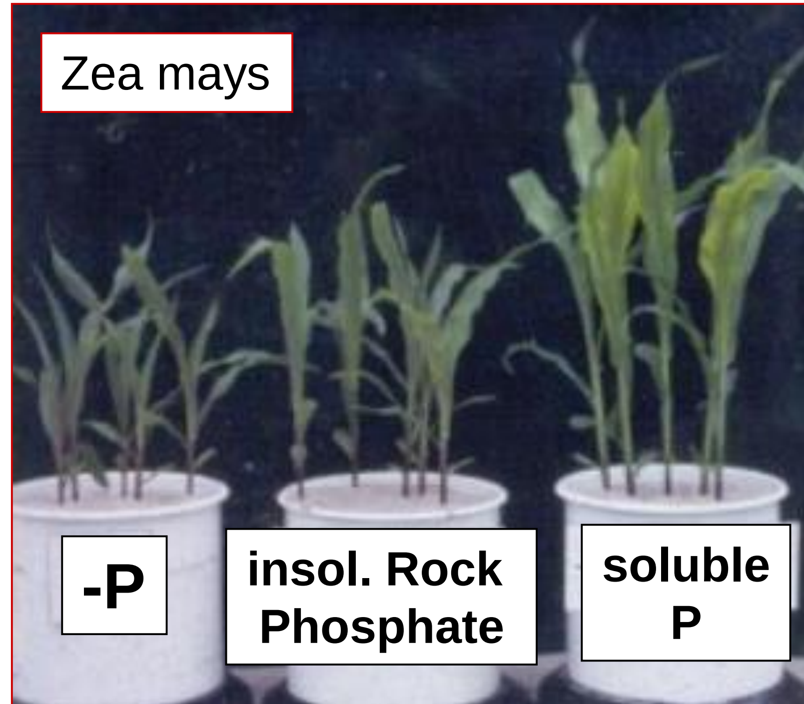
Vitalin SP11

(Combination product)

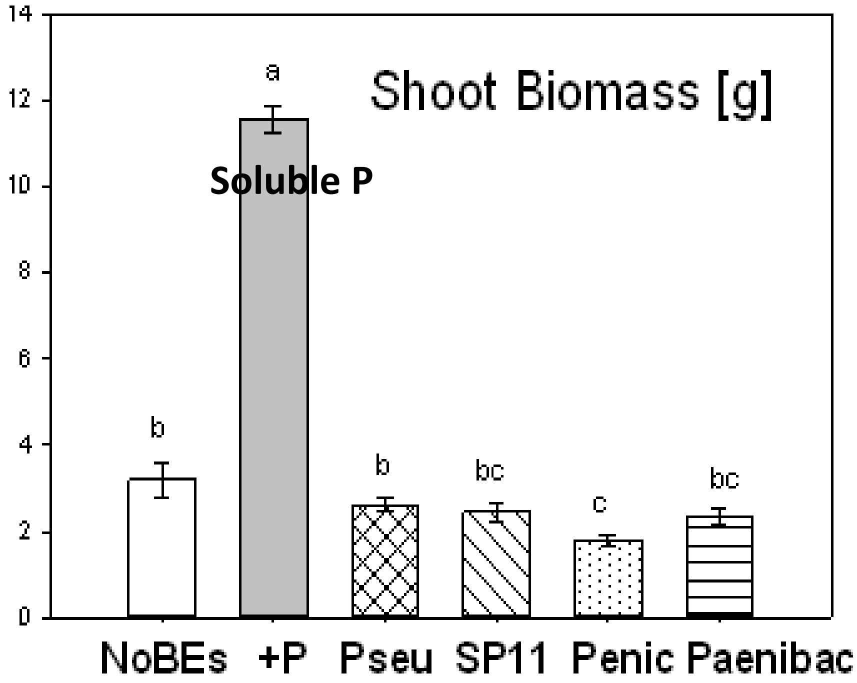
14 fungal and bacterial strains with phosphate-solubilizing potential **(PSMs**: *Trichoderma, Penicillium, Pseudomonas, Bacillus, Paenibacillus, Burkholderia and Streptomyces)* identified in the BIOFECTOR project

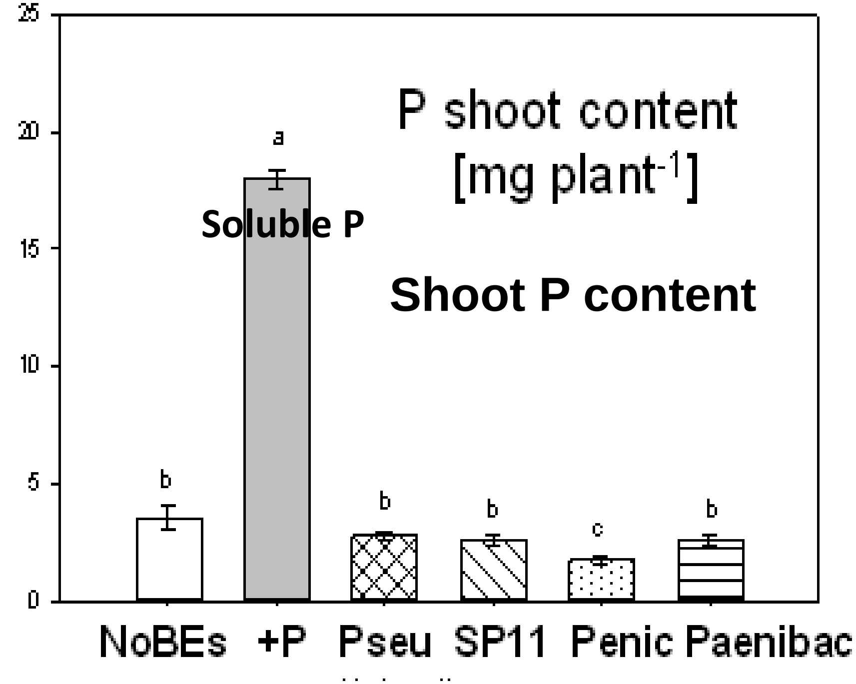
Requirements for testing PSM-mediated plant growth promotion





- **Microbial strains with P-solubilising potential**
- **Test plant without P-solubilizing potential**
- **Soil with limited plant-available (soluble) P levels but rich in spatingly soluble P sources**





