Visegrad Fund

Education of young scientist in ecologically friendly agriculture through WB6-W4 networking

Contents

Application of Polish *Trichoderma* biocontrol strains in vegetable production

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Introduction

Introduction of mineral fertilizers and pesticides into agriculture in XX century caused significant improvement in food production overall the world. However, after decades of intensive farming, serious problems with soil and water pollution, soil degradation, and residues of harmful compounds in food have appeared. Ploughing of fields with heavy equipment combined with excessive use of fertilizers and reduced crop rotation has considerably degraded soil structure, bio-functionality and biodiversity. Estimation of the area of arable soils in the world, which have been degraded so far is difficult, and the information in the literature indicates about 30 - 40%. Such soils are subject to erosion, which is much faster than the rate of humus layer creation. It is very serious problem, especially under drought conditions in global warming. Moreover, reducing the biodiversity of agricultural environments encourages the spread of pathogens and plant pests, resulting in the increasing use of pesticides. The strong pressure of pesticides, in turn, causes pathogens to become resistant to the most commonly active substances used in crop protection (Brent and Hollomon 2007).

In such conditions, the cultivation of crops requires more and more efforts to get satisfactory and high quality yield. Microbial activity in soil is a crucial factor of its functionality and plant productivity. In order to restore natural balance in soil ecosystem, microbial inoculants are involved to improve farming techniques in sustainable agriculture (Vimal et al. 2017, Gouda et al. 2018, Qiu et al., article in press). In recent years the popularity of microbial inoculants has increased, as new technics of screening and examining of plant-microbial interactions have been involved. It was found, that about 1% - 35% of microorganisms isolated from soil environment is able to show

antagonistic activity to inhibit the growth of pathogens *in vitro,* and the proportion of isolates which express plant growth promoting traits was found up to 2/3 of the cultivable population (Berg 2009). However, only sparse isolates from this pool have attributes that make them suitable for commercial application in agriculture. In fact, a few groups of microorganisms are more widely used as biopesticides or biofertilizers due to their ability to protect plants and promote growth. Among them, the most popular are bacteria *Bacillus* and *Trichoderma* fungi, which are able to survive in highly competitive environments.

The species of *Trichoderma* are known as saprotrophs or mycoparasites which are common in soil ecosystem. During the last years, these fungi have attracted particular interest because of their bioactivity against economically important fungal diseases of crop plant

Why *Trichoderma*?

Trichoderma **are particularly invasive fungi and good colonizers of the new sites. They interact with plants and phytopathogens using multiple mechanisms and a wide array of secondary metabolites. Moreover, they are easy to produce and to store for prolonged time.**

<u>The attributes of *Trichoderma* for agricultural use</u>:

Widespread occurrence - *Trichoderma* intensively colonizes the environment, including the root zone and internal tissue of plants, due to the ability to use very different substances as a source of nutrients, as well as to produce a wide range of metabolites: extracellular proteins, lytic enzymes, antibiotics, volatile compounds (VOCs).

Trichoderma **spp. can be found in many ecosystems, in different climate zones. These are some of the most frequently isolated fungi from the soil (about 10^3 cfu/g of soil).**

High reproducibility, intensive colonization of the new environment after introduction, and ability to survive in different, also unfavorable conditions.

Multiple mechanisms of action.

Production of numerous secondary metabolites.

In agriculture *Trichoderma* act as plant bio-stimulators and biocontrol agents.

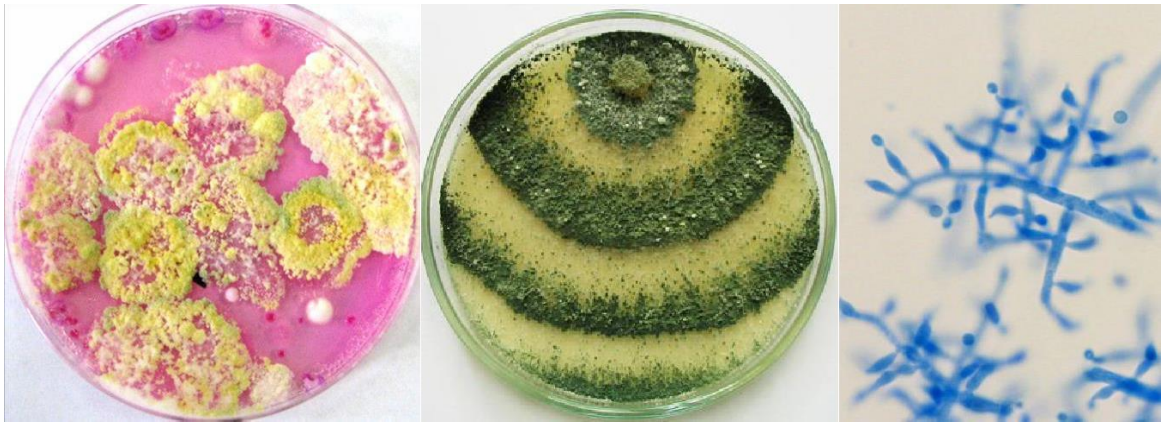
Easy mass production - intensive growth in artificial breeding conditions and production of numerous spores.

Trichoderma **have a good shelf life during storage.**

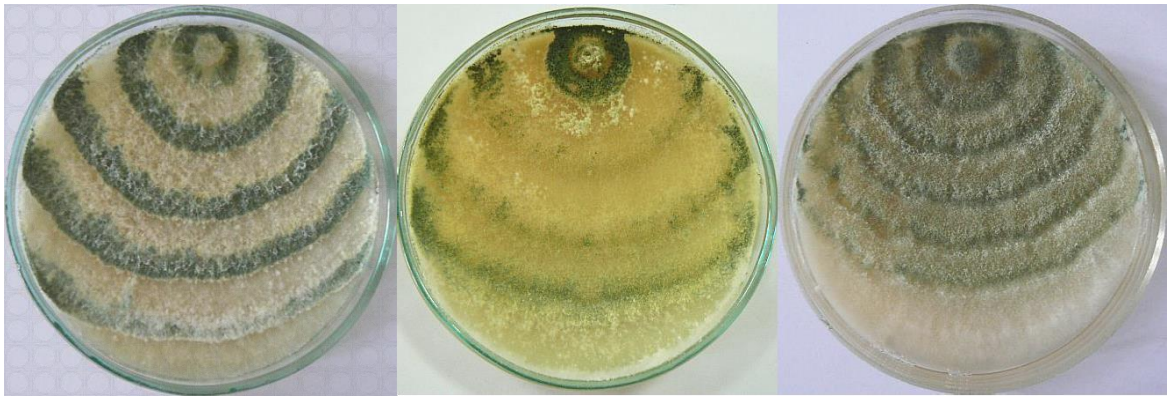
Trichoderma **spp. possess innate resistance to most agricultural chemicals, including fungicides, although individual strains differ in their resistance.**

Trichoderma **have numerous properties that can be used also in the industry (e.g. paper, textile, food and feed industry),**

Images of *Trichoderma*



Fot. 1. From the left: colonies of *Trichoderma* spp. isolated from soil on rose bengal agar; growth of *Trichoderma* culture on malt extract agar; *Trichoderma* conidiophores in microscope.



T. atroviride ***T. harzianum*** ***T. virens***

Fot. 2. Growth of *Trichoderma* isolates on PDA

Mechanisms of action and secondary metabolites production

Many years of intensive research have shown that *Trichoderma* can be an alternative method in plant protection to pesticides as well as a factor supporting plant growth in stress conditions. Such properties of these fungi depend on their ability to produce diverse secondary metabolites and on the use a wide range of mechanisms of action affected plants as well as microorganisms in soil:

increasing the availability and the uptake of nutrients difficult to access for plants production of phytohormones stimulating growth,

induction of resistance mechanisms in plants,

competition for nutrients and space,

antibiosis,

mycoparasitism.

Thanks to such wide abilities, *Trichoderma* fungi can effectively compete in variable environments with other microorganisms, and at the same time interact with plants. As a results of these interactions both organisms benefit.

The screening of beneficial isolates usually starts from laboratory examination of antibiotic and mycoparasitic properties, ability to compete for nutrients, and then plant growth stimulation and induction of plant resistance to biotic and abiotic stresses, which are studied in controlled conditions. However, in field environment, plant growth stimulation and induction of resistance may play the major role in *Trichoderma* activity. The benefit of using these fungi for plants rely on rapid establishment of stable community in the rhizosphere, where *Trichoderma*-plant interactions take place (Mendoza-Mendoza et al. 2018). The other mechanisms (competition, mycoparasitism, antibiosis) may play supporting role in these rhizosphere-interactions. It is important to remember, that the mode of action of *Trichoderma* in natural conditions is complex and rely on multiple mechanisms. In most cases it is impossible to separate direct effect of *Trichoderma* on plant

growth from the control of the pathogenic and other deleterious microorganisms that reduce plant growth.

<u>Brief description of *Trichoderma* mechanisms</u>:

Nutrient solubilization

***Trichoderma* can increase the access to poorly available for plants nutrients (P, Cu, Mn, Zn),**

solubility of the nutrients is due to production of organic acids by the fungi (e.g. citric or fumaric acids),

production of siderophores chelating Fe^{3+}, make it accessible for plants and limits iron necessary for fungal pathogens development.

Plant growth stimulation

enhancing nutrients solubility by *Trichoderma* increase their uptake by plants,

modulation of root architecture for improving water and nutrient uptake efficacy and their translocation within plant parts, and also aleviation of abiotic stresses (e.g. drought),

stimulation of seed germination and plant growth by production of substances that act as plant growth hormones:

indolyl acetic acid (IAA) - stimulates plant biomass, increasing the number of roots,

ACC deaminize reduces ethylene (stress factor for plants),

stimulation of photosynthesis process in plants and the rate of carbohydrate metabolism,

increase chlorophyll content.

Fot. 3. Stimulation of tomato biomass and root system by *Trichoderma.*

Induction of resistance mechanisms in plants

interaction of elicitors (low molecular weight compounds) released by *Trichoderma* hyphae may lead to the initiation of local defence reactions and to systemic changes in metabolism and immunity of plants,

the type of induced resistance may be difficult to determine due to the variety of biochemical pathways activated in plants byTrichoderma,

depending on *Trichoderma* strain, plant species and pathogen, resistance may take the form of induced systemic resistance (ISR) with jasmonic acid (JA) and ethylene (ET) as signal molecules or acquired systemic resistance (SAR) with salicylic acid (SA) as a mediator of immune reactions,

recently it has been suggested that there is a mixed ISR/SAR resistance combining activation routes of both types of resistance. This type of resistance induced by *Trichoderma* is referred to as *Trichoderma*-induced systemic resistance (TISR), which involves a wider variety of signaling routes.

The scheme of defence induction in plants:

interaction of elicitors released by *Trichoderma* with plant receptors

⬇

depolarization of the cell membrane and changes in pH leading to increased production of reactive oxygen species

⬇

activation of the basic defence mechanisms in plant:

enzymes of the antioxidant system

enzymes involved in strengthening structural defence barriers

synthesis of the secondary metabolites.

Competition

competition for nutrients - in the case of *Trichoderma* competition with pathogens for iron is mainly mentioned; *Trichoderma* produce low molecular weight protein compounds called siderophores, which have a high affinity to iron ions,

competition for space - *Trichoderma* has the ability to quickly retrieve and effectively use nutrients from a variety of sources, so it can grow faster, colonize the environment and compete with other microorganisms for space.

Antibiosis

antibiosis is one of the types of antagonism based on the secretion of various secondary metabolites that inhibit or kill other organisms,

***Trichoderma* produces numerous secondary metabolites (low molecular weight molecules) that do not directly affect their growth and**

reproduction, but play an important role as signal molecules and in the formation of interactions with other microorganisms,

Trichoderma **produces pyrones, terpenoids, polycetides, peptaiboles, terpenes and numerous volatile compounds (VOC),**

one strain of ***Trichoderma*** **can produce several different compounds,**

the qualitative and quantitative production of antibiotics may vary depending on strain and even on the stage of growth of the fungus, and is also strongly related to environmental conditions and nutrients availability.

Mycoparasitism

Trichoderma **may parasite and degrade mycelium and spores of other fungi, including pathogens,**

the degradation of pathogen structures is a result of the activity of cell-wall degrading enzyme complexes supported by antibiotics,

the stages of mycoparasitism:

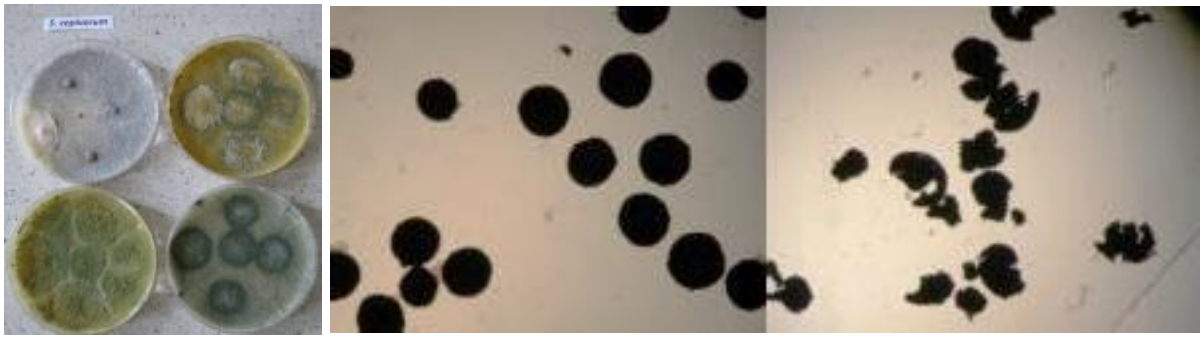
detection of other fungi and growth in their direction (chemotrophic growth),

contact with the fungus and coiling around its hyphae,

formation of apressorium-like penetration structures on the surface of the host hyphae (affinity to the lectin in the wall of the host),

production of the cell-wall degrading enzymes,

penetration into the light of host structures ending in its death.



Fot. 4. Mycoparasitism of *Sclerotium cepivorum* sclerotia by *Trichoderma.*

Detailed mechanisms of *Trichoderma* activity have been described in many review publications e.g.: Harman et al. (2004), Nawrocka and Małolepsza 2013, López- Bucio et al. (2015), Contreras-Cornejo et al. (2016), Waghunde et al. (2016) and in books e.g. Gupta et al. (2014).

Trichoderma secondary metabolites, enzymes and their functions

***Trichoderma* fungi are producers of numerous secondary metabolites such as non-ribosomal peptides, terpenoids, pyrones, polyketides, peptaibiotics, siderophores, indolic-derived compounds and volatile organic compounds (VOC) (Contreras-Cornejo et al. 2016, Lee et al. 2016, Zeilinger et al. 2016). These metabolites are not directly involved in normal growth of the fungi but support their survival in diverse environments. It should be indicated that, the prevalence and competitiveness of *Trichoderma* in nature, and their success as biocontrol agent or biostimulant is mostly related to the ability of these fungi to produce variable metabolites and their combined actions.**

The role of Trichoderma secondary metabolites **(according to Zeilinger et al. 2016):**

support of the rhizosphere competence with other microorganisms (e.g. siderophores production limits iron making it unavailable to competing microorganisms, antibiotics with antifungal and antibacterial activity),

combined action with hydrolytic enzymes in mycoparasitic activity,

involvement in self-regulation of *Tichoderma* coiling around parasitized fungal hyphae,

mediation of communication between plants and fungi,

triggering of localized and systemic responses in the plants,

attachment of *Trichoderma* to the roots,

involvement in self-protection of *Trichoderma* against adverse biotic and abiotic factors,

auto-regulatory substances regulating spore germination, colony morphogenesis and asexual development.

The production of secondary metabolites by *Trichoderma* is strain dependent and varies in relation to the equilibrium between elicited biosynthesis and biotransformation or biodegradation. The expression of metabolism-related genes is also highly related to external factors (e.g. nutrient base, pH, moisture), other organisms interactions and their signal molecules. Despite of the identification of almost 400 different metabolites produced by *Trichoderma* (Contreras-Cornejo et al. 2016), in many cases their specific activity and interactions are unknown. Moreover, not all compounds may be synthetized in laboratory conditions, thus the knowledge about biosynthesis pathways and their regulation is still limited.

The other important compounds produced by *Trichoderma* fungi are lytic enzymes such as: chitinases, proteases and glucanases (reviewed

by Viterbo et al. 2002, Hasan et al. 2014). These enzymes, mainly chitynases and glucanases, are involved in mycoparasitic properties of *Trichoderma* and play role in degradation of cell wall of parasitized fungi. The enzymes are usually extracellular, of low molecular weight and highly stable. Histochemical studies demonstrated that they cause localized lysis of cell walls at point of contact of the host with the antagonist. However, in many cases inhibition of host growth is a complex action of lytic enzymes with antibiotics.

The main functions of lytic enzymes **produced by *Trichoderma* (Hasan et al. 2014, Saravanakumar et al. 2016):**

breaking down of the polysaccharides, chitin and glucans, that are components of fungal cell walls, and destroy the cell wall integrity,

destruction of pathogens dormant structures in soil,

inactivation of the pathogenesis-related enzymes produced by pathogens and destruction of their capacity to damage plant cells,

triggering induced systemic resistance in plants.

Steps of *Trichoderma* selection for agricultural use (modified protocol described by Köhl et al. 2011):

Tab. 1. Steps for *Trichoderma* selection.

1	Choosing crops and for what purpose *Trichoderma* is to be used	
2	Selection of the source for isolation of the fungi	
3	Classification of the species (elimination of potential pathogenic species for human and mushroom)	Development of a rapid screening method for a large number of isolates, determination of their properties
4	Development of pilot formulation for initial field testing	Assessment of mass production capacity
5	Testing of the best formulations in different locations and over several growing seasons, in integration with the farming systems methods	Assessment of production costs and registration
6	Semi-technical production of the preparation	field testing

In the natural environment there are numerous microorganisms that indicate antagonistic abilities against pathogens *in vitro* or beneficial properties for plants at the stage of research. However, in many cases, in the implementation phase of the developed preparation, it appears that there are some technical problems with mass production and integration with farming techniques, or production in commercial

scale is not cost-effective. Therefore, from the very beginning, it is necessary to take efforts to assess the possibility of commercialization of planned solutions, as it is suggested in the table above.

Microbial strain intended for commercial use should meet the following requirements:

broad spectrum of action;

good multiplication on easy available and cheap materials for mass production;

stability and good viability during shelf life;

economical production;

market demand for its application.

Mass production and formulation of *Trichoderma*

Mass production of microbial inoculants in many cases is a bottle neck of successful introduction for commercial application. Transfer of laboratory scale production to industrial scale sometimes turns out complicated or cost-intensive. Among other microorganisms *Trichoderma* poses good saprophytic properties and generally are ease for mass multiplication in liquid media or on different organic materials. However, intensity of growth of various species may differ markedly depending on the specific requirement of nutrients (Fig. 1).

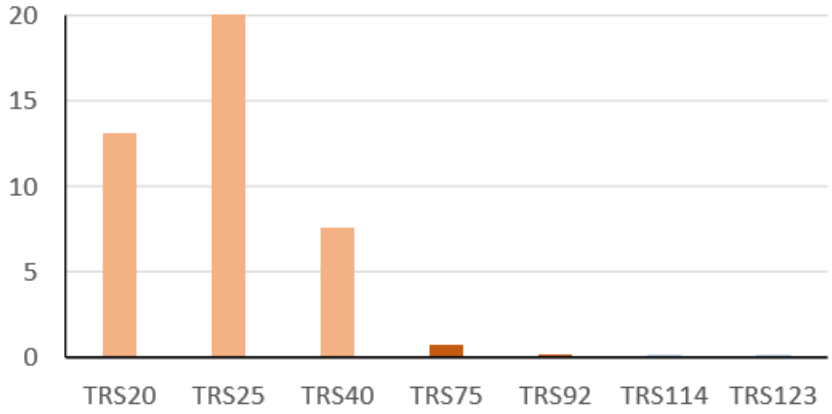


Fig. 1. The intensity of growth of various *Trichoderma* species in solid state fermentation on organic carrier. TRS20, TRS25, TRS40 – *T. atroviride*, TRS75, TRS92 – *T. harzianum*, TRS114, TRS123 – *T. gamsii.*

For mass production of *Trichoderma* liquid and solid fermentations are used (Kumar et al. 2014).