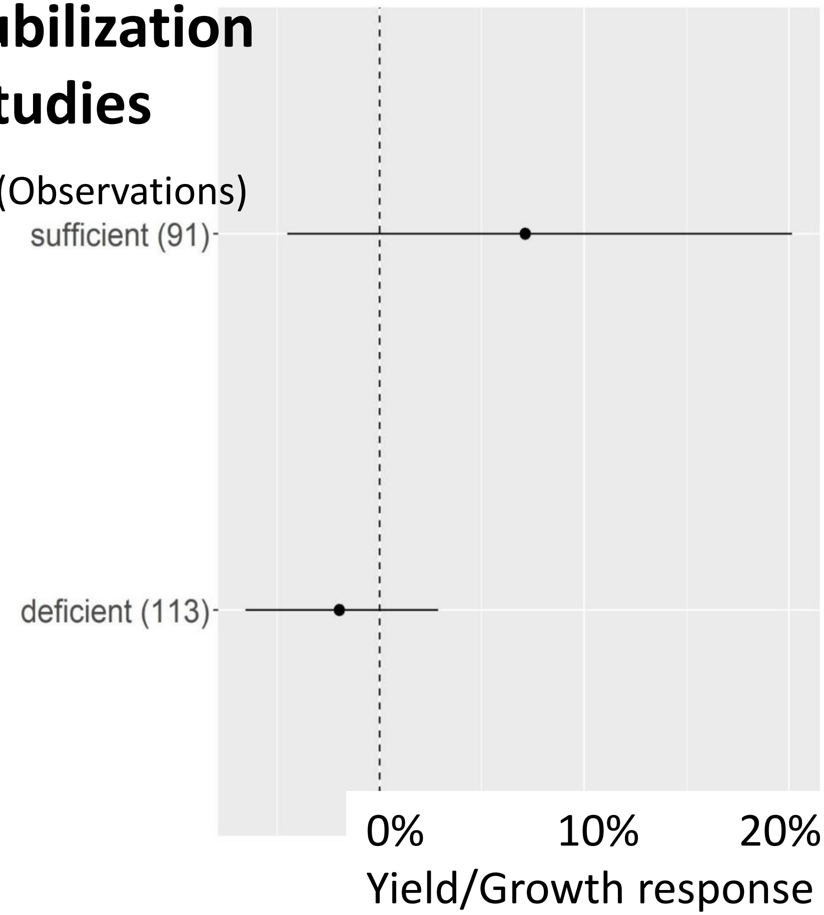
(Probst, et al., 2014)

- **No plant growth promotion in maize via P mobilisation by selected PSMs on a low-P soil pH 7.5 with insoluble Ca-P as dominant P source**

- **Similar results in 10 experiments on low-P soils with 14 BEs in 8 countries and 4 crops**

Lekfeldt et al. 2016 Chem. Biol. Technol.in Agriculture *3:22*
Thonar et al. 2017 Chem. Biol. Technol.in Agriculture *4:7*

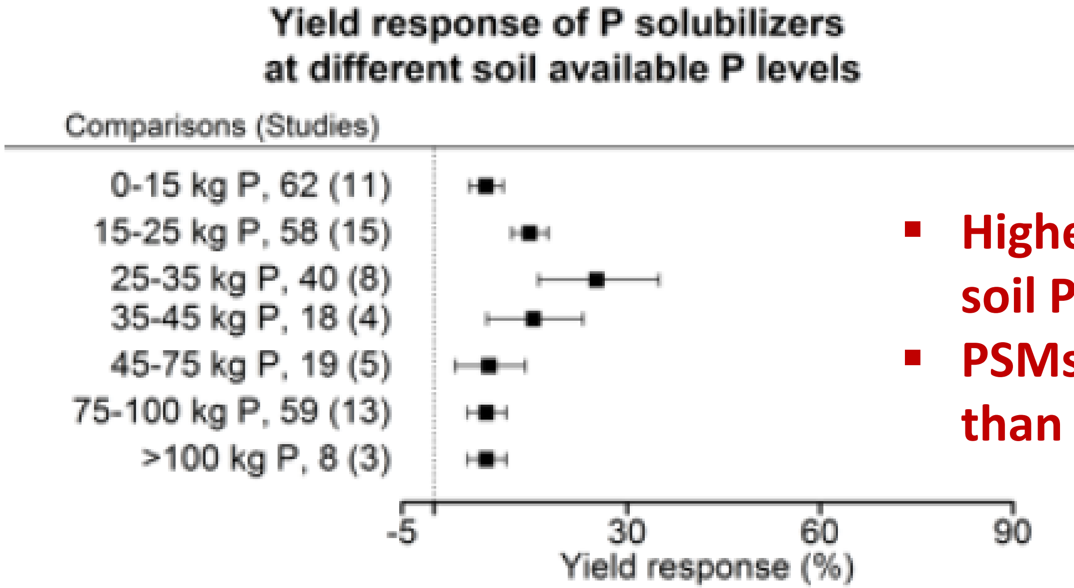
Low efficiency of PSM-assisted P solubilization in crops also confirmed in Metastudies



A) **BIOFECTOR Metastudy** (150 experiments 38 products, 3 crops)
Effect of the soil P status on growth/yield responses of microbial inoculants

Lekfeldt et al. 2017

B) **Literature survey** (160 studies)

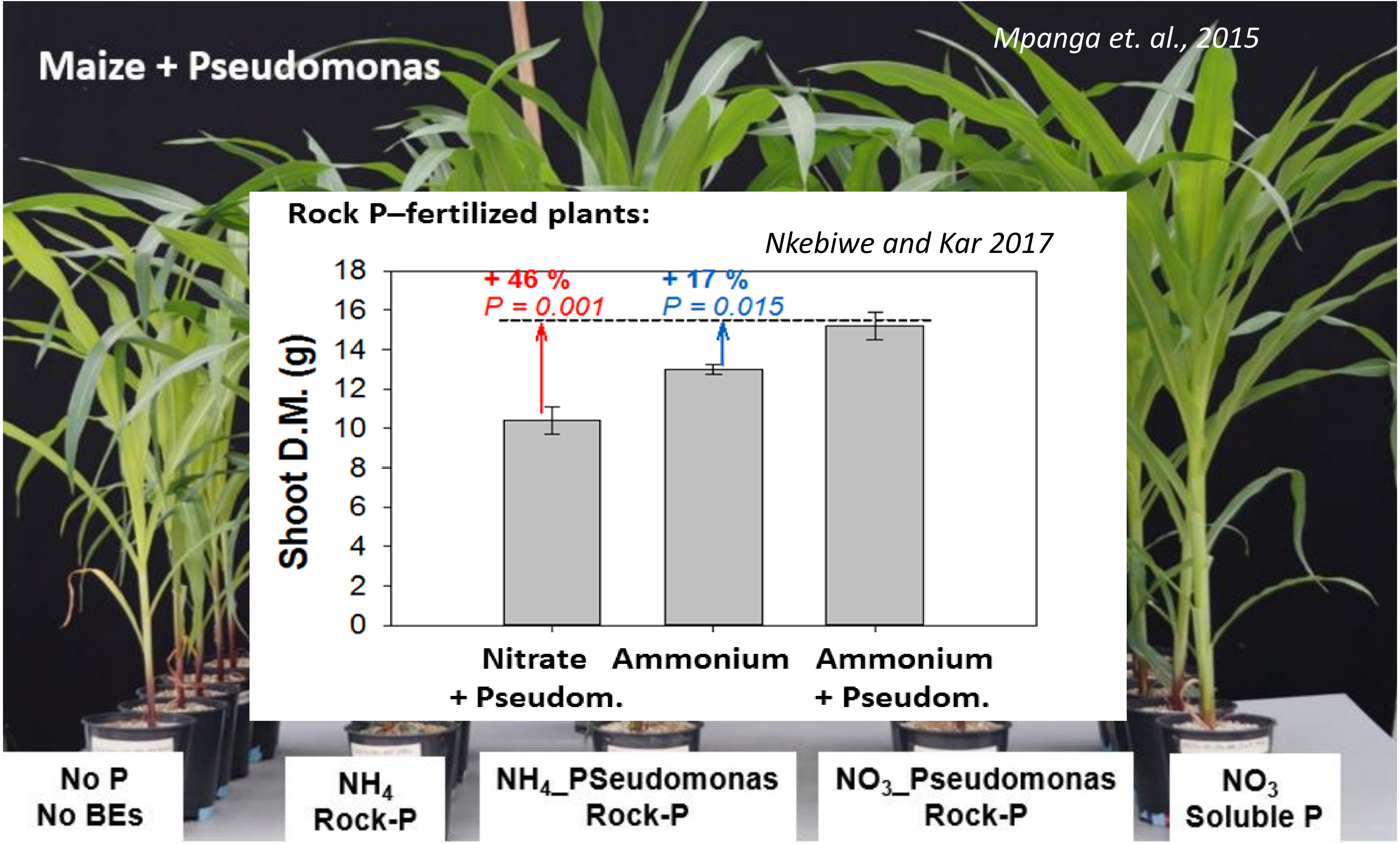


- Highest effciency at moderate available soil P levels.
- PSMs rather support soluble P acquisition than mobilization of insoluble P

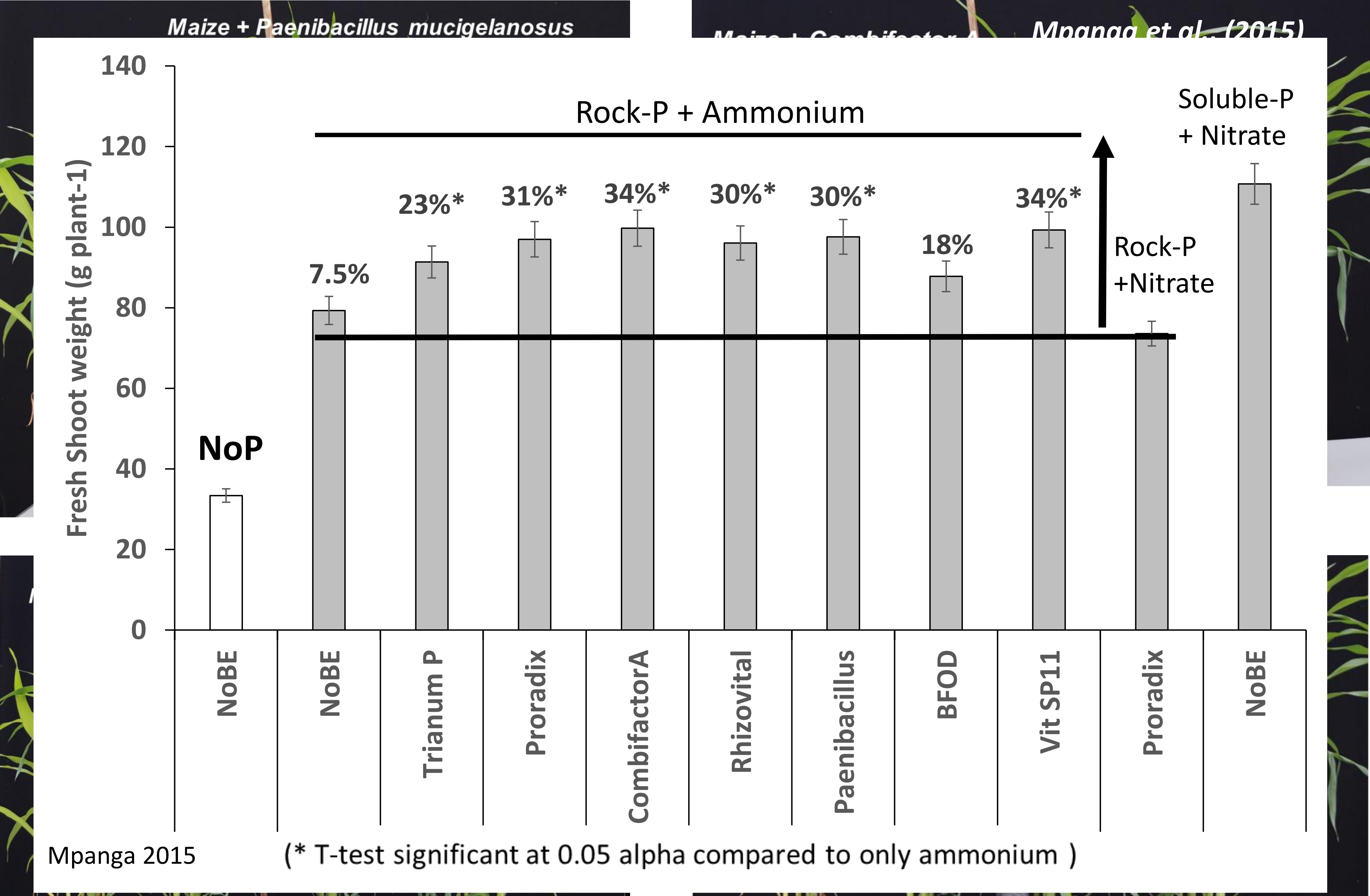
Schütz et al. 2017 Front Plant sci accepted