For liquid state fermentation (LSF) a liquid medium containing high quantity of water mixed with appropriate for fungal growth nutrients is used. Potato dextrose broth, V-8 juice, molasses-yeast medium are used for *Trichoderma* LSF. This system requires specific equipment (bioreactors).

For solid state fermentation (SSF) solid, organic, insoluble in water materials are used. These materials (e.g. cereals grains or bran, plant residues) are moistened with water, inoculated with *Trichoderma* and incubated for about two weeks to overgrown medium with fungal mycelium and to produce spores.

In the case of SSF, organic material used for fungal growth may serve as carrier for *Trichoderma*. Such preparation may be used directly for soil application or may be stored after drying. For commercial application of the fungi produced in LSF, formulation with suitable carriers to support *Trichoderma* survival should be performed. The following organic and inorganic carriers are commonly used: vermiculite-wheat

bran, wheat flour, coffee husks, sugarcane baggase, plant residues, talc, vermiculite, sodium alginate.

It is good to remember that various species and even strains of *Trichoderma* may have different survivability during storage. Therefore, viability control during shelf life is recommended.

Formulated preparations should have the following characteristics*:*

good shelf life in ambient conditions;

steady;

cheap;

not phytotoxic,

carrier for microorganism should have a precisely defined composition, and its production should be standardized;

easy to use and adopted for application with the use of common agricultural equipment;

compatible with agrochemicals.

Main factors affecting *Trichoderma* inoculations in field

Nutrient content in soil.

Amount of organic matter in soil.

Soil moisture and temperature.

Plant species cultivated.

Fertilization.

Activity of other soil microorganisms.

Effects of *Trichoderma* on plants in agriculture

Protection of plants against pathogens deleterious microorganisms and stress factors (e.g. drought, salinity).

Triggering of resistance in plants to diseases.

Increasing the availability and uptake of nutrients.

Improvement of plant growth and yield.

Positive impact on yield quality parameters.

Degradation of xenobiotics, including pesticides.

What else is there to know about the use of *Trichoderma* in plant cultivation?

Preparations with *Trichoderma* may be applied in agriculture as seed coating, seed bio-priming (treating of seeds with the spores of the fungus and then incubating them under warm and moist conditions until prior of seed emergence), seedling root dip, soil application, foliar spray.

Trichoderma **is not recommended for use in plant fertigation systems, because growing mycelium may block the flow of water and nutrients in the tubes.**

Trichoderma **may colonize the tissue covering the roots, but they do not penetrate deeper due to the activation of resistance mechanisms in plants, so they are not pathogenic to plants (Vinale et al. 2012).**

Some strains of ***Trichoderma*** **may decrease seed germination or seedling growth when are used in high concentration, because of production of plant inhibitory compounds (Contreras-Cornejo et al. 2016). Therefore, it is essential to set of maximum dose, especially for seedling production.**

The effect of ***Trichoderma*** **on plants may be modulated by the plant genotype (Tucci et al. 2011).**

In field cultivation, the most effective application technique is soil application or seed coating.

The application technique should be adapted to the species of cultivated plant (e.g. cucumber seed coating, pepper and lettuce soil application).

After soil application the preparation with ***Trichoderma*** **should be mixed with topsoil layer.**

Systematic use of ***Trichoderma*** **in field cultivation can improve its performance by stabilizing the population and enhancing plant-fungus and pathogens-fungus interactions.**

Examples of application of Polish *Trichoderma* strains in agriculture

As a result of works carried out in the framework of the project entitled "Polish Trichoderma strains in plant protection and organic waste management", No. UDA- POIG.01.03.01-00-129/09-07, several preparations containing native in Polish agriculture strains of *Trichoderma* fungi have been developed, which can be used in integrated cultivation of various vegetable species. The project was co-financed by the European Union from the European Regional Development.

All *Trichoderma* strains used for the research belong to the collection of the Research Institute of Horticulture and have been classified using biotechnological methods, and their sequences have been deposited at GenBank. All the isolates were also tested for their suitability for agriculture. These strains were used to produce biological preparations for application in vegetable cultivation. The innovation of the developed solution was the use of various organic waste from the agri-food industry as carriers for *Trichoderma* fungi.

The effects of developed preparations have been comprehensively tested in laboratory conditions, but mainly in field conditions in cultivation of several vegetable species. The effects of different *Trichoderma* isolates, carriers and application methods were evaluated. The studies were carried out on experimental plots, but also in production in horticultural farms. The effects of *Trichoderma* preparations on carrot, onion, tomato, pepper, lettuce, cucumber and potato crops were determined. The influence of selected strains of *Trichoderma* on plant yield, its structure and marketable yield quantity was investigated, as well as on the content of pro-health components in edible parts of vegetables, their sensory quality and shelf life were evaluated. The intensity and dynamics of plant diseases occurring in crops were monitored in field experiments.

The developed preparations with *Trichoderma* have a positive effect on the root systems of plants and their yield. It was found that *Trichoderma* worked best in the cultivation of solanaceous plants: tomato, pepper and potato. Positive results were also obtained with other species such as lettuce, carrot and cucumber. The bio-preparations have a positive effect on the content of pro-health components such as: lycopene in tomato fruits (increase in content even by 30%) or vitamin C in pepper, potato and lettuce. It was proved, that the applied *Trichoderma* strains have the ability to induce resistance in plants, which was reflected in significant reduction of powdery mildew on cucumbers, alternariosis on carrot leaves and potato blight. *Trichoderma* fungi used as seed coatings reduced seedlings damping- off of various vegetable species and improved plant emergence. These preparations may be an alternative to synthetic horticultural products such as mineral fertilizers and pesticides. A brief characteristic of *Trichoderma* effects on different crops is presented below.

Effects of *Trichoderma* in tomato cultivation

Tomatoes were grown in soilless cultivation, in the greenhouse;

***Trichoderma* was applied as seedling root dip before planting with suspension of dried spores obtained in solid state fermentation on organic wastes;**

The experiments were conducted in experimental greenhouse as well as in commercial production;

The best effect was obtained with *T. virens* TRS106 (significantly increased marketable yield – tab. 2 and 3);

Application of *Trichoderma* significantly increased licopen concentration in tomatoes (fig. 2).

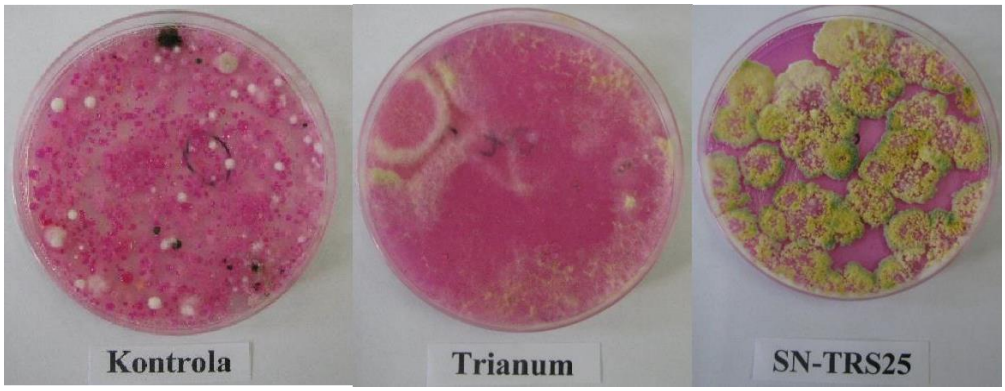
Tab. 2. Yield of tomato in the experiments conducted in Research Institute of Horticulture (Skierniewice, Poland), years 2013 – 2014

Treatment	Yield ($kg{\cdot}m^{-2}$)		
	early	marketable	total
control	19,0 a	45,4 b	47,7 a
commerial preparation	19,0 a	45,7 b	47,9 a
TRS106	20,4 a	48,2 a	50,4 a
TRS25	20,0 a	46,4 b	48,8 a

Tab. 3. Yield of tomato in the experiments conducted in the other site, in commercial production, years 2013 – 2014

Treatment	yield ($kg \cdot m^{-2}$)	
	early	marketable
control	2,8 b	29,3 b
TRS106	3,4 a	31,5 a
TRS25	3,1 b	29,8 b

Fot. 5. Ripening rate of early tomatoes in commercial production: left – control plants; right – plants treated with *Trichoderma*.



Fot. 6. Colonization of tomato roots by *Trichoderma* in soilless cultivation.

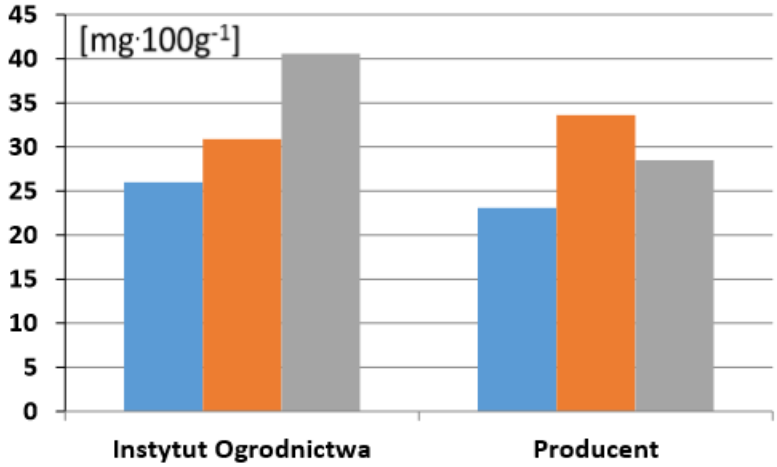


Fig. 2. Licopen concentration in tomatoes (2013 - 2014).
Left side experiments in Skierniewice, right side – experiments in commercial production.
Blue – control; orange – *Trichoderma* TRS106; grey - *Trichoderma* TRS25.

Effects of *Trichoderma* in pepper cultivation

Bell pepper plants were grown in the ground, in plastic tunnels;

***Trichoderma* was applied to the soil, before pepper transplants planting, as mycelium and spore biomass on organic carrier composed of plant residues;**

The experiments were conducted in experimental greenhouse as well as in commercial production;

The best effect was obtained with the mixture of two *Trichoderma* strains TRS123+TRS43, TRS123+TRS90 and single strain TRS43;

***Trichoderma* applications significantly increased total and marketable yield (fig. 3, fot. 7);**

Used mixtures increased also vitamin C concentration in the fruits (fig. 4).

Fot. 7. Example of one pepper harvest after soil application of *Trichoderma* (full fruiting - September). Left – control; right – fruits collected from plants treated with TRS123+90.

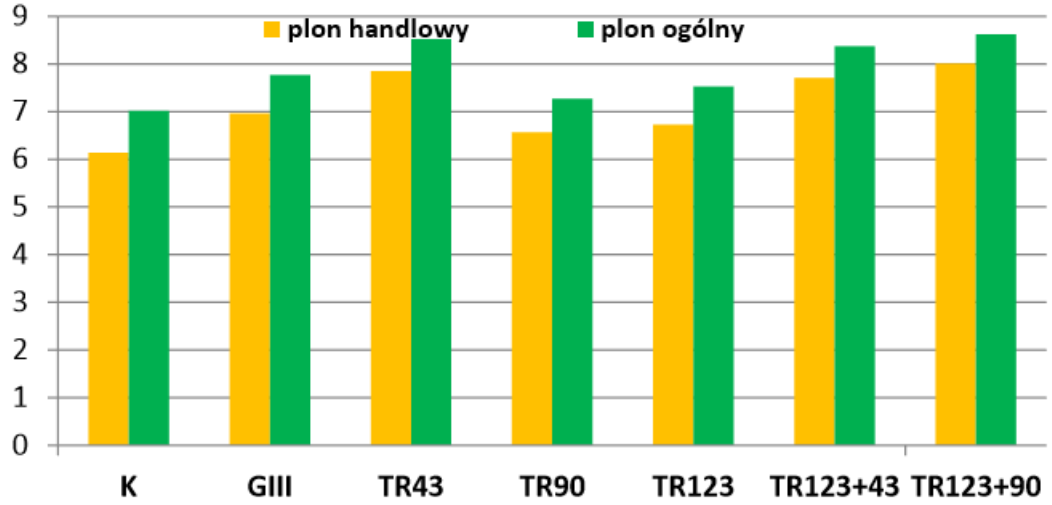


Fig. 3. The yield (kg/m^2) of pepper grown in soil treated with various strains of *Trichoderma*. Yellow bar – marketable yield, green bar – total yield. K – control, GIII – organic carrier.

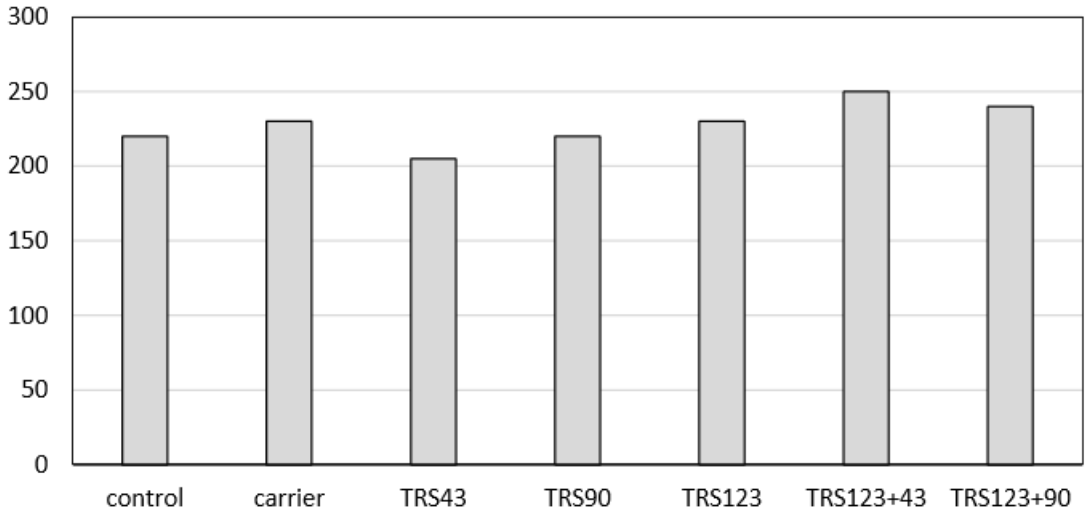


Fig. 4. Vitamin C concentration (mg/100 g) in pepper fruits after *Trichoderma* application.

Effects of *Trichoderma* in potato cultivation

Experiments with potatoes were conducted in commercial fields;

***Trichoderma* was applied to the soil, before potato planting, as mycelium and spore biomass on organic carrier composed of plant residues;**

Generally, the best and the most stabile effect was obtained with the use of single strains of *Trichoderma* TRS43 and TRS123;

The mixture of TRS43+TRS123 was also effective (fot. 8), but less than single strains (fig. 5);

***Trichoderma* applications significantly increased total, and especially marketable yield (fot. 8), the potato tubers were better formed and had a greater weight;**

the yield-forming effect in potato cultivation showed also organic carrier;

***Trichoderma* reduced incidence of potato blight on the plants (fig. 6).**

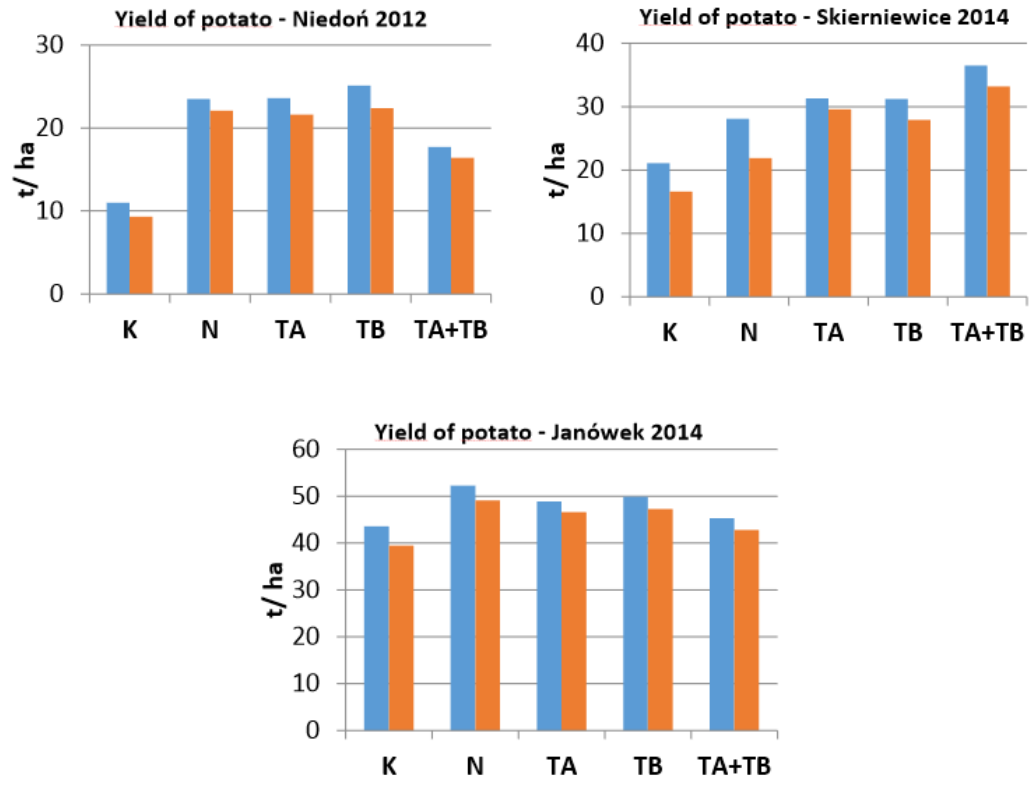


Fig. 5. Yield of potato in three field experiments: K – control, N – organic carrier, TA – TRS43, TB – TRS123, TA+TB – TRS43+TRS123;lue bar – total yield, red bar – marketable yield.

Fot. 8. Harvest of potato treated with *Trichoderma* (experiment Skierniewice, Poland).

Left – control; right – soil added with *Trichoderma* TRS43+123.

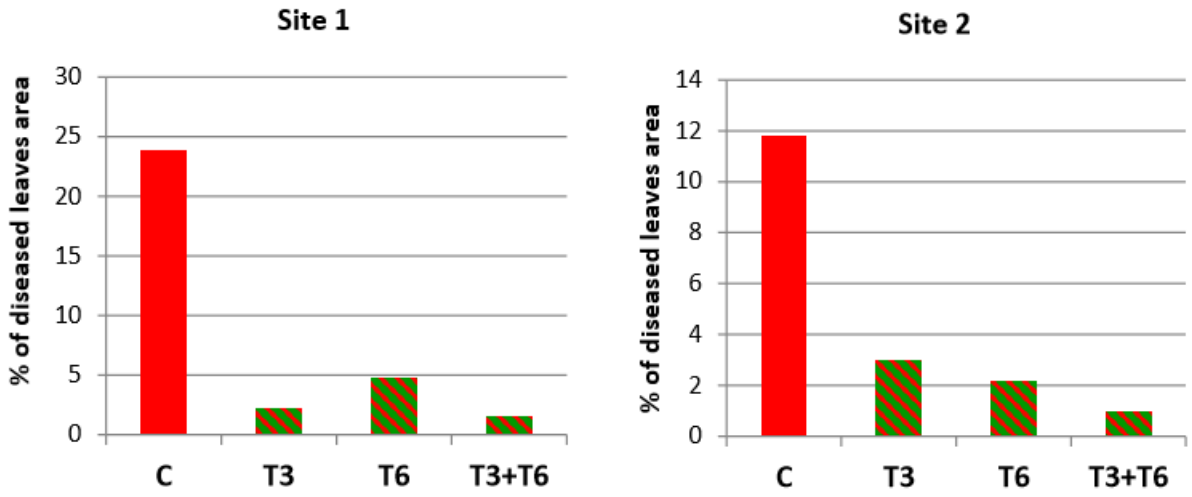


Fig. 6. Reduced incidence of potato blight on the plants treated with *Trichoderma* TRS43 (T3) and TRS123 (T6), C – control plants. Site 1 – exp. in Skierniewice, Site 2 – exp. in Janówek.