- However, similar to biological N_2 fixation the expression of the P solubilizing potential of biostimulants strongly depends on the culture conditions

Example: Microbial P soubilization affected by the form of N fertilization

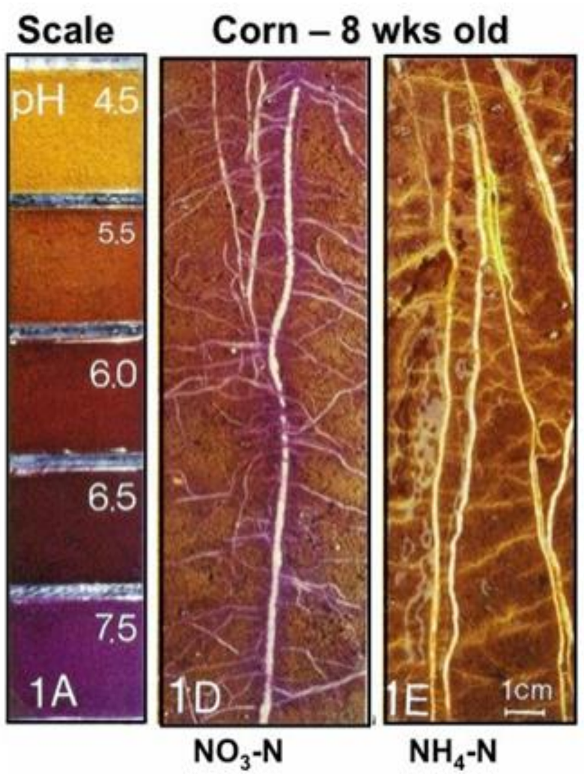


Stabilized Ammonium fertilization synergistically supports plant growth promotion in maize supplied with sparingly soluble Ca-P (Rock-P) after inoculation with *Pseudomonas sp. DMSZ13134 (Proradix)*

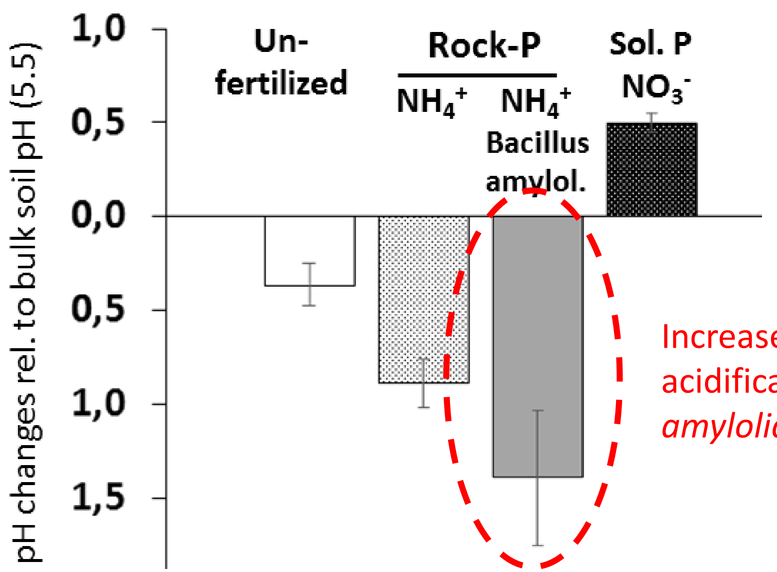


Similar synergistic ammonium effects also after inoculation with other bacteria and fungi belonging to the genera *Trichoderma, Penicillium, Pseudomonas, Bacillus, Paenibacillus, and Streptomyces*.

Benefits of ammonium fertilization for Plant- PGPM interactions

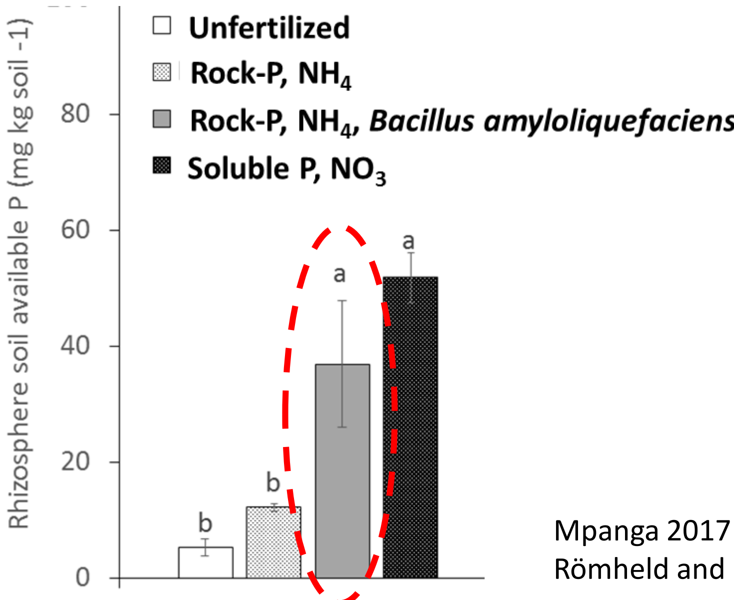


NH$_4$-induced rhizosphere pH changes in maize



Increased rhizosphere acidification by *Bacillus amyloliquefaciens*

Increased P availability in the rhizosphere



Ammonium uptake induces rhizo-sphere acidification which mediates Rock-P mobilization:

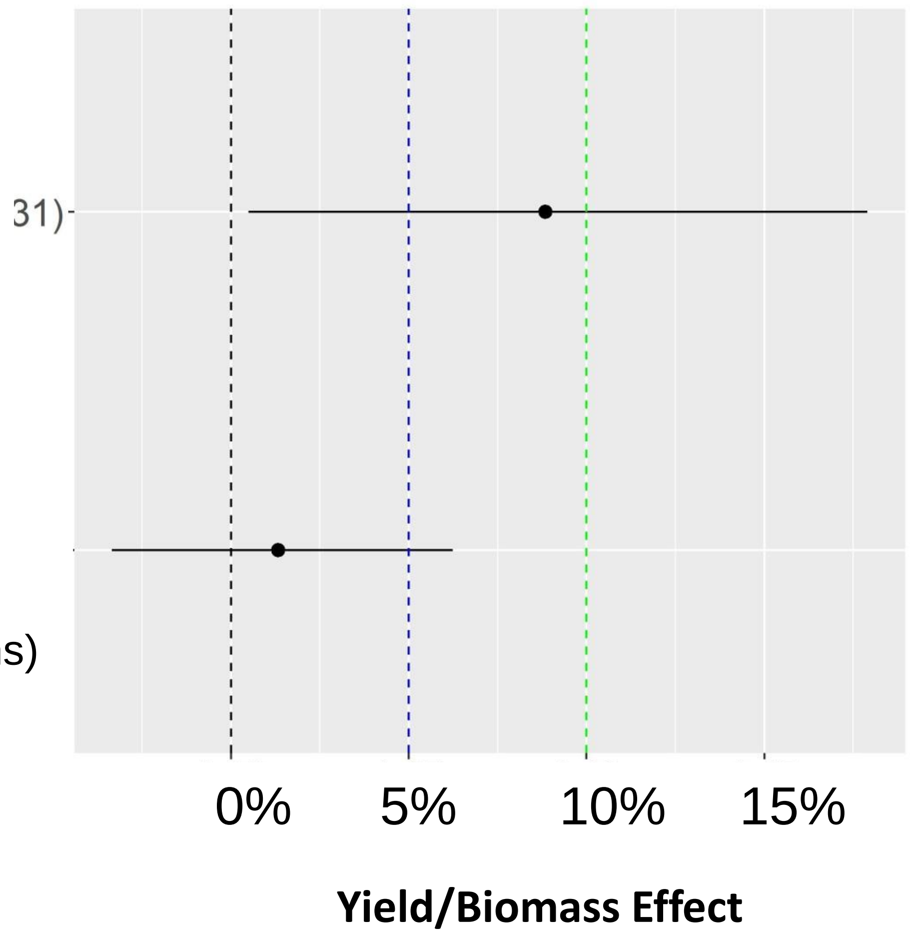
- supports activity of P solubilising BEs
- is supported also by root growth-promoting BEs due to formation of a larger acidifying root system

Mpanga 2017
Römheld and Maschner 1993

Synergistic effects of PSMs with stabilized Ammonium fertilization also confirmed by the BIOFECTOR Meta-study

Stabilized Ammonium
(31 observations)

Other soluble N fertilizers
(134 observations)



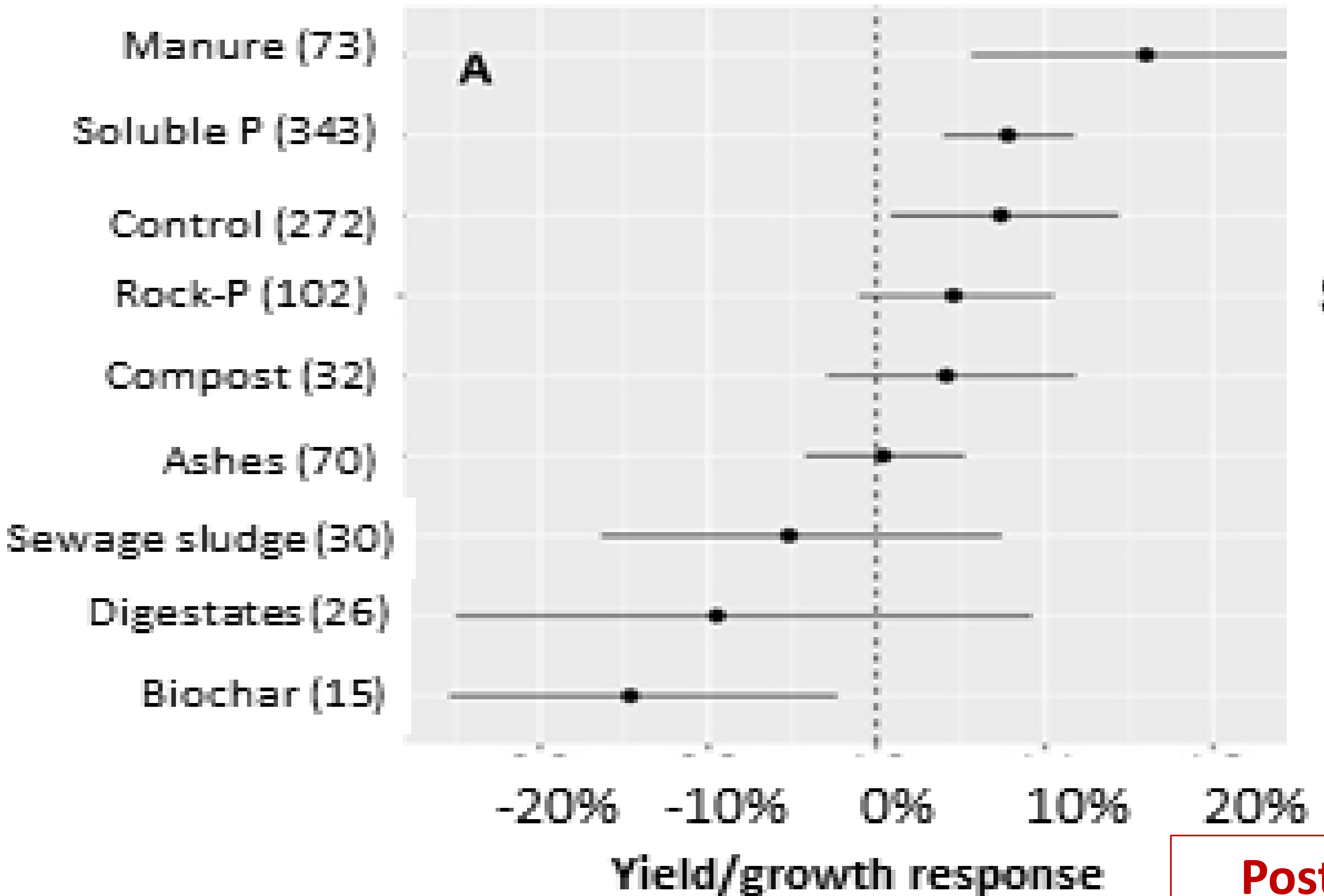
9 % effect of PSMs here

Only significant in combination with stabilized ammonium

Poster Mpanga et al.