Effects of *Trichoderma* in cucumber cultivation

Three year experiment was conducted on experimental field of the Research Institute of Horticulture in Skierniewice;

***Trichoderma* was used as seed coating or applied to the soil before cucumber sowing on organic carrier;**

Control plants were not protected against downy mildew or sprayed with fungicide immediately after first symptoms of the disease appeared.

The best effects were obtained with *T. atroviride* TRS25 used as seed coating;

This application significantly increased seed germination and delayed downy mildew incidence (fig. 7 and 8);

The effect of single application of *Trichoderma* on cucumber yield in these experiments was not significant compared to chemical spraying.

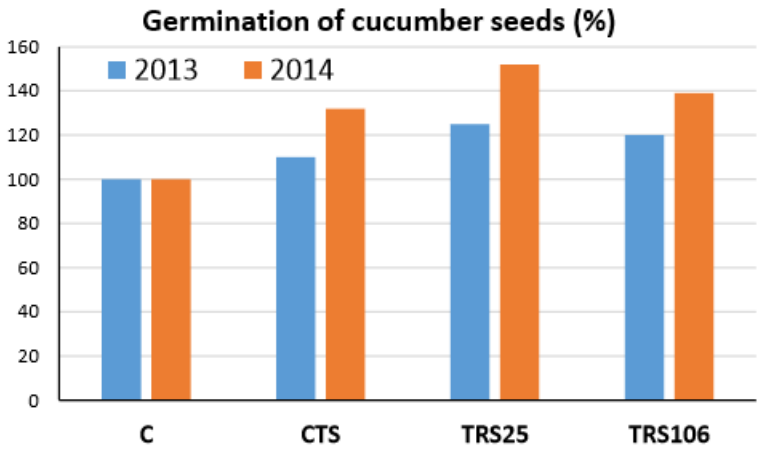
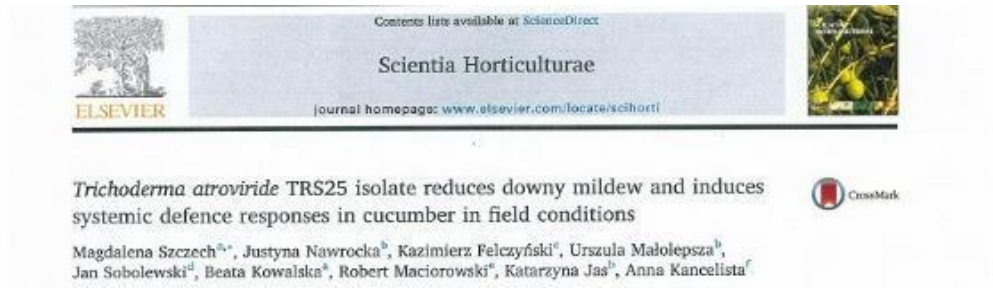


Fig. 7. Germination of cucumber seeds coated with *Trichoderma* TRS25 and TRS106, in field experiments conducted in the years 2013 – 2014. C – control, not treated seeds; CTS – seeds treated with chemical mortar.



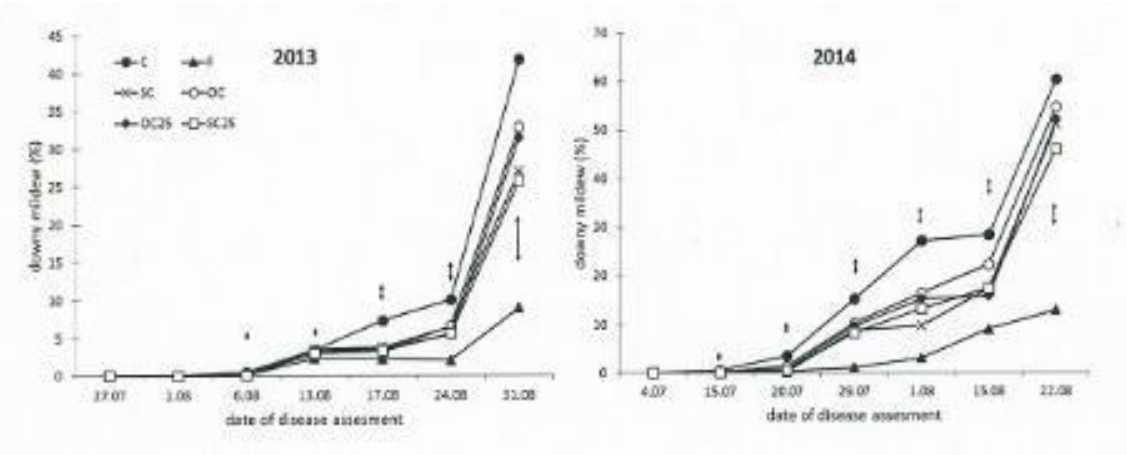


Fig. 8. Reduced incidence of downy mildew on cucumber plants with *Trichoderma* TRS25 (for more information see publication presented above).

Effects of *Trichoderma* in lettuce cultivation

The experiments with lettuce were conducted on experimental field of the Research Institute of Horticulture in Skierniewice and in commercial field;

***Trichoderma* was applied to the soil before lettuce planting on organic carrier composed of plant residues;**

In all experiments the mixtures of two *Trichoderma* strains were the most effective treatments, where the yield of lettuce was significantly higher compared to single strains and control plants;

Tables 4 and 5 present the effect of *Trichoderma* mixtures on marketable lettuce yield in "experimental" and "commercial" experiments;

***Trichoderma* enhanced biomass of lettuce roots (fot. 10);**

Vitamin C content increased in lettuce leaves treated with *Trichoderma*.

Soil application of *Trichoderma* on organic carrier also caused a significant increase in nitrate content in lettuce. However, its concentration did not exceed the acceptable limit for lettuce plants.

Tab. 4. Yield (%) and chlorophyll content in lettuce treated with the mixtures of *Trichoderma*, in the experiments conducted twice a year (spring and autumn) in the Research Institute of Horticulture in Skierniewice, years 2013 – 2014.

Treatment index	Marketable yield (%)			
	spring	autumn	spring	autumn
control	100	100	17,4	16,5
carrier	119	118	15,7	14,2
TRS43+TRS85	133	121	21,2	19,7
TRS25+TRS59	133	95	15,3	14,2
TRS25+TRS106	135	112	21,0	20,1

Tab. 5. Effect of *Trichoderma* mixture and commercial preparations (A, B) on the yield (%) of lettuce in the experiments conducted in commercial production, years 2013 – 2014.

Treatment	Year 2013	Year 2014
control	100	100
carrier	110	111
TRS59+TRS90	118	116
Commercial A	97	102
Commercial B	105	119

Fot. 9. Commercial experiments with lettuce.

Fot. 10. *Trichoderma* stimulated development of lettuce root system.

Effects of *Trichoderma* in carrot cultivation

The presented experiments with carrot were conducted on experimental field of the Research Institute of Horticulture in Skierniewice;

***Trichoderma* was used as seed coating or applied to the soil with organic carrier before carrot sowing;**

Organic carrier significantly reduced carrot germination and as a result yield of these plants (tab. 6);

Seed coating with *Trichoderma* spores preparations did not affect seed germination, but enhanced yield;

The yield increase was related to enhanced biomass of the roots (fot. 11);

Significant reduction of alternariosis on carrot leaves was observed in all experiments (fig. 12);

***Trichoderma* did not affected sensory and biochemical quality of carrot.**

Fot. 11. *Trichoderma* used as seed coating stimulated carrot root growth.

Tab. 6. Effect of *Trichoderma* and organic carrier on carrot growth and yield in field experiments (years 2013 – 2014).

Treatment	Seed germination (%)	Marketable yield (t/ha)	Root biomass (g)
control	100,0	92,8	119,5
organic carrier	57,7	73,7	135,3
TRS14 + carrier	61,6	71,2	135,5
TRS20 + carrier	62,4	64,0	123,3
TRS43 + carrier	67,1	73,5	138,0
chemical seed coating	103,0	108,2	149,5
TRS14 seed coating	101,1	100,4	144,9
TRS20 seed coating	99,4	99,3	145,0
TRS43 seed coating	108,8	96,4	132,3

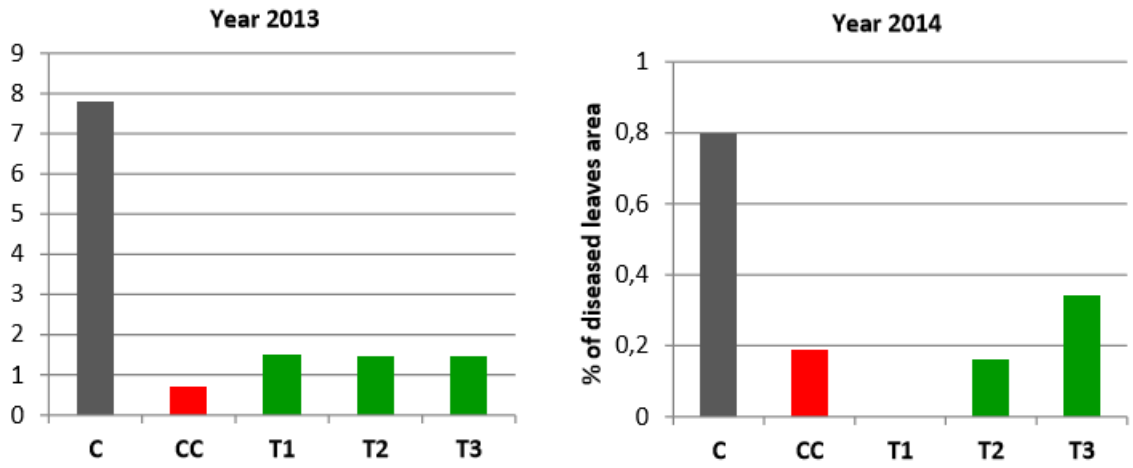


Fig. 9. Reduced incidence of alternariosis on carrot leaves after seed coating with *Trichoderma*. C – control, CC chemical treatment, T1 – TRS14, T2 – TRS20, T3 – TRS43.

References

Berg G., 2009. Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. Appl. Microbiol. Biotechnol. 84: 11 – 18.

Brent K.J. and Hollomon D.W., 2007. Fungicide resistance in crop pathogens: How can it be managed? FRAC Monograf: http://www.frac.info/docs/default-source/publications/monographs/monograph-1.pdf.

Contreras-Cornejo H.A., Macías-Rodríguez L., del-Val E., Larsen J., 2016. Ecological functions of *Trichoderma* spp. and their secondary metabolites in the rhizosphere: interactions with plants. FEMS Microbio. Ecol., 92: 1 – 17.

Gouda S., Kerry R.G., Das G., Paramithiotis S., Shin H.S., Patra J.K., 2018. Revitalization of plant growth promoting rhizobacteria for sustainable development in agriculture. Microbiological Research, 206: 131 -140.

Harman G.E., Howell C.R., Viterbo A., Chet I., Lorito M., 2004. *Trichoderma* species

– opportunistic, avirulent plant symbionts. Nat. Rev. Microbiol., 2: 43 – 56.

Hasan S., Gupta G., Anand S., Kaur H., 2014. Lytic enzymes of Trichoderma: their role in plant defense. Inter. J. Appl. Res. Stud., 3 (2): 1 – 4.

Köhl J., Postma J., Nicot P., Ruocco M., Blum B., 2011. Stepwise screening of microorganisms for commercial use in biological control of plant-pathogenic fungi and bacteria. Biological Control, 57: 1 – 12.

Kumar S., Thakur M., Rani A., 2014. *Trichoderma*: mass production, pormulation, quality control, delivery and its scope in commercialization in India for the management of plant diseases. Afr. J. Agric. Res., 9 (53): 3838 – 3852.

Lee S., Yap M., Behringer G., Hung R., Bennett J.W., 2016. Volatile organic compounds emitted by *Trichoderma* species mediate plant growth. Fungal Biol. Biotechnol., 3: 1 – 14.

López-Bucio J., Pelagio-Flores R., Herrera-Estrella A., 2015. *Trichoderma* as biostimulant: exploiting the multilevel properties of a plant beneficial fungus. Sci. Hortic., 196: 109 – 123.

Mendoza-Mandoza A., Zaid R., Lawry R., Hermosa R., Monte E., Horowitz B.A., Mukherjee P.K., 2018. Molecular dialogues between *Trichoderma* and roots: role of the fungal secretome. Fungal Biology Reviews, 32: 62 – 85.

Qiu Z., Egidi E., Liu H., Kaur S., Singh B.K., (XXXX). New frontiers in agriculture productivity: optimized microbial inoculants and in situ microbiome engineering. Biotechnology Advances: https://doi.org/10.1016/j.biotechadv.2019.03.010

Reino J.L., Guerrero R.F., Hernández-Galán R., Collado I.G., 2008. Secondary metabolites from species of the biocontrol agent *Trichoderma*. Phytochem. Rev., 7: 89 – 123.

Saravanakumar K., Fan L., Fu K., Yu C., wang M., Xia H., Sun J., Li Y., Chen J., 2016. Cellulase from *Trichoderma harzianum* interacts with roots and triggers induced

systemic resistance to foliar disease in maize. Scientific Reports/6:35543/ doi:101038/srep35543

Waghunde R.R., Shelake R.M., Sabalpara A.N., 2016. *Trichoderma*: a significant fungus for agriculture and environment. Afr. J. Agric. Res., 11 (22): 1952 – 1965.

Vimal S.R., Singh J.S., Arora N.K., Singh S., 2017. Soil-plant-microbe interactions in stressed agriculture management: a review. Pedosphere, 27 (2): 177 – 192.

Vinale F., Sivasithamparam K., Ghisalberti E.L., Ruocco M., Woo S., Lorito M., 2012. *Trichoderma* secondary metabolites that affect plant metabolism. Nat. Plant Com., 7 (11): 1545 – 1550.

Viterbo A., Ramot O., Chernin L., Chet I., 2002. Significance of lytic enzymes from *Trichoderma* spp. in the biocontrol of fungal plant pathogens. Antonie van Leeuwenhoek, 81: 549 – 556.

Zeilinger S., Gruber S., Bansal R., Mukherjee P.K., 2016. Secondary metabolism in

***Trichoderma* – chemistry meets genomics. Fungal Biol. Rev., 30 (2): 74 – 90.**

Recommended books

Gupta V.G., Schmoll M., Herrera-Estrella A., Upadhyay R.S., Druzhinina I., Tuohy M. (Eds.) (2014) Biotechnology and Biology of *Trichoderma*. Elsevier

Mukherjee P.K., Horwitz B.A., Singh U.S., Mukherjee M., Schmoll M. (Eds.) (2013)

***Trichoderma* biology and applications, CAB International**

Recommended additional articles

Calvo P., Nelson L., Kloepper J.W., 2014. Agricultural uses of plant biostimulants.

Plant Soil, 383: 3 – 41

Identification and monitoring of *Trichoderma* in field soil

Materials prepared by Grzegorz Bartoszewski

General Introduction

Over-usage of pesticides in agricultural ecosystem raises worries about biological potential of the soil and level of pesticide residues in food and feed

There is urgent need to develop and introduce to farmers' practice ecologically-friendly efficient plant protection methods

Ecologically-friendly pest/pathogen control strategies could be developed based on microorganisms – they offer many opportunities for plant protection

Microorganisms are applied in agricultural practices as Biological Control Agents (BCA) to manage plant pathogens and/or Plant Growth Promoting Factors (PGPF) to help in crop adoption to the changing environmental conditions – to keep yield and its quality, induce resistance/tolerance to stresses, improve water and nutrients usage efficiency, lower production costs

***Trichoderma* fungi are microorganisms that show many beneficial effects in crop production as BCA and/or PGPF - Weindling in 1932 mentioned for the first time that *Trichoderma* could be used as BCA**

There are *Trichoderma*-based biopreparations already available on the market, however there are now many studied on *Trichoderma* to develop novel biopreparations/applications for agriculture

Important features of *Trichoderma* fungi

***Trichoderma* fungi are ubiquitous and common in ecosystems – their major ecological role is decomposition of organic matter**

***Trichoderma* fungi are frequently isolated from soil - more than 10 species are isolated from the soil, forest trees, wooden materials and other environments**

***Trichoderma* are characterized by huge biodiversity – thus taxonomy of *Trichoderma* is complex and complicated**

There are many beneficial sides of *Trichoderma*, but it can be also harmful – it has to be carefully studied before application

Important features of *Trichoderma*

<u>Beneficial sides of *Trichoderma*</u>:

Biotechnological applications, *T. reesei* is good source of carbohydrate active enzymes, eg. cellulases, enzymes for biofuel production

Certain *Trichoderma* strains are applied in agricultural, as biological agent to control pathogens (BCA), plant growth promoting factor – phytostimulant (PGPF), bioremediation agent, several species: *T. harzianum, T. virens, T. atroviride, T. asperellum,* and others

No plant pathogenic *Trichoderma* reported

***Trichoderma* can be bioindicator of soil health**

***Trichoderma* fungi are relatively easy to isolate and cultivate in controlled conditions to prepare biocontrol formulations - fast-growing, producing-well conidia, thus production of biopreparations is relatively simple and easy**

Harmful sides of *Trichoderma*:

Some strains can feed on fungi and cause green mould disesase of cultivated mushrooms (*T. aggressivum, T. pleurotum, T. pleuroticola),*

Examples of clinical cases of opportunistic infections in immunocompromised patients are documented (*T. longibrachiatum, T. bissettii, T. citrinoviride*)

Trichoderma taxonomy

Genera name _Trichoderma_ for many years coexisted with _Hypocrea_: _Trichoderma_ is used for asexual form (anamorpha) and _Hypocrea_ for sexual form (telomorpha)

For the first time name _Trichoderma_ was used in 1794 and _Hypocrea_ in 1825 – now, since principle "one fungus = one name" (One Fungus = One Name Conference, on 19–20 April 2011 in Amsterdam) is applied, name _Trichoderma_ is commonly accepted.