Lekfeldt et al. 2017

Highly selective effects of microbial biostimulants also in combination with organic P fertilizers

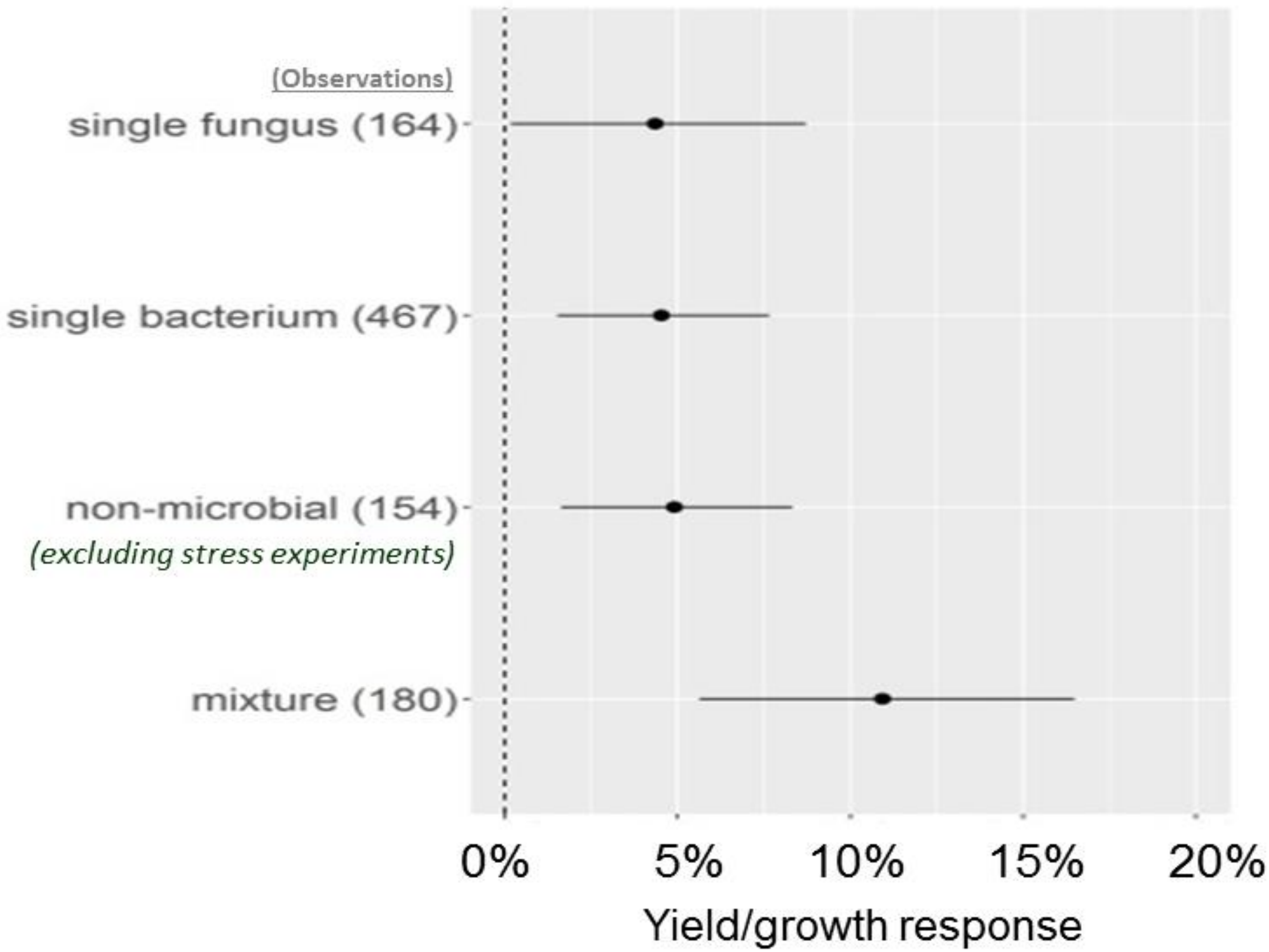


Lekfeldt et al. 2017

Poster Posta et al.

Assessment of Consortium Products

Better performance of combination (consortium products) as compared with single agents ?



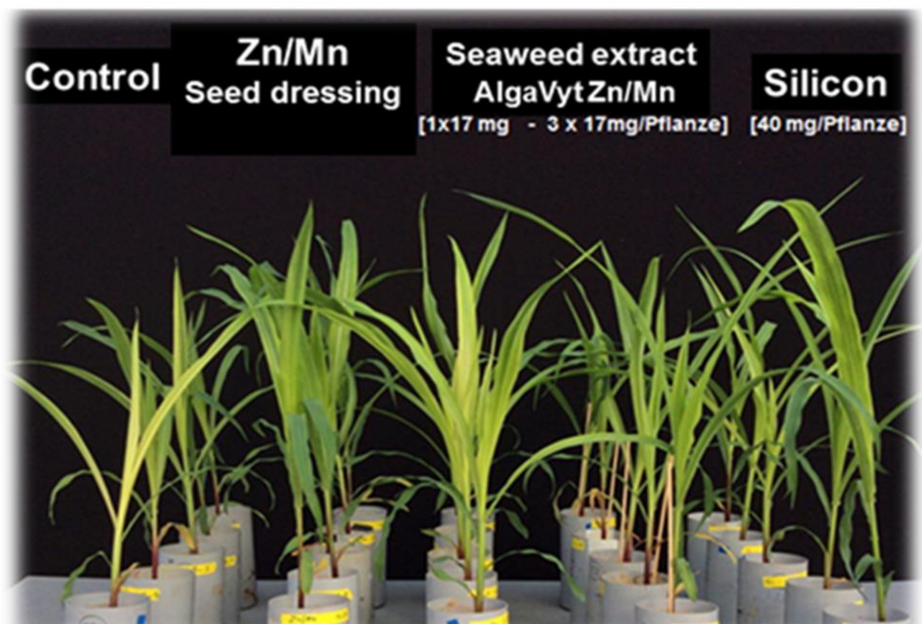
The effect of single or combination product (mixture) application on the mean effect of microbial inoculants on plant growth/yield. A total of 965 observations from BIOFECTOR pot and field experiments were included into the analysis (Lekfeldt et al., 2017)

Example:

Bio-stimulants for improved cold stress tolerance during early growth in maize
- Synergistic Effects -

Poster
Moradtalab et al.