Primary _Trichoderma_ taxonomy was based on morphological features – morphological species were distinguished (Rifai, 1969)

Progress in _Trichoderma_ taxonomy resulted in species grouping and introduction of sections (Bissett, 1991)

Application of DNA-based fingerprinting methods allowed for more precise species grouping

Breakthrough in _Trichoderma_ taxonomy was development of DNA barcoding system _Trich_OKEY i _Tricho_BLAST http://isth.info/ (Kopchinskiy, Druzhinina, Kubicek, 2005)

Trichoderma DNA-based molecular taxonomy is continuously adjusted – now more than 227 species are distinguished and new species are being discovered, most of the species are known only to taxonomist

Trichoderma genomes are now available for several species contributing to _Trichoderma_ classification (JGI Genome Portal http://genome.jgi.doe.gov), genome size range: 9-14 Mb, the genomes of most of the species are still not known

Genomics based methods in combination with genome-wide phylogenetic analysis can be now applied _Trichoderma_

Trichoderma taxonomy – examples of species classification

Kingdom: *Fungi*

Subkingdom: *Dikarya*

Phylum: *Acomycota*

Class: *Sordariomycetes*

Order: *Hypocreales*

Family: *Hypocreaceae*

Genus: *Trichoderma / Hypocrea (anamorpha/telomorpha)*

Section: *Pachybasium*

Species: eg. *Trichoderma crassa*, *Trichoderma virens, Trichoderma cerinum, Trichoderma harzianum* species complex - now at least 14 species

Section: *Longibrachiatum*

Species: eg. *Trichoderma longibrachiatum,*
Trichoderma reesei

Section: *Trichoderma*

Species: eg. *Trichoderma atroviride, Trichodrma koningii, Trichodrma koningiopsis*

<u>Several other sections also exist</u>

Trichoderma species identified in Poland – example of diversity

T. afroharzianum*, *T. aureoviride*, *T. atrobrunneum*, *T. atroviride*, *T. brevicompactum*, *T. cerinum*, *T. citrinoviride*, *T. crassum*, *T. fertile*, *T. gamsii*, *T. hamatum*, *T. harzianum* sensu stricto, *T. koningii*, *T. koningiopsis*, *T. lentiforme*, *T. longibrachiatum*, *T. longipilis*, *T. polysporum*, *T. pseudokoningii*, *T. rossicum*, *T. spirale*, *T. strigosum*, *T. tomentosum*, *T. velutinum*, *T. virens*, *T. viride*, *T. viridescens

Some aspects related to existing _Trichoderma_ diversity

diversity overall – reproductive barriers not considered – species are grouped on phylogenetic trees in 16 clades and several single lineages

within-species – reproductive isolation of the species in force – changes accumulated in population

unique to the strain - useful for the BCA/PGPA identification/monitoring in the environment – difficult to identify

DNA-based molecular identification of _Trichoderma_ - major steps in methodology

- preparation of fungal cells

- DNA isolation

- identification of DNA variability – many approaches

- molecular techniques to monitor DNA changes

- bioinformatics analysis, eg. sequence analysis, Bayesian phylogeny

- genera/species/strain identification or classification

Preparation of fungal cells for DNA isolation

- fungi cultivation usually in laboratory conditions

- fungal culture have to be established from single spore

- solid media culture – easy to establish culture

- liquid media culture – easy to collect cells by centrifugation

- fungal cells could be deep frozen and stored for DNA isolation

DNA isolation methodology

- several methods available - usually optimization step is needed

- simple methods: cells are boiled in the buffer to release DNA, quick and cheap method, DNA quantity vary and quality is low, occasionally used

- CTAB based methods: developed by Aldrich and Cullis (1993) for plant tissues, DNA quantity usually high, further purification step is recommended, relatively cheap, efficient and easy, often used

- column-based methods: available commercially as a set/kit, DNA quality is high, quantity could be low/medium depending on the set, relatively expensive, easy to use, often used

- RNA and protein removal - usually RNase A and proteinase K treatment steps are included in the protocols

DNA based techniques used in *Trichoderma* taxonomy and genetic variability studies

- DNA fingerprinting techniques

- DNA sequence-based or barcoding

- Genome-wide based sequence analysis

DNA Fingerprinting based on hybridization

- RFLP - DNA Restriction Fragment Length Polymorphism method – targeting variability within mitochondrial or chromosomal DNA, expensive and difficult, historical technique

DNA fingerprinting methods based on polymerase chain reaction (PCR)

- RAPD – Random Amplified Polymorphic DNA method – one of the simplest techniques – based on random primers – relatively cheap and easy to apply, problems with reproducibility, rarely used now

- AFLP – Amplified Fragment Length Polymorphism method – difficult technique - based on random primers and reduction of the genome complexity, not used now

- rep-PCR – based on ERIC/REP/BOX primers designed for tandemly repeated DNA regions (microsatellites), fast and easy method, good for preliminary assessment and grouping of *Trichoderma* isolates, better reproducibility than RAPD (longer primers)

- SCAR – Sequence Characterized Amplified Regions – based on specific primers, can be used for specific strain detection, single set or multiplexed primes are used, can be applied in combination with Real-Time PCR/TaqMan technique, many possibilities, frequently used

- PCR-RFLP – simplified RFLP method – genomic region is amplified and digested by restriction enzymes to monitor polymorphism, often used

- other techniques are also available

Barcoding used *Trichoderma*

- MLST – Multi Locus Sequence Typing – based on PCR amplification of taxonomic loci and analysis of amplified DNA sequences = barcodes, commonly used in bacteria/fungi taxonomy

- ITS1 and ITS2 - commonly used for genera identification in fungi, low variability of DNA sequence is limitation for species identification

- TEF1 – widely used for *Trichoderma* species identification, common standard for species identification

- RBP2 – very useful and recently becoming more widely used

Most common barcodes used for *Trichoderma* taxonomy and molecular phylogeny

Barcode	Description
ITS1	Internal Transcribed Spacer 1 located between 18S and 5.8S rRNA genes
ITS2	Internal Transcribed Spacer 2 located between 5.8S and 28S rRNA genes
TEF1	Translation Elongation Factor 1-alpha gene fragment barcode is usually intron 4 (intron 5 is also used)
RBP2	RNA polymerase II gene fragment barcode is fragment of the exon
*chi*18-5	chitinase 42 kDa gene barcode is fragment of the large exon
CAL1	fragment of the calmodulin 1 gene *cal1*
ACT1	fragment of the gene encoding actin 1

Monitoring of *Trichoderma* in the soil – why important?

To monitor longevity of applied BCA/PGPF *Trichoderma* strains

Help to understand dependencies of fungal/microbial communities in the soil towards crop-beneficial soil microorganism management

Usually soil samples are collected from the field before and after *Trichoderma* application to monitor fungal/microbial population including applied *Trichoderma* strain(s)

Microbiological and DNA-based approaches are used, including metagenomics concept

Monitoring of *Trichoderma* in soil

microbiological approach

Soil samples collection – precise methodology has to be developed, number of samples/repeats, individual vs. pooled samples, etc.

Samples treatment with the buffer and preparation of dilution series

Plating of the samples on medium, eg. Martin's solid medium (1950), PDA (Potato Dextrose Agar)

Colony assessment and *Trichoderma* colonies counting - for validation molecular identification of representative colonies is recommended

Calculation of colony forming units (CFU) per gram of soil

Monitoring of *Trichoderma* in soil

DNA-based approach

Soil sampling from the field – e.g. before and after *Trichoderma* application to monitor population size, longevity of BCA/PGPF strain(s)

DNA isolation from soil samples - choice and optimization of DNA isolation method is usually needed – efficient homogenization of the sample is important

Designing of the approach and application of PCR-based method, for example multiplex-PCR or Real-Time TaqMan with specific primers/probes to detect *Trichoderma* species or strains

Metagenomic approach to describe fungal population – mycobiome or overall microbial population – microbiome

Next Generation Sequencing (NGS) methods are used in metagenomics studies – Illumina MiSeq is common choice

Usually it is possible to estimate the population of the fungi at the genera level –at the species level it can be difficult

Recommended books and book chapters

Gupta VG, Schmoll M, Herrera-Estrella A, Upadhyay RS, Druzhinina I, Tuohy M (Eds.) (2014) Biotechnology and Biology of *Trichoderma*. Elsevier,

Atanasova L, Druzhinina IS, Jaklitsch WM (2013) Two hundred *Trichoderma* species recognized on the basis of molecular phylogeny. In: Mukherjee PK, Horwitz BA, Singh US, Mala M, Schmoll M (Eds.), *Trichoderma*: biology and applications, CABI Publishing, 10-42

Recommended articles

Kredics L, Chen L, Kedves O, et al. (2018) Molecular tools for monitoring *Trichoderma* in agricultural environments. Front. Microbiol. 9:1599 - review

Chaverri P, Branco-Rocha F, Jaklitsch W, et al. (2015) Systematics of the *Trichoderma harzianum* species complex and the re-identification of commercial biocontrol strains. Mycologia 107: 558-590 – case study

Oskiera M., Szczech M., Stepowska A., Smolinska U., Bartoszewski G. (2017) Monitoring of *Trichoderma* species in agricultural soil in response to application of biopreparations. Biol. Control 113: 65–72 – case study

Recommended Web-pages

***Trichoderma* taxonomy ISTH web page:** http://isth.info/

JGI Genome Portal http://genome.jgi.doe.gov/

THE USE OF TRICHODERMA IN ALLEVIATION OF PLANT TOLERANCE TO ABIOTIC STRESS

Plant-microbe interaction in the rhizosphere related to soil quality

Dejana Pankovic, Educons University

Soil has the major function to act as a supporting medium for plants or food crops feeding the human and animal population. It is fragile and easily degraded when misused and mismanaged and always remains at receiving end of all irresponsible anthropogenic activities. It is gradually renewable but over the human time scale of decades to centuries therefore the preservation of soil quality is of utmost importance for human and environmental health.

Soil quality research is traditionally focused primarily on chemical and physical properties however, soil biological properties can serve as early and sensitive indicators of agro-ecosystems in response to soil management practices (Figure 1).

Plant roots are modifying rhizosphere environment. Some processes are shared both by roots and associated microorganisms such as: exudation of soluble compounds, water uptake, nutrient mobilization by roots and microorganisms, rhizosphere-mediated soil organic matter decomposition, and the subsequent release of CO_2 through respiration. Rhizosphere processes are important as they utilize approximately 50 % of the energy fixed by photosynthesis in terrestrial ecosystems and contribute roughly 50 % of the total CO_2 emitted from terrestrial ecosystem. Root exudates are mainly composed of water soluble sugars, organic acids, and amino acids, but also contain hormones, vitamins, amino compounds, phenolics and sugar phosphate esters It is considered that 40 % of the total amount of carbohydrates produced by photosynthesis is released into the soil surrounding roots.

Microbes are also affecting the rhizosphere zone. Their abundance is affected by several ecological factors: moisture, temperature, organic matter, rhizosphere structure of plant, and plant diversity but also by crop management practices like tillage, irrigation, nutrient, organic and green manuring, mulching. Microbes play a key role in nutrient

cycling and energy flow and have main importance in biological nitrogen fixation and phosphorus solubilization (phosphate solubilizing bacteria) and phosphorus mobilization (arbuscular mycorrhizal fungi).

Important measure of soil quality are the following dynamic physical soil quality indicators*:* bulk density, aggregate stability, hydraulic conductivity and water holding capacity. Bulk density is considered as one of the most important dynamic property of soil. It is altered by cultivation, compression, animal grazing, agricultural machinery, weather and others. It also can serve as an indicator of soil compaction and relative restriction to root growth. Agregate stability is an important measure of soil quality for crop establishment, water infiltration and resistance to erosion and compaction. However, hydraulic conductivity reflects the rate at which water flows through soil, and affects many properties of the soil, including infiltration, drainage, nutrient flow within the soil, and soil erosion. Water holding capacity is the amount of water retained by the soil after it has been saturated and depends on previous physical characteristics of soil.

Rhizosphere processes are important in carbon dynamics. Carbon mineralization and subsequent release of CO_2 is governed either by respiration activity of root tissues or microbial cells present in the root vicinity. Soil respiration is the production of CO_2 or consumption of O_2 as a result of the metabolic processes of living organisms at the rhizosphere and in the soil. It is a useful measure of soil activity and decomposition. Soil organic matter is the key quality factor of soil. About 95 % of the total soil organic matter is non-living, therefore, relatively stable or resistant to change. Microbial biomass carbon is a relatively small (1–4 % of total soil organic carbon). This is labile fraction that quickly responds to C availability and also strongly influenced by management practices and system perturbations.

Rhizosphere processes are also important in soil nutrient dynamics. Nitrogen is the primary nutrient element to crop plants controlling their vegetative and reproductive growth. It supplies majorly from soil available nitrogen either organic or inorganic forms, constantly converts to readily-available forms (NH_4^+, NO^{3-}), mostly carried out by native microbial populations in soil. Inorganic N compounds are not stable in soil and are subjected to volatilization and leaching. In most soils small proportion of the total phosphorus is present in available form due to its low solubility. Soil available potassium also determines soil quality. Available micronutrients, such as Zn and Cu, can be depleted by intensive cropping with high yielding varieties.

Biological properties of the soil include size and diversity of the microbial, macro and microfaunal biomass, enzyme quantities and activities and soil respiration. Four enyzmes are particularly important: dehydrogenases, phosphatates, β–Glucosidase and urease.

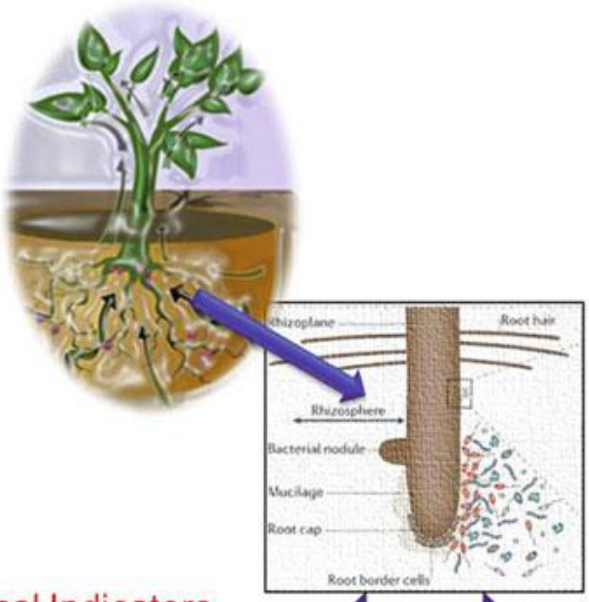
Dehydrogenases reflect the total range of oxidative activity of soil microflora and are a good indicator of microbiological activity. Phosphatases catalyse hydrolysis of esters and anhydrides of phosphoric acid, so they play critical roles in P cycles in soil ecosystems.

β–Glucosidase catalyse reactions of cellulose degradation, releasing glucose as a source of energy that maintains metabolically active microbial biomass in soil. However as a free enzyme in soil solution, it normally has a time-limited activity, so rapidly degrades and denatures.

Urease enzymes catalyse urea hydrolysis which breaks into CO_2 and NH_3. As a result, nitrogen losses take place by NH_3 volatilization and soil pH increase. Originates mainly from plants and microorganisms found as both intra- and extra-cellular. It represents up to 63 % of

total activity in soil, but is rapidly degraded in soil by proteolytic enzymes.

Soil microbes co-operate in the rhizosphere and improve soil quality by formation of soil particles. Soil particles are bound together by bacterial products and by hyphae of saprophytic and arbuscular mycorrhizal fungi, into stable microaggregates (ranges in between 2 and 20 µm in diameter). Larger micro-aggregates (ranges in between 20 and 250 µm in diameter) are formed with bacterial polysaccharides acting as binding agents. Micro-aggregates are bound into macro-aggregates (higher than 250 µm in diameter), with bacterial polysaccharides acting as binding agents and arbuscular mycorrhizal mycelia increasing the size of macro-aggregates.



Soil Physical Indicators

- Bulk Density
- Water Holding Capacity
- Aggregate stability
- Hydraulic Conductivity

Soil Chemical Indicators

- SOC, TOC, DOC
- Available N, P, K
- Micronutrients (Fe, Mn, Zn, Cu)

Soil Biological Indicators

- Soil Enzymes
- Microbial Biomass Carbon
- Soil Respiration
- Metabolic Quotient

Figure 1. Soil quality indicators of the rhizosphere soil (Bhaduri et al., 2015).

Trichoderma species and their interaction with plants in alleviation of drought

***Trichoderma* species are widely known for their antagonistic properties and are among the most studied biocontrol agents controlling plant pathogens. As a result of plant-*Trichoderma* interaction, various enzymes, avirulence-like gene products and low molecular weight compounds are released from both fungal and plant cell. Elicitors from *Trichoderma* are responsible for the expression of several gene families encoding proteins related to the plant defense response system.**

Plant growth promotion and abiotic stress tolerance have also been reported (Contreras-Cornejo et al., 2015). However, mechanisms of abiotic stress tolerance, such as drought in plants in interaction with *Trichoderma* spp. remain unclear.

It is known that plant adaptation to drought involves regulation of stomata opening, which modulates the rate of transpiration water loss and carbon dioxide uptake.

When water is available in the soil, plants will absorb water through its roots. This water will be used by the plant or released through transpiration by open stomata in the leaves. (Figure 2 A). CO_2 and oxygen are being absorbed and released through the open stomata so process of photosynthesis may function normally. If the water is

limited in the soil, plants try to prevent water loss. Water loss through transpiration can be reduced by closing the stomata in the leaves, which is regulated by phytohormones such as abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA) or ethylen (ET) (Figure 2 B). In this case water is preserved but when the stomata are closed CO_2 exchange is not possible and photosynthesis will decrease, therefore the growth of the plant will decrease.

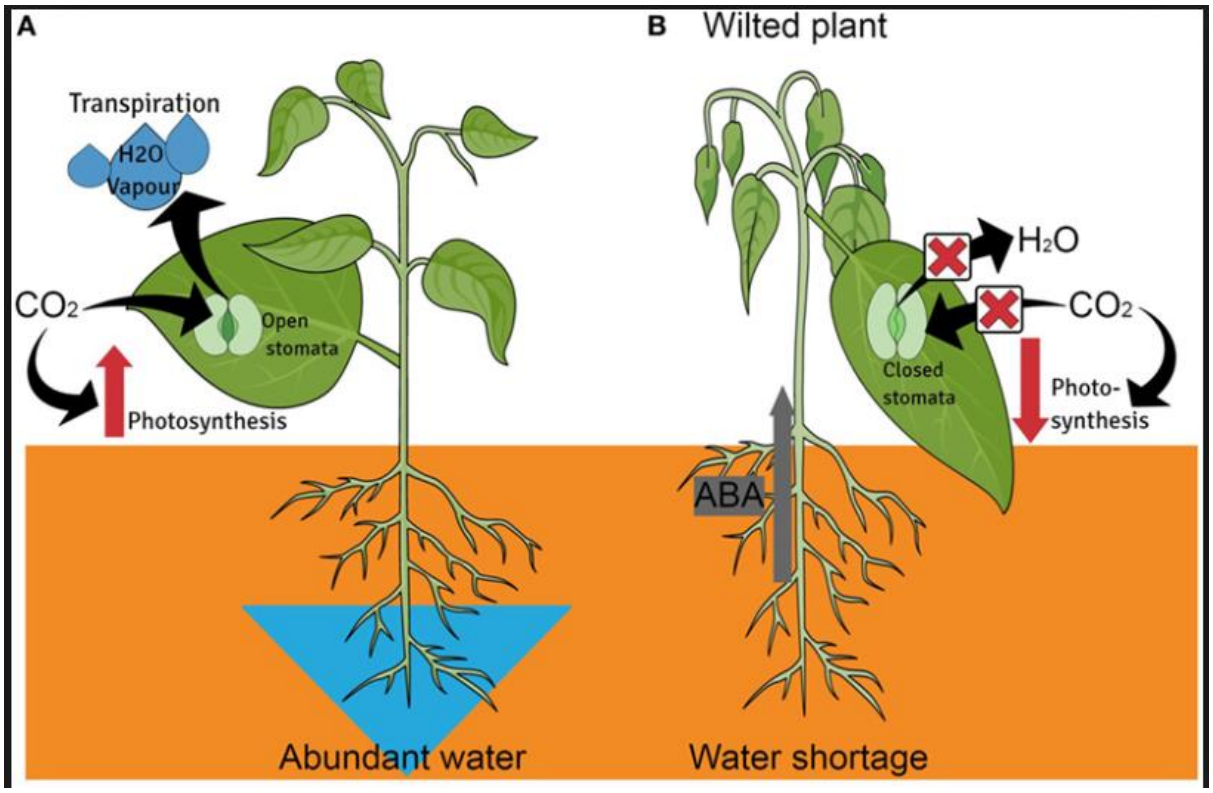


Figure 2- Internal defenses of plants under water stress (van der Vyver and Peters, 2017)

We have studied the influence of *Trichoderma* on drought tolerance of tomato plants in two experiments. In the first experiment early reactions of small tomato plantlets to Trichoderma-drought treatment were examined, and in the second one we investigated late reactions of tomato at the phase of 10 leaves, to Trichoderma-drought treatment.

In both experiments we have applied the *Trichoderma* strain isolated from agricultural soil, identified as *T. brevicompactum* and deposited in the Szeged Microbiological Collection (www.szmc.hu) as SZMC 22661 (Racic et al., 2017). This species produces alamethicins, that could enhance biocontrol abilities, therefore the members of this clade were suggested as biocontrol agents. However it produces trichodermin - trichothecene-type terpenoid toxin with fungitoxic and phytotoxic activities. Generally, the investigations about the effects of *T. brevicompactum* on plants are very limited, when compared to other taxa like *T. viride* or the *T. harzianum* species complex.

Experiment 1 Early reactions to Trichoderma-drought treatment

Tomato plantlets (*Solanum lycopersicum* Mill. cv. Ailsa Craig) were grown under controlled conditions with optimal water supply (75% soil water capacity) until the 4th leaf developmental phase. Plants were subjected to three treatments: cessation of watering, addition of the *T. brevicompactum* SZMC 22661 spore suspension (8 x 10^6 CFU) to the root zone, and combination of both treatments (Figure 3).

Measurements were performed 2, 6, 24 and 48 hours from the onset of treatments.

Figure 3. Tomato plants used for the investigation of the early reactions to *T. brevicompactum* application.

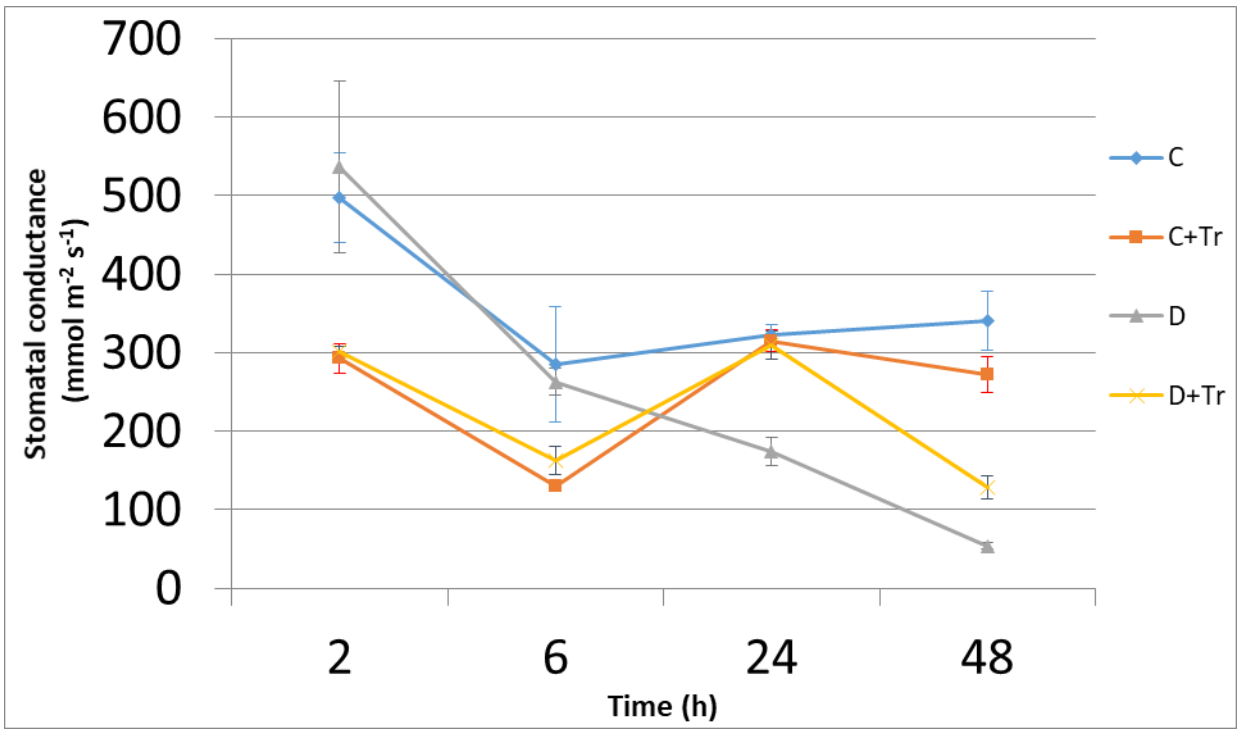


Figure 4. Early reactions of stomatal conductance to Trichoderma-tomato interaction under different water supply. C refers to optimally watered plants; C+Tr refers to optimally watered plants treated with *T. brevicopmactum*; D refers to plants that were not watered any more and D+Tr refers to plants that were not watered but were treated with *T. brevicopmactum*.

The decreased stomatal conductance that appeared already 2 h after application of Trichoderma, was recovered again to the values of control plants 24 h after the begining of treatment (Figure 4). Exactly at this time point plants have started to experienced the decreased soil water content (Table 1). The decrease of stomatal content was coupled with decreased leaf water potential (Figure 5).

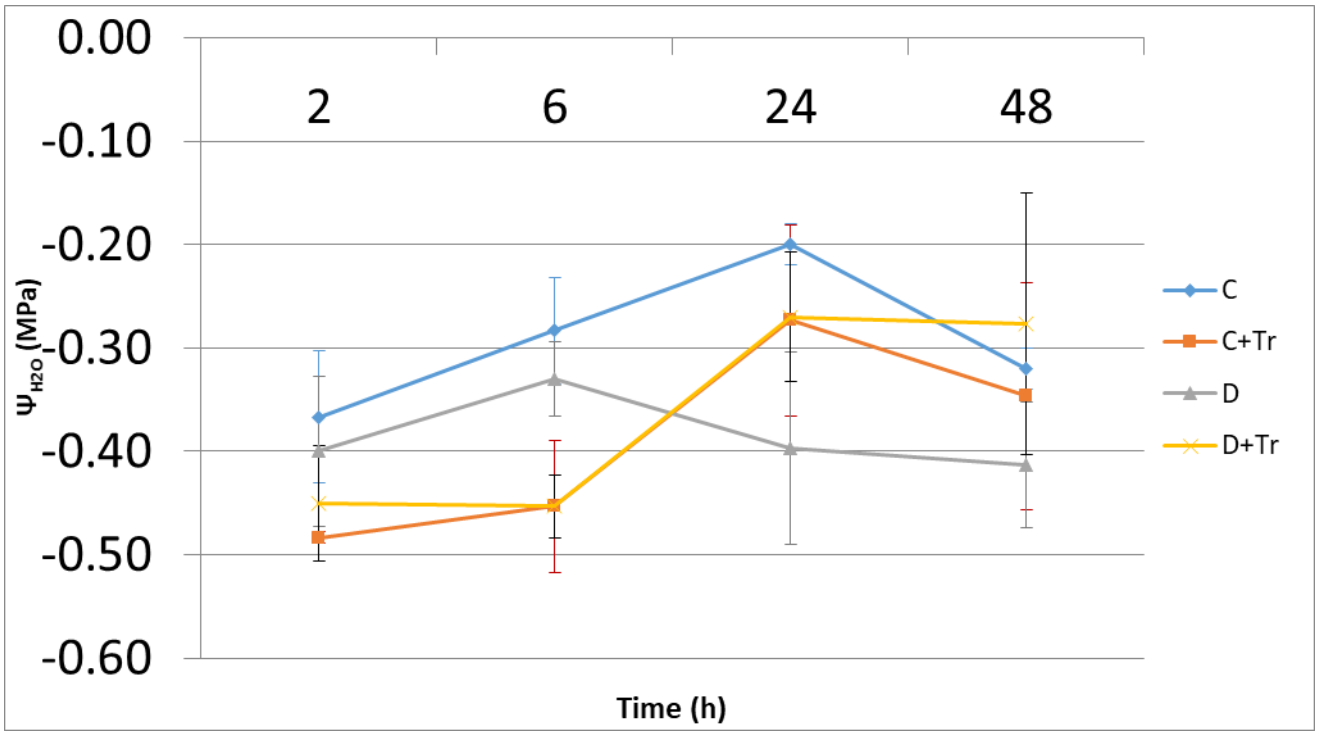


Figure 5. Early reactions of leaf water potential (ψ_{H2O}) to Trichoderma-tomato interaction under different water supply. C refers to optimally watered plants; C+Tr refers to optimally watered plants treated with *T. brevicopmactum*; D refers to plants that were not watered any more and D+Tr refers to plants that were not watered but were treated with *T. brevicopmactum*.

Table 1. Soil water content (SWC) determined at 6h, 24 h and 48h after the simulatenous addition of *T. brevicompactum* and cessation of watering of tomato plants

SWC			
Treatments	**6h**	**24h**	**48h**
C	**28.6 ± 4.4**	**34.4 ± 1.6**	**35.7 ± 3.1**
C + Tr	**29.3 ± 1.5**	**43.3 ± 0.1**	**38.9 ± 0.8**
D	**30.2 ± 0.5**	**27.7 ± 2.3**	**11.7 ± 0.1**
D + Tr	**29.1 ± 2.7**	**23.6 ± 2.4**	**20.1 ± 0.8**

Mean values ± standard deviation for soil water content (SWC) ($p<0.05$).

Experiment 2 Late reactions to Trichoderma-drought treatment

Tomato plants (Lycopersicon esculentum Mill. cv. Ailsa Craig) were grown under controlled conditions with optimal water supply (75% soil water capacity) until the 6th leaf developmental phase and transplanted to bigger pots. Seven days after transplantation *T. brevicompactum* isolate was added to half of the plants. At the stage of 9-10 leaves, half of the T treated and T untreated plants were not watered any more. Measurements of physiological parameters were

done 17 days after the addition of Trichoderma and seven days after the start of drought treatment (Figure 6).

Figure 6. Tomato plants from the second experiment at the harvesting period.

Of all measured morpho-physiological parameters leaf RWC, SLA and plant DW decreased with drought significantly. This decrease was significantly attenuated with the addition of *T. brevicompactum.* (Table 2).

Table 2. Morpho-physiological parameters (soil water content =SWC; chrorophyll content = CHL; Flavonol content =FLAV; nitrogen balance index = NBI; relative water content =RWC; specific leaf area = SLA; dry

weight= DW) measured on tomato plants subjected to different treatments. (C: optimal watering; C+T: optimal watering and *T. brevicompactum*; D: drought; D+T: drought and *T. brevicompactum).*

	SWC	CHL	FLAV	NBI	RWC	SLA (m^2/kg)	DW g
C	33.98 ± 2.07	25.16 ± 1.02	0.05 ± 0.01	467.4 ± 54.1	51.51 ± 7.01	43.01± 1.22	5.43 ± 0.41
C+T	35.13 ± 3.08	24.02 ± 0.24	0.05 ± 0.01	465.4 ± 64.79	60.11 ± 6.29	49.01± 1.1	7.96 ± 0.34
D	14.38 ± 3.27	26.78 ± 1.71	0.07 ± 0.01	478.33 ± 85.47	38.32 ± 1.25	38.72 ± 2.93	4.63 ± 0.61
D+T	10.24 ± 2.61	28.63 ± 0.73	0.06 ± 0.01	517.5 ± 59.87	44.91 ± 6.08	36.82 ± 3.06	7.13 ± 2.13

This was connected with decreased stomatal conductance (Figure 7) and higher ABA content in the leaves (Figure 8). However, ABA content in the roots was lower (Figure 8). ABA content was in relation to the expression level of NCED1 gene, involved in ABA synthesis (Figure 9).

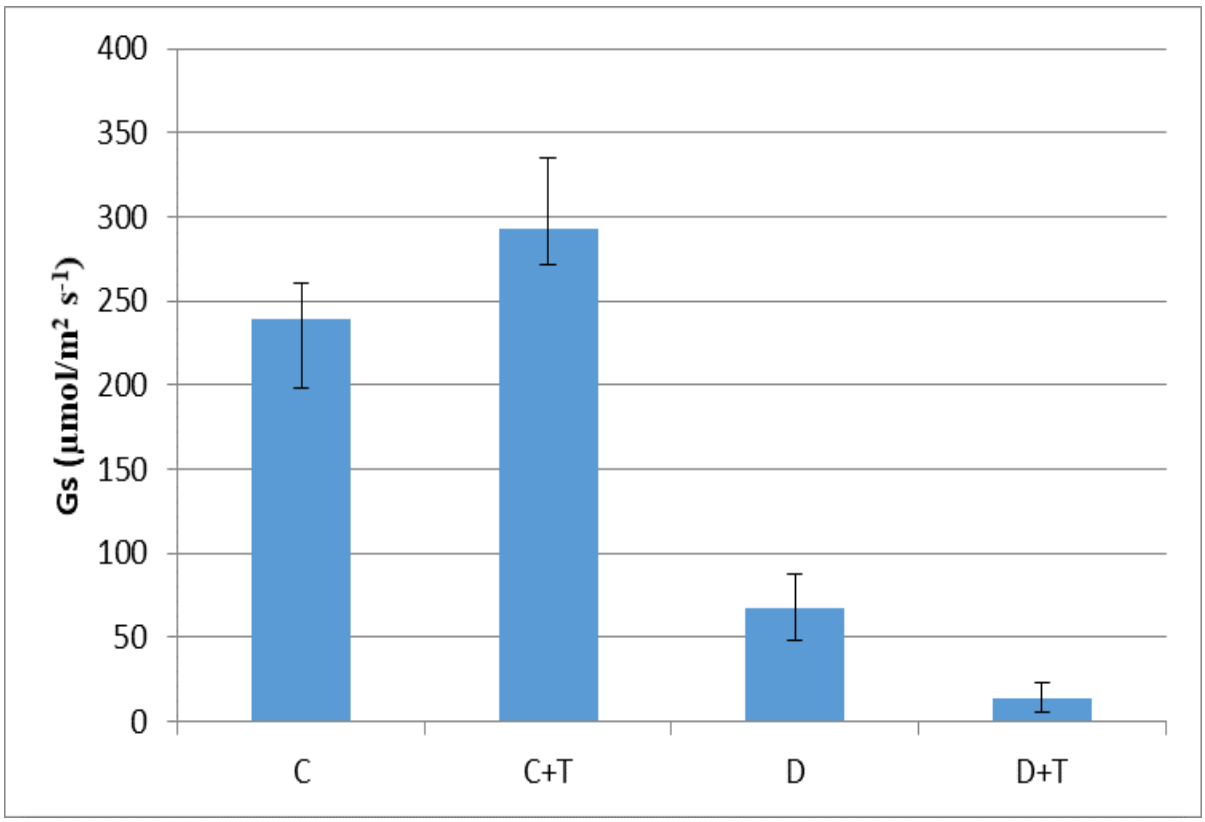


Figure 7. Stomatal conductance of tomato plants subjected to different treatments. C: optimal watering; C+T: optimal watering and *T. brevicompactum*; D: drought; D+T: drought and *T. brevicompactum*

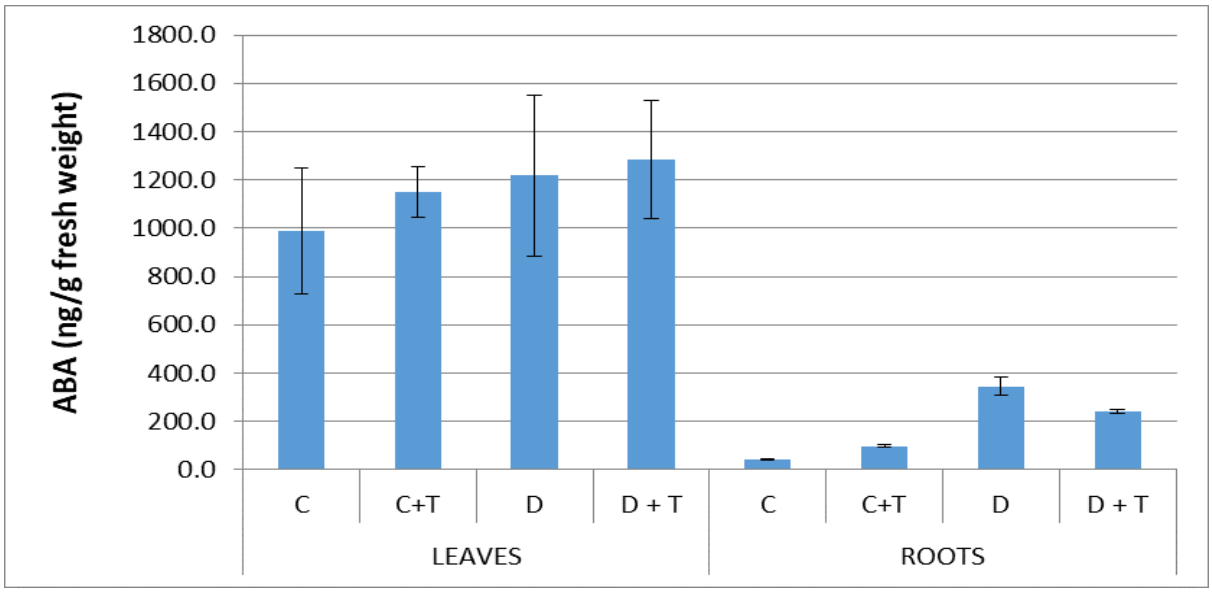


Figure 8. Abscisic acid (ABA) content in leaves and roots of tomato plants subjected to different treatments. C: optimal watering; C+T: optimal watering and *T. brevicompactum*; D: drought; D+T: drought and *T. brevicompactum.*

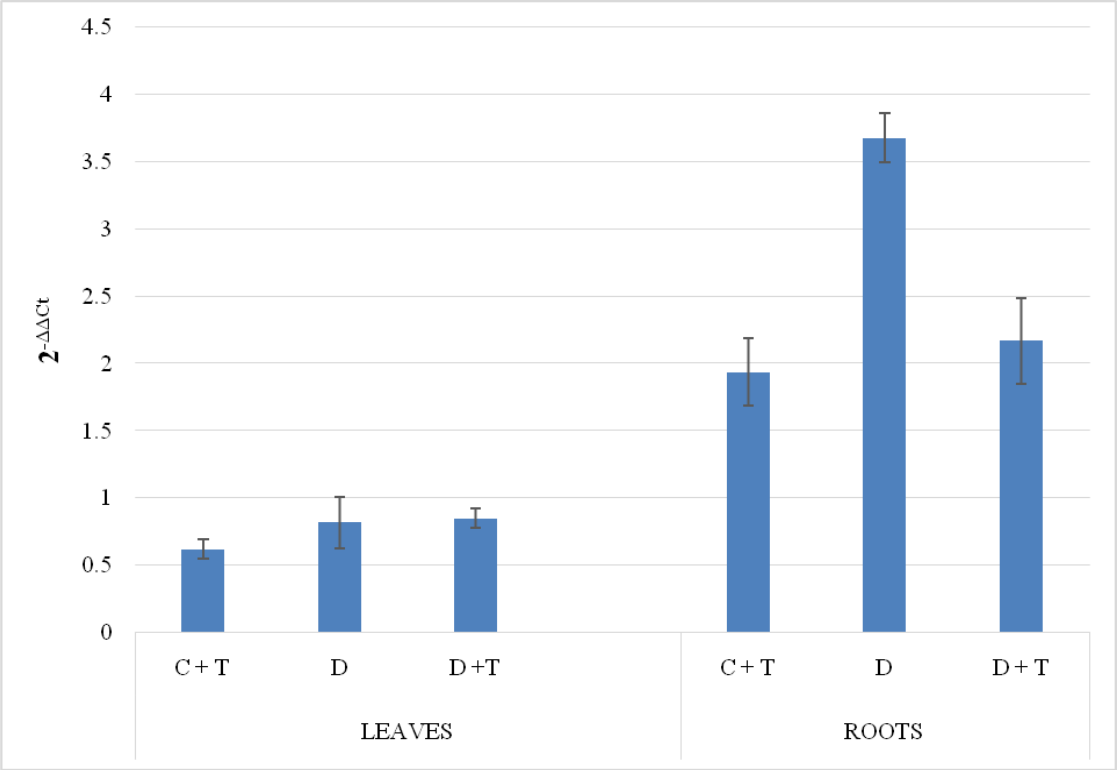


Figure 9. *qPCR analysis of NCED1 gene expression levels in tomato roots and leaves with or without the addition of Trichoderma. C: optimal watering; C+T: optimal watering and T. brevicompactum; D: drought; D+T: drought and T. brevicompactum.*

Table 3 illustrates early versus late reactions of tomato plantlets to Trichoderma-drought treatment. Stomatal conductance and ABA content are important physiological parameters that react fast and are connected with better tolerance of plants to drought.