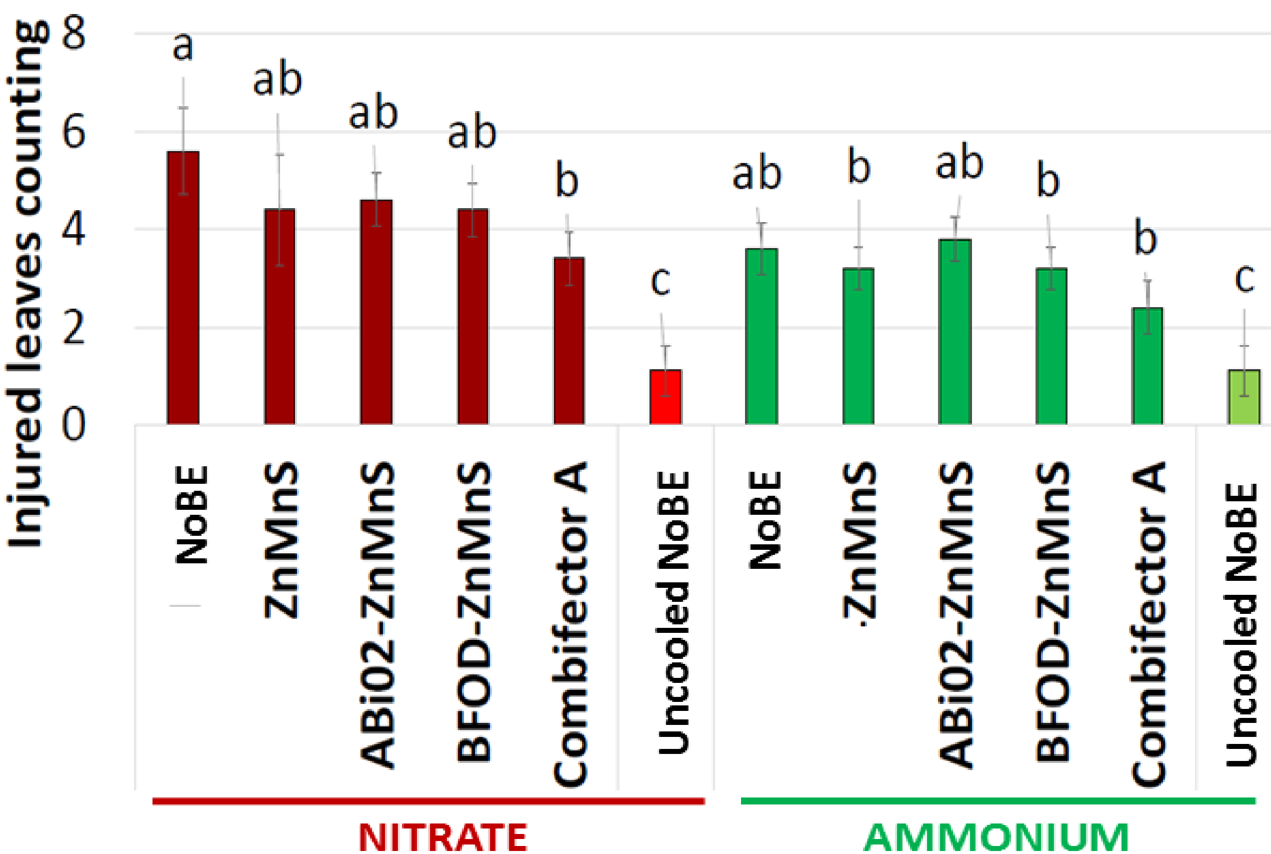
Maize ;2 weeks
12°C soil temperature



Bradacova et al., 2016 Chem. Biol. Technol. Agric. *3:19*

Number of damaged leaves

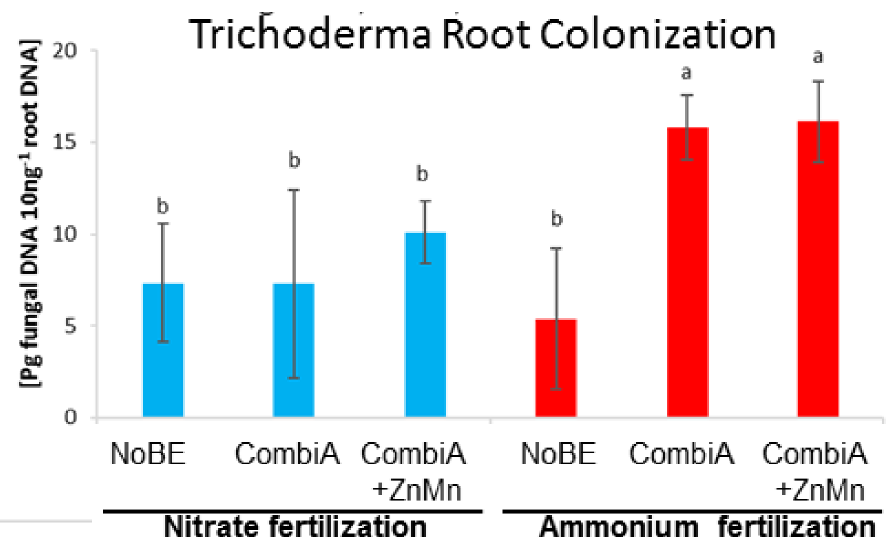
(cold stress experiment maize – silty loam pH 6.8, 14d 12°C)



- Ammonium fertilization showed synergistic protective effects in combination with Zn/Mn seed dressing (ZnMnS) and microbial Inoculants:
  - **ABI02** (*Bacillus atrophaeus*) **BFOD** (Penicillium sp.),
  - **Combifector A:** Bacillus/Trichoderma/Pseudomonas + Micronutrients
- Leaf damage declined in the order :
  ABI02+ZnMn > ZnMn > BFOD+ZnMn > CombifectorA > uncooled control

Synergistic activation of metabolic stress defence lines by fertilizer effects and different components of the consortium product CombiA

% change relative to NO_3 fertilization	NH_4 only	CombiA	CombiA Zn/Mn
Root length	n.s	+ 101	+159
Oxidative stress			
Leaf damage	**- 33**	**- 33**	**- 62**
SOD	+23	+51	+ 66
POD	+29	+58	+ 58
Antioxidants	+15	+46	+ 46
Phenolics	+13	+38	+130
Cryo-Protectants			
Proline	n.s	+104	+102
Sugars	n.s	+ 72	+ 34
Nutrient status: Zn	+72	n.s.	+133
Stress Priming			
ABA	+36		
Salicylic acid	+28		
Jasmonic acid	+42		
Indole acetic acid	+48		



- Ammonium fertilization but not Zn/Mn promotes root colonization with the CombiA strain of Trichoderma