Table 3. Early versus late reactions of tomato plantlets to Trichoderma-drought treatment

Early reactions	Late reactions
Plants responded to *Trichoderma* treatment by significant decrease of Gs already 2h after application.	**Under control conditions, Gs was 22% higher in *Trichoderma*-treated plants, this was coupled by increased RWC of leaves (17% higher than in untreated plants) and doubled ABA content in roots**
This was coupled with decreased ψ_{H2O} and increased ABA content in both leaves and roots.	**The effect of *Trichoderma* was opposite under drought, i.e. Gs was 79% lower in the treated plants, but RWC was higher**
Higher Gs of droughted plants that were in contact with *Trichoderma* was in accordance with higher ψ_{H2O} and lower ABA, both in roots and leaves in comparison to plants without *Trichoderma* treatment	**In the roots of droughted, untreated plants, the ABA content increased more than 8-fold, but in *Trichoderma*-treated plants it increased to a lower extent (5.8 times) in comparison to control plants**

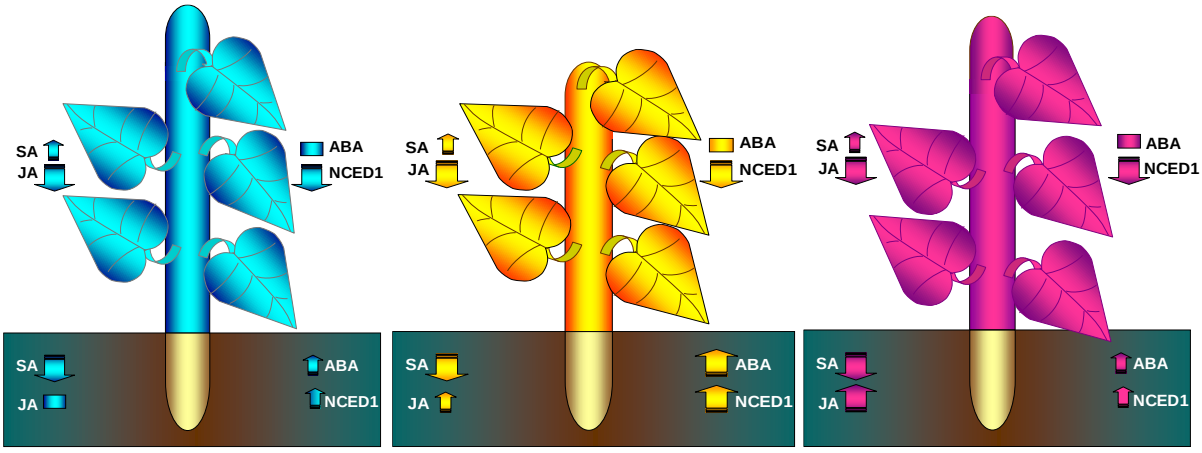


Figure 10 Model of long term of Trichoderma effect on amelioration of tomato drought tolerance. Colours illustrate the stomatal opening, from the highest (blue), medium (yellow) to the lowest (pink). Signalling pathways were regulated in the opposite direction in leaves and roots. (Racic, Vukelic et al., 2018).

The results of second experiment led us to propose the model of Trichoderma amelioration of drought tolerance(Figure 10) which is connected with the highest upregulation of JA signalling pathway marker genes, medium upregulation of NCED1 gene that contributed to medium ABA content in roots, the lowest stomatal conductance and lower inhibition of plant growth (C) (Racic, Vukelic et al., 2018).

The effect of T. harzianum on metal uptake of tomato plants

Seed of tomato plants from organic production, were grown in a growth cabinet at control conditions (LI: 250µmolm^{-2}s^{-1}, day/night T: 23/17^{o}C, RH: 60% and PP: 14^{h}). Experiments started when the tomato plants were in the phase of established six leaves and continued until the 10th leaf appeared (Figure 11). The plants were sown in the mixture of soil:compost (1:5 mass ratio) in pots (depth of 24 cm). *Trichoderma* was applied by root dip method, at the time of

transplanting. The concentration of metals (Cd, Co, Cu, Cr, Fe, Mn, Ni, Se, Zn) was measured in different parts of tomato plants (roots, stem and leaves) by ICP-OES method.

Figure 11. Tomato plants before the harvest, in the experiment were the effect of T. harzianum on metal uptake was examined.

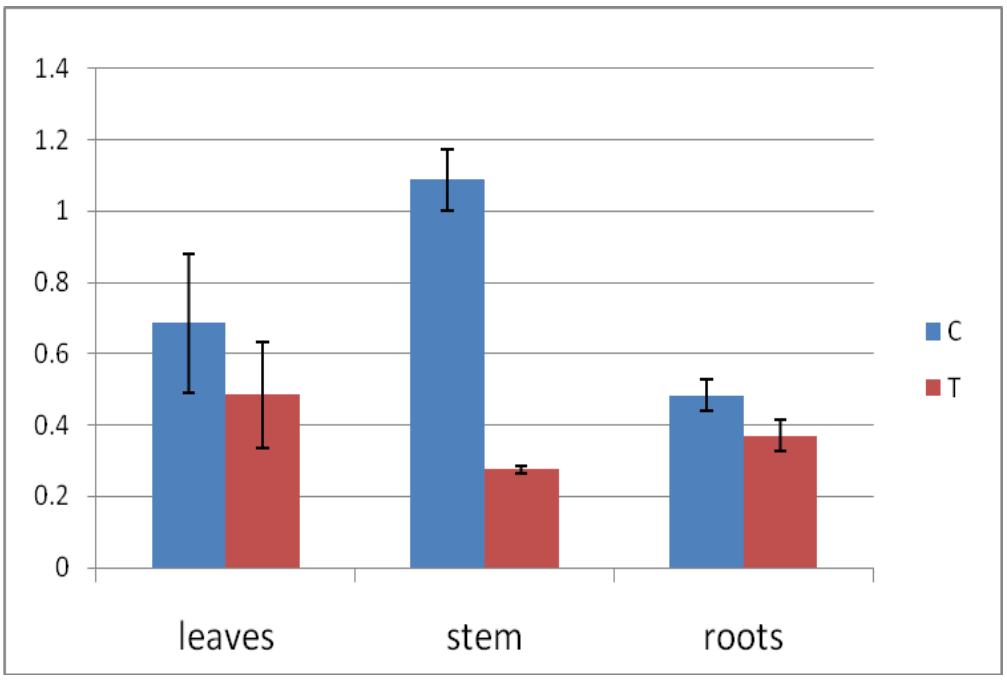


Figure 12. The content of Cd (mg/kg) in different plant parts in Trichoderma treated (T) and untreated plants (C).

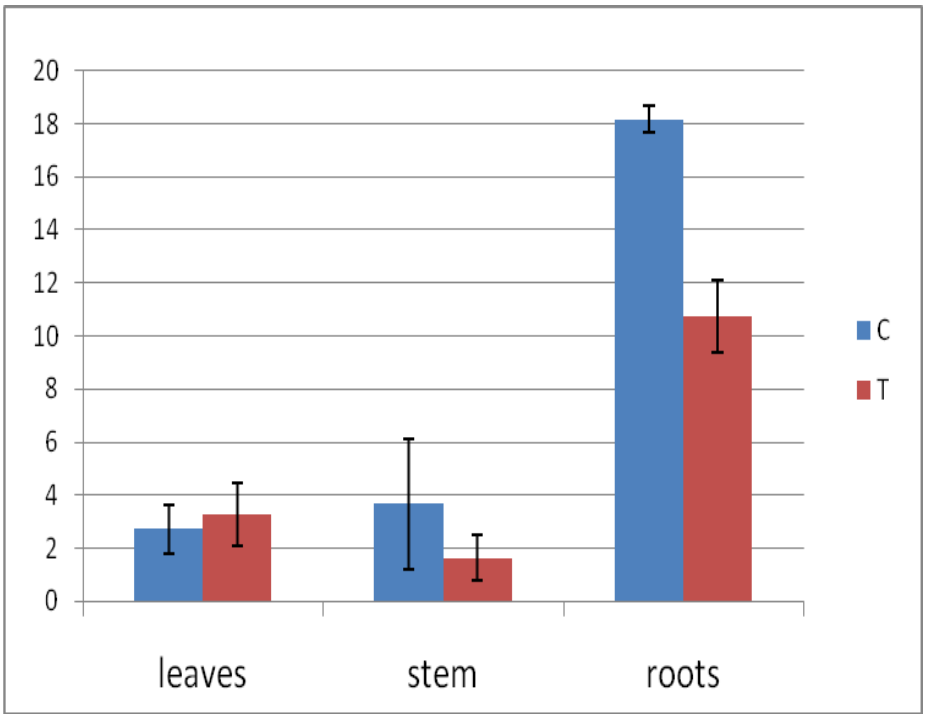


Figure 13. The content of Ni (mg/kg) in different plant parts in Trichoderma treated (T) and untreated plants (C).

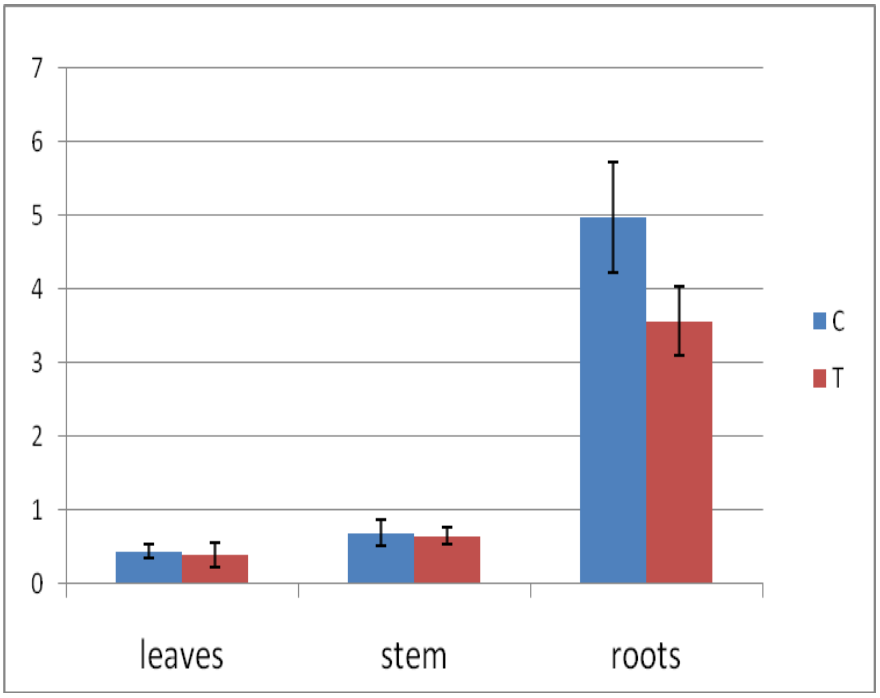


Figure 14. The content of Cr (mg/kg) in different plant parts in Trichoderma treated (T) and untreated plants (C).

In the presence of *Trichoderma* the content of toxic elements (Cd, Ni and Cr) in different plant organs is decreased (Figures 12, 13 and 14), which suggests it can reduce heavy metal availability to plants.

Other elements such as Co, Cu, Fe, Se, Zn were also examined, but their content was not affected by Trichoderma treatment.

Conclusion:

The application of T. brevicompactum to Ailsa Craig tomato plants is beneficial for plant drought tolerance.

T. harzianum reduces the availability of heavy metals to plants, therefore the uptake of Cd, Ni and Cr is decreased, as well as their content in different plant parts.

References:

Bhaduri, D., Pal, S., Purakayastha, T. J., Chakraborty, K., Yadav, R. S., & Akhtar, M. S. (2015). Soil quality and plant-microbe interactions in the rhizosphere. In *Sustainable agriculture reviews* (pp. 307-335). Springer, Cham

van der Vyver C and Peters S (2017) How Do Plants Deal with Dry Days?. Front. Young Minds. 5:58. doi: 10.3389/frym.2017.00058

Prokić Lj., Racić G., Vukelić I., Bojović M., Radić D., Jovanović Lj., Panković D.Correlations between ABA content and physiological parameters up to 48 h after Trichoderma-Tomato interaction and drought. 3rd International Conference on Plant Biology (22nd SPPS Meeting). 9-12. june 2018, Belgrade

Racić G., Körmöczi P., Kredics L., Raičević V., Mutavdžić B., Vrvić M. M., Panković D. (2017) Effect of the edaphic factors and metal content in soil on the diversity of *Trichoderma* spp. *Environmental Science and Pollution Research*, *24*(4), 3375-3386.

Racić, G., Vukelić, I., Prokić, L., Ćurčić, N., Zorić, M., Jovanović, L., Panković, D. (2018). The influence of Trichoderma brevicompactum treatment and drought on physiological parameters, abscisic acid content and signalling pathway marker gene expression in leaves and roots of tomato. Annals of applied biology, 173(3), 213-221.

Vukelić, I., Racić, G., Bojović, M., Prokić, Lj., Jovanović, Lj., Panković, D. (2017): Early changes in physiological parameters after Trichoderma - Tomato interaction in water stress conditions. COST WG1 / EPPN2020 workshop, 29th – 30th September, Institute of Field and Vegetable Crops, Novi Sad, Serbia, p. 122.

Are biocontrol agents clinically relevant? – Genus *Trichoderma* in the spotlight

Materials prepared by László Kredics

Importance of the genus *Trichoderma*

<u>Beneficial side:</u>

Ecological role: degradation of plant residues in the soil

Biotechnological significance: cellulose degradation (*T. reesei*)

Agricultural application: biological control of plant pathogenic fungi (*T. harzianum, T. virens, T. atroviride, T. asperellum, T. longibrachiatum*)

<u>Harmful side:</u>

Green mould disease of cultivated mushrooms (*T. aggressivum, T. pleuroti, T. pleuroticola*)

Clinical importance: opportunistic infections in immunocompromised patients (*T. longibrachiatum*)

Clinical relevance of the genus *Trichoderma*

The genus is on the growing list of emerging fungal pathogens.

Patients at risk:

- **immunocompromised transplant recipients**
- **patients undergoing continuos ambulatory peritoneal dialysis (CAPD)**
- **patients with HIV infection**

Number of known cases:

- **around 100**
- **growing from year to year**

Examples: Skin lesion caused by *T. longibrachiatum* Munoz et al. (1997)

11-year-old male

Severe aplastic anemia

Skin lesion on the medial aspect of the wrist of a pediatric patient with aplastic anemia

Central eschar formation with a surrounding white discoloration, edema and erythema

Skin biopsy specimen showed septate hyphal elements with irregular forms, arranged in a radial fashion

Intravenous amphotericin B

Bone marrow transplantation

Patient healed

Examples: Necrotizing stomatitis caused by *T. longibrachiatum*

Myoken et al. (2002)

Female neutropenic patient (Hiroshima survivor) with malignant lymphoma

Intensive chemotherapy

Oral lesions

Necrotizing stomatitis

Septate fungal hyphae showing dichotomous branching at acute angles in necrotic gingiva

Oral itraconazole, oral rinses with amphotericin B

Pulmonary infiltration

Patient died

Examples: *T. harzianum* infection in a renal transplant recipient

Guarro et al. (1999)

68-year-old man

Chronic renal failure

Renal transplantation

Headache, neurological symptoms

Patient died

Post mortem diagnosis: disseminated *Trichoderma* infection

Methenamine silver stain of a brain abscess showed a radiating pattern of *T. harzianum* hyphae

Periodic acid-Schiff stain revealed branching pattern of hyphae from a lung lesion

Examples: Sinusitis due to *T. longibrachiatum*

Molnár-Gábor et al. (2013)

29-year-old female, Hungary, Szeged

Immunocompetent status

Frontal and occipital headache for more than 15 years

Worsening nasal blockage

CT revealed inhomogenous shadow in the sphenoid sinus

Surgical removal of fungal mass (2 operations)

Topical amphotericin B, regular suction

Patient recovered

Recent otitis and keratitis cases due to *Trichoderma*

56-year-old, otherwise healthy female, Croatia

Discomfort, scaling and mild pruritus limited to the external auditory canal with fluffy and grayish-green discharge

Topical terbinafine

Complete recovery

65-year-old South Indian male

Diabetic, asthmatic

Complaints of pain and redness in the right eye

Central full thickness corneal infiltrate with feathery margins surrounded by immune ring with hypopyon and cataractous lens

Topical voriconazole and natamycin, intrastromal and intracameral voriconazole, oral ketoconazole

therapeutic keratoplasty along with lens extraction and anterior vitrectomy

Heart infections due to *Trichoderma*

71-year-old Hungarian man with previous aortic valve implantation

increased inflammation parameters, profuse diarrhea, fever, reduced renal function and elevated liver enzymes

Clinical samples taken from the removed aortic valve and the wall of the aorta during his reoperation revealed filamentous fungal growth

Hyphal elements in infected tissues

Intravenous voriconazole

Patient died to another cause

75-year-old Hungarian woman

Pacemaker implant

Infection of the pacemaker sac

Extraction of the device

Morphology-based identification of clinical *Trichoderma* isolates

Section	**Species**
Longibrachiatum	***T. longibrachiatum***
	T. citrinoviride
	T. pseudokoningii
	T. reesei
Pachybasium	***T. harzianum***
Trichoderma	***T. viride***
	T. atroviride
	T. koningii

??Are really all of them potential opportunists??

Clinical *Trichoderma* isolates examined at the Department of Microbiology, University of Szeged

Species	Strain	Isolated from
T. longibrachiatum	UAMH 9515	peritoneal effluent, Newfoundland, Canada
	ATCC 201044	skin lesion, TX, USA
	ATCC 208859	HIV+ patient, TX, USA
	CBS 446.95	lung of a man, Vienna, Austria
	CNM-CM 2171	cutaneous feet skin lesions, Spain
	CPK 2882	sinus lavage of a rhinosinusitis patient
	CPK 2880	blood culture of leukaemia patient
	CPK 2881	stool of a pediatric patient
T. pseudokoningii	IP 2110.92	brain biopsy, Villejuif, France
	UAMH 7955	sinus, Pennsylvania, USA
	UAMH 7956	lung, liver, intestinal wall, Iowa, USA
T. citrinoviride	UAMH 9573	peritoneal catheter, Newfoundland, Canada
T. koningii	CNM-CM 382	peritoneal fluid, Las Palmas, Spain
T. harzianum	CBS 102174	brain and lung abscesses, Spain
T. viride	CNM-CM 1798	blood culture, Spain
	CNM-CM 2277	sputum of a patient with TBC, Spain

Strain identification by ITS-sequence analysis

Primers: ITS1, ITS4

Product: about 600 bp fragment (ITS1 – 5.8S rDNA – ITS2)

Sequence analysis: *TrichO*key 2.0 (http://www.isth.info)

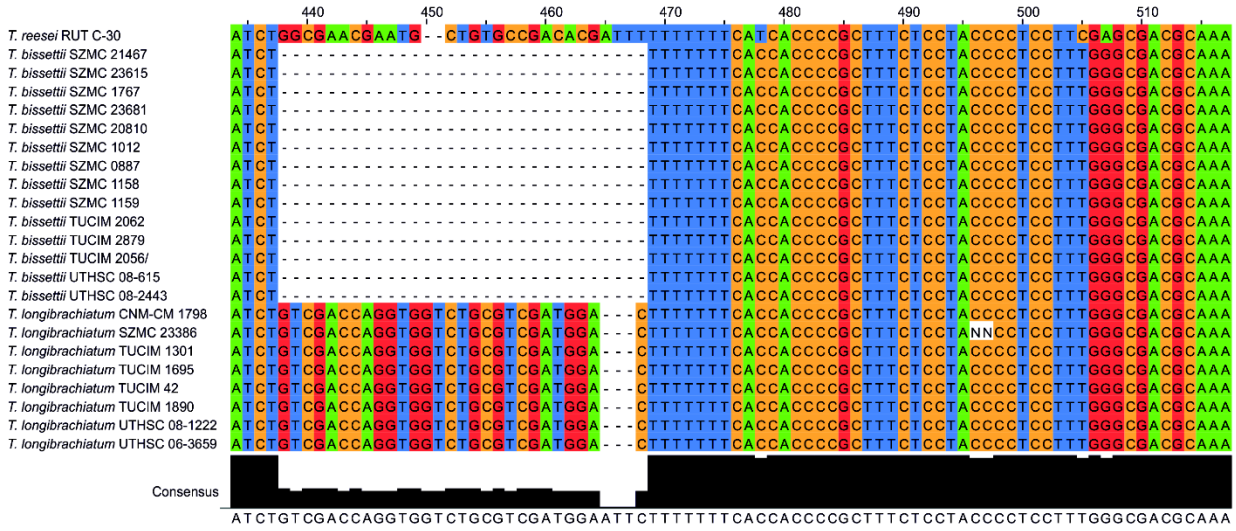
Strain	Species	Identity
CBS 102174	*T. harzianum*	confirmed
ATCC 201044	*T. longibrachiatum*	confirmed
ATCC 208859	*T. longibrachiatum*	confirmed
UAMH 9515	*T. longibrachiatum*	confirmed
CBS 446.95	*T. longibrachiatum*	confirmed
CNM-CM 2171	*T. longibrachiatum*	confirmed
CPK2880	*T. longibrachiatum*	confirmed
CPK 2881	*T. longibrachiatum*	confirmed
CPK 2882	*T. longibrachiatum*	confirmed
IP 2110.92	*T. pseudokoningii*	reidentified
UAMH 7955	*T. pseudokoningii*	reidentified
UAMH 7956	*T. pseudokoningii*	reidentified
UAMH 9573	*T. citrinoviride*	reidentified
CNM-CM 382	*T. koningii*	reidentified
CNM-CM 1798	*T. viride*	reidentified
CNM-CM 2277	*T. viride*	reidentified

All reidentified strains proved to belong to the species duplet *T. longibrachiatum/T. orientale*, sequence analysis of a *tef1* fragment revealed that both of these species are clinically relevant.

Recent taxonomic developments

- *T. longibrachiatum* previously considered to be a uniform species

- Sandoval-Denis et al. (2014): morphology, ITS (internal transcribed spacer), *tef1* (translation elongation factor 1α), *chi18-5*, endochitinase) sequence analysis: *T. longibrachiatum* + *T. bissettii* sp. nov.



Section of the *tef1* sequence alignment of *T. longibrachiatum* and *T. bissettii* strains with the diagnostic indel inside the 4th large intron allowing their differentiation. Numbers above the alignment indicate postitions related to the start position of the *tef1* coding region in *T. reesei* RUT C-30

Antifungal susceptibilities of clinical and environmental *Trichoderma* isolates

- Minimal inhibitory concentration (µg/mL) of antifungal drugs (determined by Etest modified for moulds)

- Resistance to ITC, FLC, PSC (clinical, environmental)

Strain	Source	AMB	AND	CSP	MCF	ITC	FLC	PSC	VRC
***T. longibrachiatum* SZMC 23386**	keratitis	1	0.44	0.29	0.04	>32	>256	>32	5
T. longibrachiatum TUCIM 42	tea plantation	2.5	0.38	0.38	0.064	>32	64	>32	2
***T. bissettii* SZMC 21467**	otitis externa	32	0.0435	32	0.0275	32	>256	32	1
***T. bissettii* SZMC 23615**	endocarditis	4	>32	0.23	2	>32	>256	>32	1.5
***T. bissettii* SZMC 23681**	pacemaker	1.5	0.25	0.078	0.064	>32	>256	>32	0.75
T. bissettii SZMC 1158	wheat rhizosphere	4	0.25	0.38	0.032	>32	>256	>32	3
T. bissettii SZMC 1767	mushroom compost	7	0.25	0.5	0.064	>32	>256	>32	2

AMB: amphotericin B, AND: anidulafungin, CSP: caspofungin, MCF: micafungin, ITC: itraconazole, FLC: fluconazole, PSC: posaconazole, VRC: voriconazole

Conclusions

- Identification of clinical *Trichoderma* isolates should be confirmed with sequence-based methods

- Pathogenic *Trichoderma* strains are restricted almost exclusively to species of section *Longibrachiatum*

- *tef1* or other markers are needed to clearly separate *Trichoderma longibrachiatum* from *T. orientale* and *T. bissettii*

- drug of choice for treatment: voriconazole

- Environmental habitats (including agricultural systems) are potential sources of human infections

- Members of section *Longibrachiatum* should be excluded from biocontrol

Recommended literature

Kredics L, Antal Z, Dóczi I, Manczinger L, Kevei F, Nagy E. Clinical importance of the genus *Trichoderma*. A review. Acta Microbiol Immunol Hung. 2003;50(2-3):105-17.

Hatvani L, Manczinger L, Vágvölgyi C, Kredics L. *Trichoderma* as a human pathogen. In: Mukherjee, PK; Horwitz, BA; Singh, US; Mukherjee, M; Schmoll, M (szerk.) *Trichoderma* - Biology and Applications. Wallingford, UK: CAB International, (2013) pp. 292-313.

Munoz FM, Demmler GJ, Travis WR, Ogden AK, Rossmann SN, Rinaldi MG. *Trichoderma longibrachiatum* infection in a pediatric patient with aplastic anemia. J Clin Microbiol. 1997 Feb;35(2):499-503.

Myoken Y, Sugata T, Fujita Y, Asaoku H, Fujihara M, Mikami Y. Fatal necrotizing stomatitis due to *Trichoderma longibrachiatum* in a neutropenic patient with malignant lymphoma: a case report. Int J Oral Maxillofac Surg. 2002 Dec;31(6):688-91.

Guarro J, Antolín-Ayala MI, Gené J, Gutiérrez-Calzada J, Nieves-Díez C, Ortoneda M. Fatal case of *Trichoderma harzianum* infection in a renal transplant recipient. J Clin Microbiol. 1999 Nov;37(11):3751-5.

Molnár-Gábor E, Dóczi I, Hatvani L, Vágvölgyi C, Kredics L. Isolated sinusitis sphenoidalis caused by *Trichoderma longibrachiatum* in an immunocompetent patient with headache. J Med Microbiol. 2013 Aug;62(Pt 8):1249-52. doi: 10.1099/jmm.0.059485-0.

Druzhinina IS, Komoń-Zelazowska M, Kredics L, Hatvani L, Antal Z, Belayneh T, Kubicek CP. Alternative reproductive strategies of *Hypocrea orientalis* and genetically close but clonal *Trichoderma longibrachiatum*, both capable of causing invasive mycoses of humans. Microbiology. 2008 Nov;154(Pt 11):3447-59. doi: 10.1099/mic.0.2008/021196-0.

Sandoval-Denis M, Sutton DA, Cano-Lira JF, Gené J, Fothergill AW, Wiederhold NP, Guarro J. Phylogeny of the clinically relevant species of the emerging fungus *Trichoderma* and their antifungal susceptibilities. J Clin Microbiol. 2014 Jun;52(6):2112-25. doi: 10.1128/JCM.00429-14.

Bad moulds vs. good mushrooms: mould diseases in mushroom cultivation

Materials prepared by Lóránt Hatvani

Mushroom cultivation

Oyster mushroom (*Pleurotus ostreatus*):

Human food

Enzymes (industry)

Biodegradation

Bioconversion

Medicinal mushroom

Shiitake (*Lentinula edodes*):

Food

Medicinal mushroom

Button mushroom (*Agaricus bisporus*):

Food

Medicinal mushroom???

The problem

Pest and diseases: viruses, bacteria, nematodes, insects…

***Moulds*: huge yield loss, economic importance**

Dry bubble:

Affected mushroom: *A. bisporus*

Causal agent: *Lecanicillium fungicola* (var. *fungicola, aleophilum*) (Berendsen *et al.* 2010)

Hungary, Serbia, Ireland: *L. fungicola* var. *fungicola*

Wet bubble:

Affected mushroom: *A. bisporus*

Causal agent: *Mycogone perniciosa* (Sharma and Singh 2003)

Hungary, Serbia, Croatia: *M. perniciosa*

Cobweb:

Affected mushroom: *A. bisporus*

Causal agent: *Cladobotryum mycophilum* (McKay *et al.* 1999)

Hungary, Serbia: *C. mycophilum*

Spain, Korea: king oyster mushroom (*Pleurotus eryngii*) is also affected (Gea *et al.* 2011, Kim *et al.* 2012)

"White" mould:

Affected mushroom: *A. bisporus* (Hungary, 2016)

Causal agent: *Trichoderma decipiens*

Green mould:

Affected mushrooms: *A. bisporus*, *P. ostreatus*, *L. edodes*

Causal agents: *Trichoderma* species

***A. bisporus*:**

Causal agent: *T. aggressivum* f. *aggressivum*, f. *europaeum* (Samuels *et al.* 2002)

Hungary (2004-2005): T. *aggressivum* f. *europaeum* (Hatvani *et al.* 2007)

Croatia (2010): *T. harzianum*!!! (Hatvani *et al.* 2012)

Hungary (2015-2016): *T. aggressivum* f. *aggressivum*!!!
(Hatvani *et al.* 2017)

Symptoms of green mould on *A. bisporus* (András Geösel)

<u>*P.* ostreatus:</u>

Causal agent: *T. pleuroti*, *T. pleuroticola*
(Park *et al.* 2006, Komoń-Zelazowska *et al.* 2007)

Spain, Croatia: *T. pleuroti* + *T. harzianum*!!!

Hungary (2015): *T. aggressivum* f. *aggressivum*!!!

<u>*L.* edodes:</u>

Causal agent: *T. harzianum*!!! (Hungary, Serbia)

Infection: Why???

- Presence/lack of pathogens

- Mushroom (variety, fitness)

- External factors (temperature, pH, fungicides, compost bacteria...)

- etc.

Pest management

<u>Means:</u>

- Physical

- Chemical

- Biological

- Integrated (IPM): combination

<u>Important steps:</u>

- Isolation, identification and characterization of the causal agents

- Laboratory-scale experiments (small, medium)

- Field trials

Dry bubble:

Host: ***A. bisporus***

Causal agent: ***Lecanicillium fungicola*** **var.** ***fungicola***
(sequence analysis of the ITS [internal transcribed spacer] region)

Potential control: temperature

Strain	Colony growth (mm/day)			AI*	
	20 °C	25 °C	30 °C	20 °C	25 °C
L. fungicola IP2	3.30	2.00	0	65	27
L. fungicola XT2	3.50	1.95	0	69	45
A. bisporus	1.60	1.75	1.50	-	-

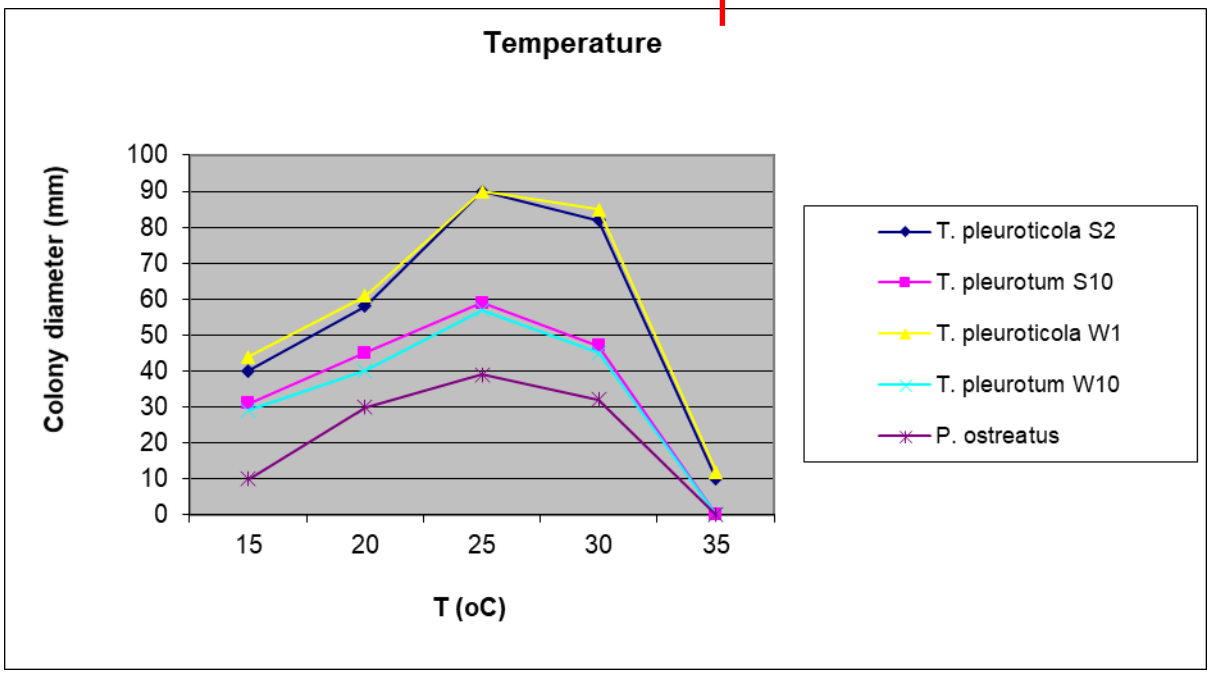
AI*: aggressivity index (originally described as biocontrol index, Szekeres *et al.* 2006)

Green mould:

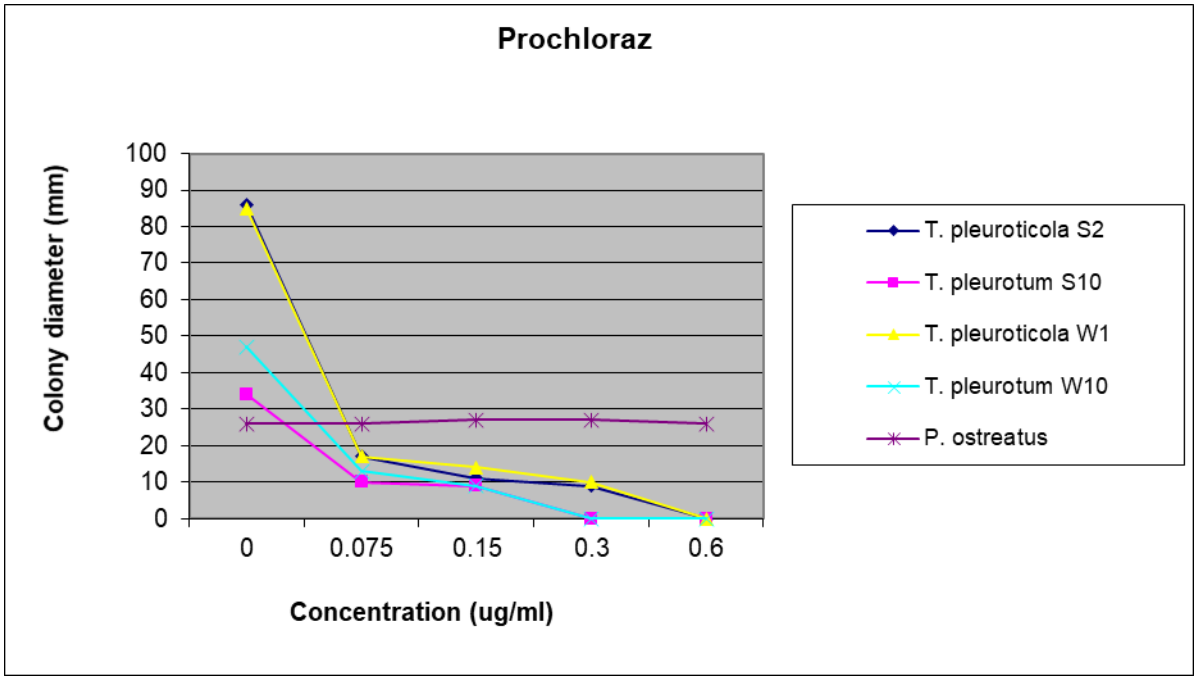
Host: ***P. ostreatus***

Causal agents: ***Trichoderma pleuroticola***, ***T. pleuroti*** **(ITS)**

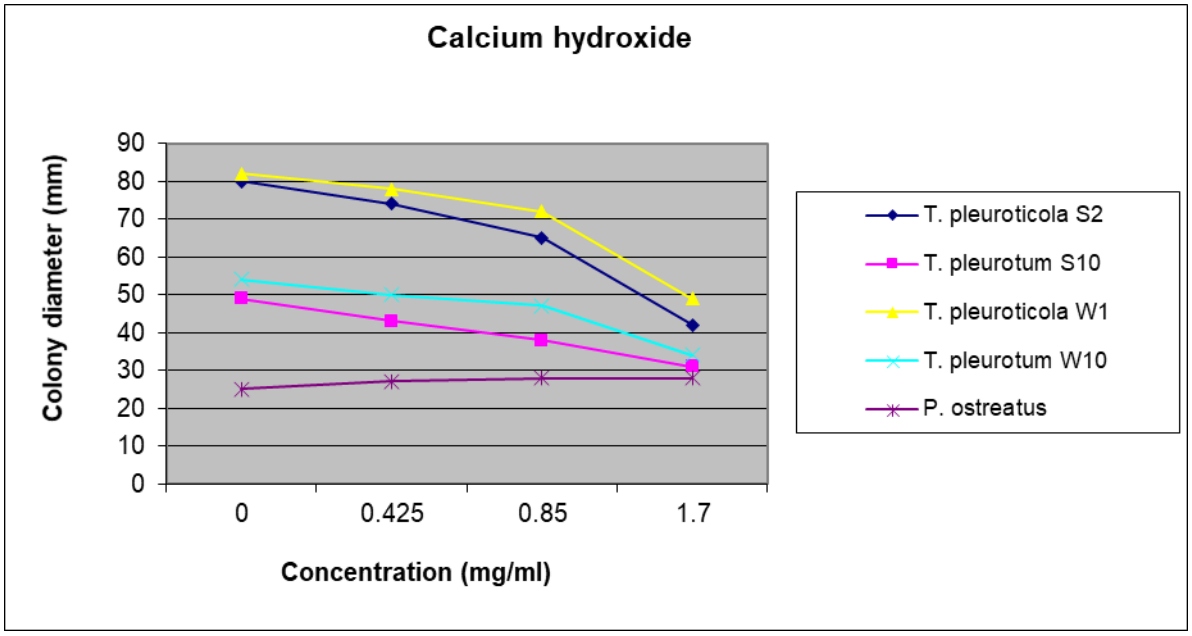
Temperature: overlapping profiles ↑ not applicable



Potential control 1: Prochloraz-Mn (fungicide)

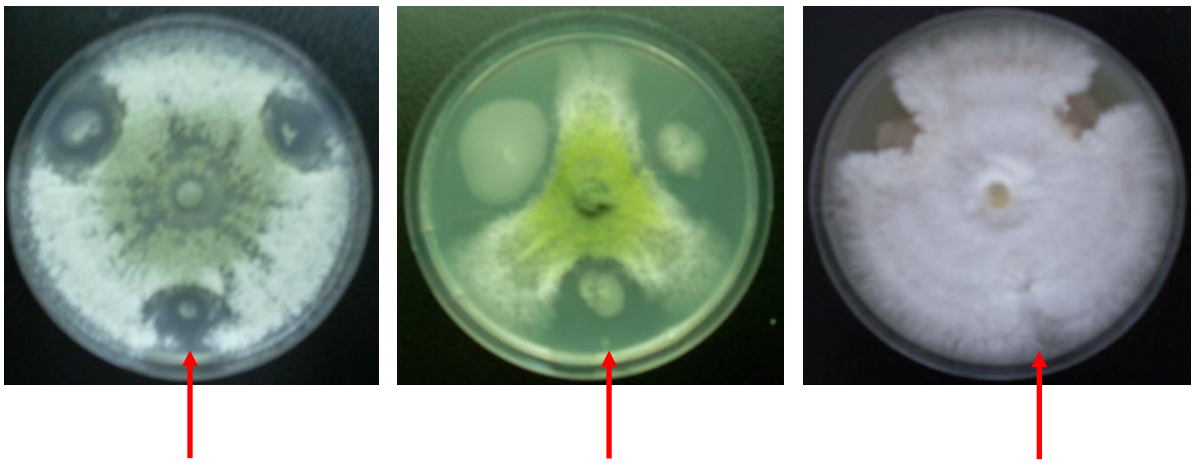


Potential control 2: Calcium hydroxide



Potential control 3: antagonistic bacteria (*Bacillus subtilis*)

T. pleuroticola ***T. pleuroti*** ***P. ostreatus***



Conclusions

Different mould diseases of cultivated mushrooms:

dry bubble, wet bubble, cobweb, white mould, green mould …

Widening spectrum of causal agents:

Trichoderma aggressivum* f. *aggressivum*, *T. harzianum*, *T. decipiens*, *Cladobotryum mycophilum

Difficult physical control (temperature)

Chemical control: calcium hydroxide, prochloraz

Biological control possibilities (antagonistic bacteria)

Inreasing need for efficient and safe biological or IPM strategies!!!