Moradtalb, Ahmed and Geistlinger 2017

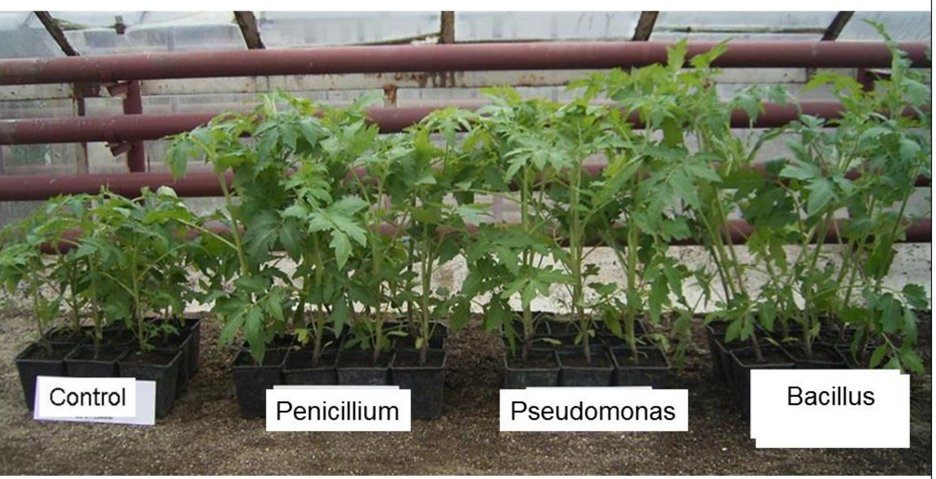
Tomato, low P soil, 50% P fertlization level

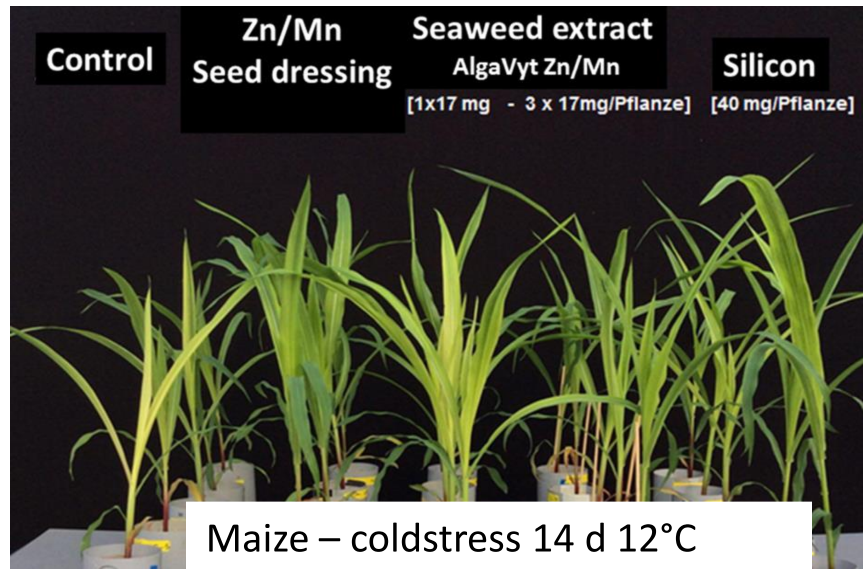
Control Trichoderma Proradix FZB42 P. jessenii

Maize, low P soil, 50% P fertlization level

P. jessenii FZB42 Proradix Trichoderma Control

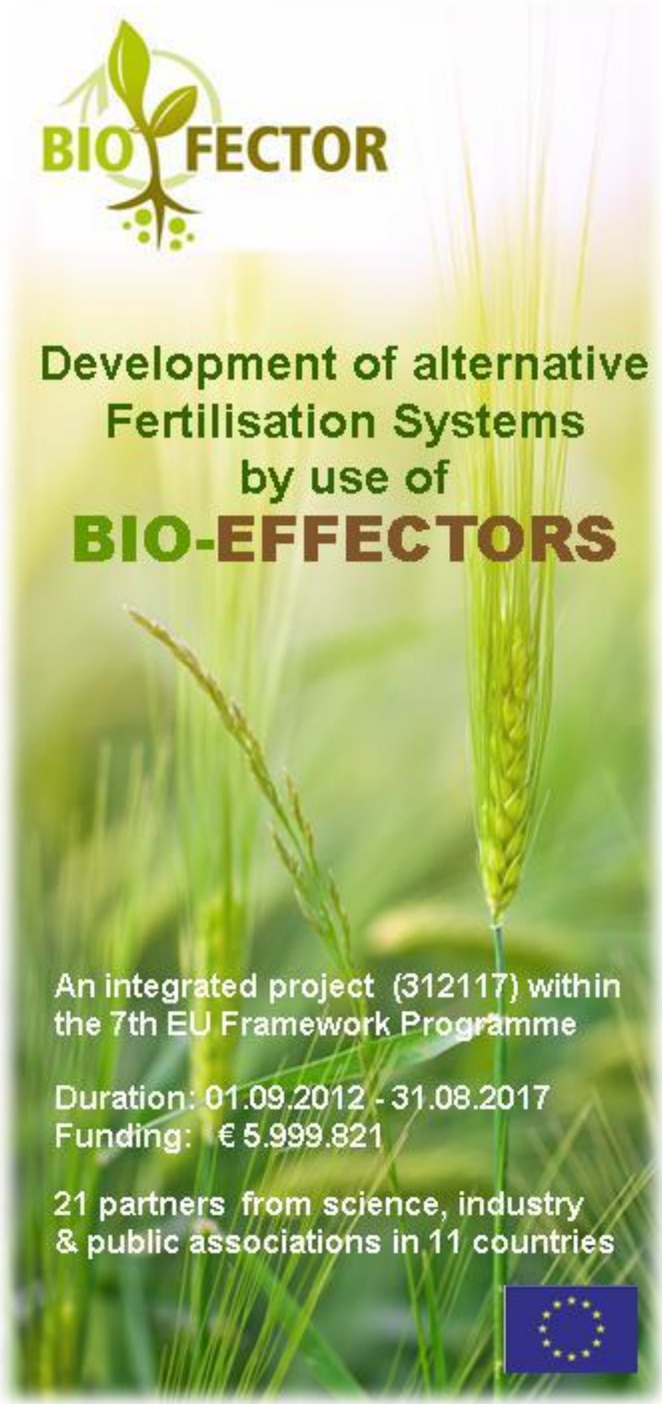
Organic Recycling Fertilisers – Composted Manures





The right set of circumstances (**RSC)**

Under suitable application conditions, Biostimulants of different origin exhibit similar effects → underlines importance of environmental factors



Conclusions

- The efficiency of **biostimulants** is largely determined **by specific application conditions**

„The right Set of Circumstances"(RSC)
No general responses !

- Under **RSC** conditions significant und reproducible effects can be expected for various BEs of different origin

- Our challenge is a clearer definition and under-standing of the **RSC** condtions, and to find management tools to meet these requirements for practice implementation