Recommended literature

Berendsen RL, Baars JJ, Kalkhove SI, Lugones LG, Wösten HA, Bakker PA. (2010). Pathogen profile: *Lecanicillium fungicola*, causal agent of dry bubble disease in white-button mushroom. *Mol Plant Pathol*. 11(5): 585-595. doi: 10.1111/j.1364-3703.2010.00627.x

Gea FJ, Navarro MJ, Suz LM. (2011). First Report of *Cladobotryum mycophilum* causing cobweb on cultivated king oyster mushroom in Spain. *Plant Dis.* 95(8): 1030. doi: 10.1094/PDIS-03-11-0255

Hatvani L, Antal Z, Manczinger L, Szekeres A, Druhzinina IS, Kubicek CP, Nagy A, Nagy E, Vágvölgyi C, Kredics L. (2007). Green mold diseases of *Agaricus* and *Pleurotus* spp. are caused by related but phylogenetically different *Trichoderma* species. *Phytopathology.* 97(4): 532–537. doi: 10.1094/PHYTO-97-4-0532

Hatvani L, Kredics L, Allaga H, Manczinger L, Vágvölgyi C, Kuti K, Geösel A. (2017). First report of *Trichoderma aggressivum* f. *aggressivum* green mold on *Agaricus bisporus* in Europe. *Plant Dis.* 101(6): 1052-1053. doi: 10.1094/PDIS-12-16-1783-PDN

Hatvani L, Sabolić P, Kocsubé S, Kredics L, Czifra D, Vágvölgyi C, Kaliterna J, Ivić D, Đermić E, Kosalec I. (2012). The first report on mushroom green mould disease in Croatia. *Arh Hig Rada Toksikol.* 63(4): 481-487. doi: 10.2478/10004-1254-63-2012-2220

Kim MK, Lee YH, Cho KM, Lee JY. (2012). First report of cobweb disease caused by *Cladobotryum mycophilum* on the edible mushroom *Pleurotus eryngii* in Korea. *Plant Dis.* 96(2): 1374. doi: 10.1094/PDIS-01-12-0015-PDN

Komoń-Zelazowska M, Bissett J, Zafari D, Hatvani L, Manczinger L, Woo S, Lorito M, Kredics L, Kubicek CP, Druzhinina IS. (2007).

Genetically closely related but phenotypically divergent *Trichoderma* species cause worldwide green mould disease in oyster mushroom farms. *Appl Environ Microbiol.* 73(22): 7415–7426. doi: 10.1128/AEM.01059-07

McKay GJ, Egan D, Morris E, Scott C, Brown AE. (1999). Genetic and morphological characterization of *Cladobotryum* species causing cobweb disease of mushrooms. *Appl Environ Microbiol.* 65(2): 606-610.

Park MS, Bae K S, Yu SH. (2006). Two new species of *Trichoderma* associated with green mold of oyster mushroom cultivation in Korea. *Mycobiology.* 34(3): 111–113. doi: 10.4489/MYCO.2006.34.3.111

Samuels GJ, Dodd SL, Gams W, Castlebury LA, Petrini O. (2002). *Trichoderma* species associated with the green mold epidemic of commercially grown *Agaricus bisporus*. *Mycologia.* 94(1): 146-170.

Sharma VP, Singh C. (2003) Biology and control of *Mycogone perniciosa* Magn. causing wet bubble disease of white button mushroom. *J Mycol Plant Pathol.* 33(2): 257-264.

Szekeres A, Leitgeb B, Kredics L, Manczinger L, Vágvölgyi C. (2006). A novel, image analysis-based method for the evaluation of *in vitro* antagonism. *J Microbiol Methods.* 65(3): 619–622.

Good moulds vs. bad mushrooms: towards the biological control of *Armillaria* root rot

Materials prepared by Csaba Vágvölgyi, György Sipos and László Nagy

Microorganisms invading living trees

Bacterial and fungal invaders of living trees must overcome

the innate immune system of the host tree

the presence of potentially toxic antibacterial and/or antifungal compounds

the low nitrogen and phosphorous content in the woody environment

the complexity of the organic substrates like lignin and phenols

The most effective invaders are the pathogenic wood-decay fungi.

The genus *Armillaria*

Basidiomycota, Agaricales, Physalacriaceae

More than 70 species, regular native associates of forest ecosystems worldwide

Predominantly diploid rather than dikaryotic vegetative phase

Largest and oldest terrestrial organisms - humongous fungus (*A. ostoyae*) in Oregon (USA): 965 hectares, 600 tons, several thousands of years

Rhizomorphs: unique multicellular structures (*Armillaria* & *Guyanagaster*), apical polarized growth, made up of 4 distinct hyphal layers

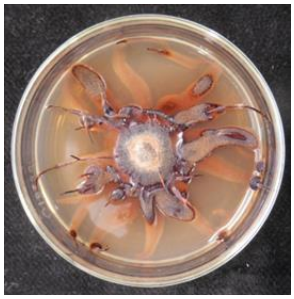
Most *Armillaria* species are facultative necrotrophs

Colonizing and killing the root cambium

Transition to saprobic phase: decomposing dead woody tissues

More than 500 plant host species

Dispersal strategy: lower-than expected mutation rate - clonal stability



Life strategy	Conifers	Deciduous trees (e.g., oak species)
Pathogenic	*Armillaria ostoyae*	*Armillaria mellea*
Saprotrophic / Opportunistic pathogen	*Armillaria cepistipes* *Armillaria borealis*	*Armillaria gallica*

An integrative approach

Genome sequencing of parasitic and saprophytic *Armillaria* species, comparative genomics

Transcriptomic comparison of different developmental stages

Proteomic analysis

Collection and identification of *Armillaria* samples from infected and healthy forests

Metagenomic analysis of *Armillaria*-associated microbiomes

Isolation and identification of bacteria and fungi from *Armillaria*-associated microbiota

Selection and characterization of potential BCAs for the control of *Armillaria* root rot

Complex investigation of the interactions in the *Armillaria* – host – BCA system

Development of environment-friendly management strategies, field experiments

Full *Armillaria* genomes published

***A. mellea* (Collins et al. 2013), *A. fuscipes* (Wingfield et al. 2016), *A. ostoyae*, *A. cepistipes*, *A. gallica*, *A. solidipes* (Sipos et al. 2017)**

What we learned from *Armillaria* genomes?

- Lineage-specific innovations

- The position of *Armillaria* species in the Physalacriaceae was confirmed, with *Guyanagaster* and *Cylindrobasidium* as their closest relatives

- Expansion of protein coding gene repertoires in *Armillaria* spp.

- Separation just in the Cenozoic geological era

- There is a significant genome expansion in *Armillaria*, affecting several pathogenicity-related genes, lignocellulose-degrading enzymes

and lineage-specific genes expressed during rhizomorph development

- 442 genes showed greater than fourfold elevated expression over vegetative mycelium and relatively constant expression across rhizomorph and fruting samples, suggesting they are linked to complex multicellularity in *A. ostoyae*

- Rhizomorph development draws extensively on fruiting body genes, fruting body development could have been the cradle for the evolution of rhizomorphs in *Armillaria*

- Genes encoding components of the endoplasmic reticulum protein posttranslational import system are upregulated in rhizomorphs

- This may indicate an intensified biogenesis and cargo of extracellular proteins along the secretory pathway

- Genes involved in chitin synthesis and remodeling are differentially used by *A. ostoyae* in a development- and tissue-specific manner

- Hydrophobins were mostly overexpressed in the vegetative mycelia

- Four hydrophobins reached their highest expression in rhizomorphs

- Cerato-platanins and expansins might act as first-line cell-lysis weaponry during invasion

Studying the *Armillaria* problem in Central Europe

- Collection of *Armillaria* fruting bodies in Northwest Hungary and Austria

- Morphological examination of *Armillaria* fruiting bodies

- Species-level identification of *Armillaria* isolates:

- 39 *Armillaria* strains received and deposited in the Szeged Microbiology Collection (SZMC)

- Sequence-based identification of *Armillaria* isolates

- Translation elongation factor 1α (*tef1)*

- Confirmed species:

A. gallica

A. mellea

A. cepistipes

A. ostoyae

- Remarkable genetic diversity within *A. gallica*

- Hardwood-specific species were found in the Keszthely Hills

- *A. mellea* and *A. gallica* were abundant in a severely damaged forest area

- A large *A. gallica* colony was detected in the Sopron Hills

- Conifer-specific *Armillaria* species were isolated from the Rosalia Hills, Austria

The microbiota associated with *Armillaria*

The assembly of the "*Armillaria*-associated microbiota" might be dependent:

on the host genotype

on its phenotypic traits

or on the forest environment

Isolation and species-level identification of potential BCAs: *Trichoderma*

Total number of *Trichoderma* isolates: 115

Molecular identification by ITS and *tef1* sequence analysis

51 out of 115 *Trichoderma* strains identified and deposited in SZMC

Detected species:

***T. simmonsii* (11)**

***T. koningii* (9)**

***T. atroviride* (8)**

***T. virens* (4)**

***T. harzianum* (4)**

***T. gamsii* (3)**

***T. asperellum* (3)**

***T. hamatum* (2)**

***Trichoderma* sp. (2)**

***T. paratroviride* (1)**

***T. tomentosum* (1)**

***T. atrobrunneum* (2)**

***T. crassum* (1)**

Isolation and species-level identification of potential BCAs: pseudomonads

***Pseudomonas* strains identified based on a region of the *rpoB* gene**

29 *Pseudomonas* strains identified and deposited in SZMC

Detected species:

- *P. mandelii* (11)

- *Pseudomonas* sp. (8)

- *P. jessenii* (4)

- *P. koreensis* (2)

- *P. fluorescens* (4)

Isolation and species-level identification of potential BCAs: bacilli and other bacteria

Bacilli and other bacteria identified based on the 16S rRNA gene

11 bacterial strains identified and deposited in SZMC

Detected species:

- ***Bacillus cereus*** **(2)**
- ***Bacillus mycoides*** **(1)**
- ***Paenibacillus alginolyticus*** **(2)**
- ***Paenibacillus castaneae*** **(1)**
- ***Paenibacillus*** **sp. (2)**
- ***Burkholderia*** **sp. (1)**
- ***Lysinibacillus*** **sp. (1)**
- ***Streptomyces*** **sp. (1)**

Screening for *Trichoderma* strains with biocontrol potential

Trichoderma **strains confronted with *Armillaria* in all combinations on PDA plates**

Biocontrol Index (BCI) values calculated based on image analysis of plate photographs

BCI = (area of *Trichoderma* colony / total area occupied by the colonies of both *Trichoderma* and *Armillaria*) × 100

BCI values of *Trichoderma* strains against *A. gallica* isolates

	A. gallica 5/1	*A. gallica* 12/2A	*A. gallica* 14/2	*A. gallica* 15/2
T. virens 13/1	100.00	100.00	100.00	100.00
T. atroviride 13/2	37.51	100.00	100.00	100.00
T. harzianum 13/3	100.00	100.00	100.00	100.00
T. harzianum 14/1	100.00	85.37	100.00	100.00
T. citrinoviride 14/2	52.83	95.05	100.00	78.70
T. citrinoviride 14/3/2	52.52	100.00	100.00	100.00
T. hamatum 12/1	100.00	79.88	100.00	100.00
Trichoderma sp. 12/2	77.23	100.00	100.00	89.75
T. virens 12/3	100.00	74.27	100.00	100.00
T. virens 3/1	100.00	100.00	100.00	100.00
T. virens 3/2	100.00	100.00	100.00	100.00
T. virens 3/3	100.00	100.00	100.00	100.00
T. virens 5/1	100.00	100.00	100.00	100.00
T. virens 5/2	100.00	100.00	100.00	100.00
T. crassum 5/3/1	73.76	89.12	100.00	100.00
T. virens 5/3/2	100.00	100.00	100.00	100.00

Screening for bacterial strains with biocontrol potential

Colony diameters (cm) of *A. gallica* isolates in the presence of different *Pseudomonas* strains

	A. gallica 5/1	*A. gallica* 12/2A	*A. gallica* 14/2	*A. gallica* 15/2
Control	1.9*	1.6*	1.7*	1.4*
P. mandelii 3/2	0.9	1.1	1.4	1.0
P. putida 3/3	1.1	1.0*	1.3	1.0
Pseudomonas sp. 3/4	1.3	1.1	1.4	1.1
P. mandelii 3/5	1.3	1.1	1.3	0.9
Pseudomonas sp. 3/6	1.3	1.1	1.2	1.0
Pseudomonas sp. 3/7	1.1	1.3	1.4*	1.0
P. fluorescens 13/1	1.1	0.0	0.8	0.7
P. mandelii 13/2	1.1	0.7	0.4*	0.5
Pseudomonas sp. 13/5	1.1	1.2	1.1	0.8
Pseudomonas sp. 13/6	1.3	1.1	1.2	1.0
Pseudomonas sp. 13/7	1.5	1.0	1.4*	0.4
Pseudomonas sp. 13/8	1.0	1.1	1.1	1.0
Pseudomonas sp. 12/4	1.2*	0.8*	1.2	0.9
P. fluorescens 3T1	1.2	1.0	1.0*	0.5

Characterization of BCA candidates:

siderophore production

phosphorous mobilization

indole acetic acid production

Interaction studies:

antagonism assays

stem invasion assays

seedling growth promotion assays

Conclusions

Comparative genomics revealed an expansion of protein coding genes in the genus *Armillaria*

Transcriptomic data suggest that fruiting body formation might have been the cradle of rhizomorph formation

Genes differentially expressed in different developmental stages of *Armillaria* could be identified

***Armillaria gallica* shows a remarkable genetic diversity in Central Europe**

Metagenome analysis may shed light on possible bacterial endosymbionts of *Armillaria*

Rhizomorph-associated bacteria and fungi with biocontrol potential could be selected

Studies on the interactions of the pathogen-host-BCA system are expected to shed light on mechanisms of pathogenicity, biocontrol and plant growth promotion

Recommended literature

Collins C, Keane TM, Turner DJ, O'Keeffe G, Fitzpatrick DA, Doyle S. Genomic and proteomic dissection of the ubiquitous plant pathogen, *Armillaria mellea*: toward a new infection model system. J Proteome Res. 2013 Jun 7;12(6):2552-70. doi: 10.1021/pr301131t

Wingfield BD, Ambler JM, Coetzee MP, de Beer ZW, Duong TA, Joubert F, Hammerbacher A, McTaggart AR, Naidoo K, Nguyen HD, Ponomareva E, Santana QS, Seifert KA, Steenkamp ET, Trollip C, van der Nest MA, Visagie CM, Wilken PM, Wingfield MJ, Yilmaz N. IMA Genome-F 6: Draft genome sequences of *Armillaria fuscipes, Ceratocystiopsis minuta, Ceratocystis adiposa, Endoconidiophora laricicola, E. polonica* and *Penicillium freii* DAOMC 242723. IMA Fungus. 2016 Jun;7(1):217-27. doi: 10.5598/imafungus.2016.07.01.11.

Sipos G, Prasanna AN, Walter MC, O'Connor E, Bálint B, Krizsán K, Kiss B, Hess J, Varga T, Slot J, Riley R, Bóka B, Rigling D, Barry K, Lee J, Mihaltcheva S, LaButti K, Lipzen A, Waldron R, Moloney NM, Sperisen C, Kredics L, Vágvölgyi C, Patrignani A, Fitzpatrick D, Nagy I, Doyle S, Anderson JB, Grigoriev IV, Güldener U, Münsterkötter M, Nagy LG. Genome expansion and lineage-specific genetic innovations in the forest pathogenic fungi *Armillaria*. Nat Ecol Evol. 2017 Dec;1(12):1931-1941. doi: 10.1038/s41559-017-0347-8.

Control of Varroa Mite (Varroa Destructor) on Honey Bees by micro biological agents

MATERIALS PREPARED BY SLADJAN RASIC

General Introduction

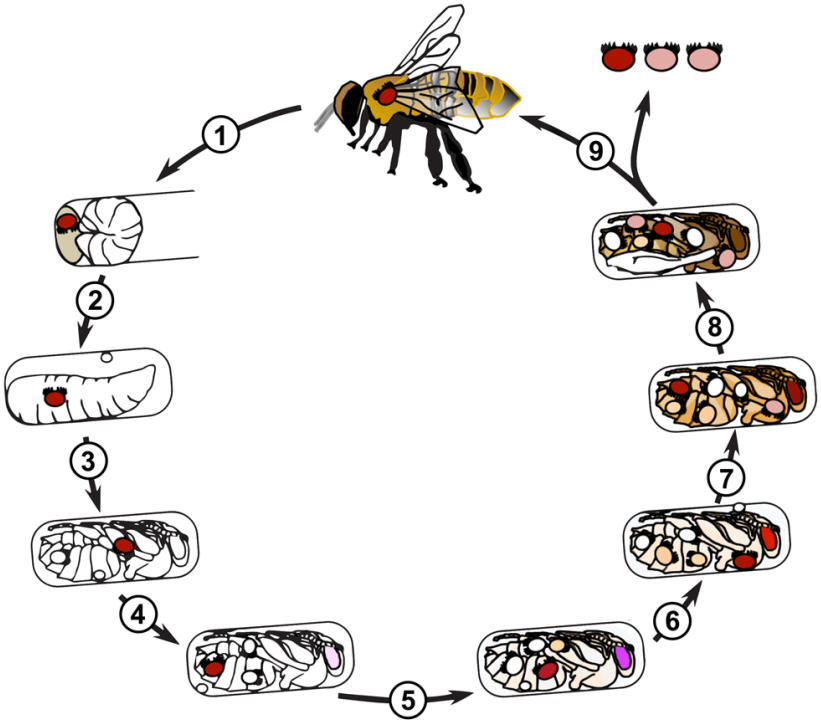
Very rapidly after *Varroa destructor* invaded apiaries of *Apis mellifera*, the devastating effect of this mite prompted an active research effort to understand and control this parasite. Over a few decades, varroa has spread to most countries exploiting *A. mellifera*. As a onsequence, alarge number of teams have worked with this organism, developing a diversity of research methods. Often different approaches have been followed to achieve the same goal. The diversity of methods made the results difficult to compare, thus hindering our understanding of this parasite.

***Varroa* is a mite with a head of the size of the cheek, with 8 legs, brown to reddish brown. It is fed to the haemolymph of bees. This mite is present in almost all countries of the world except for the Australian continent.**

Varroa destructor

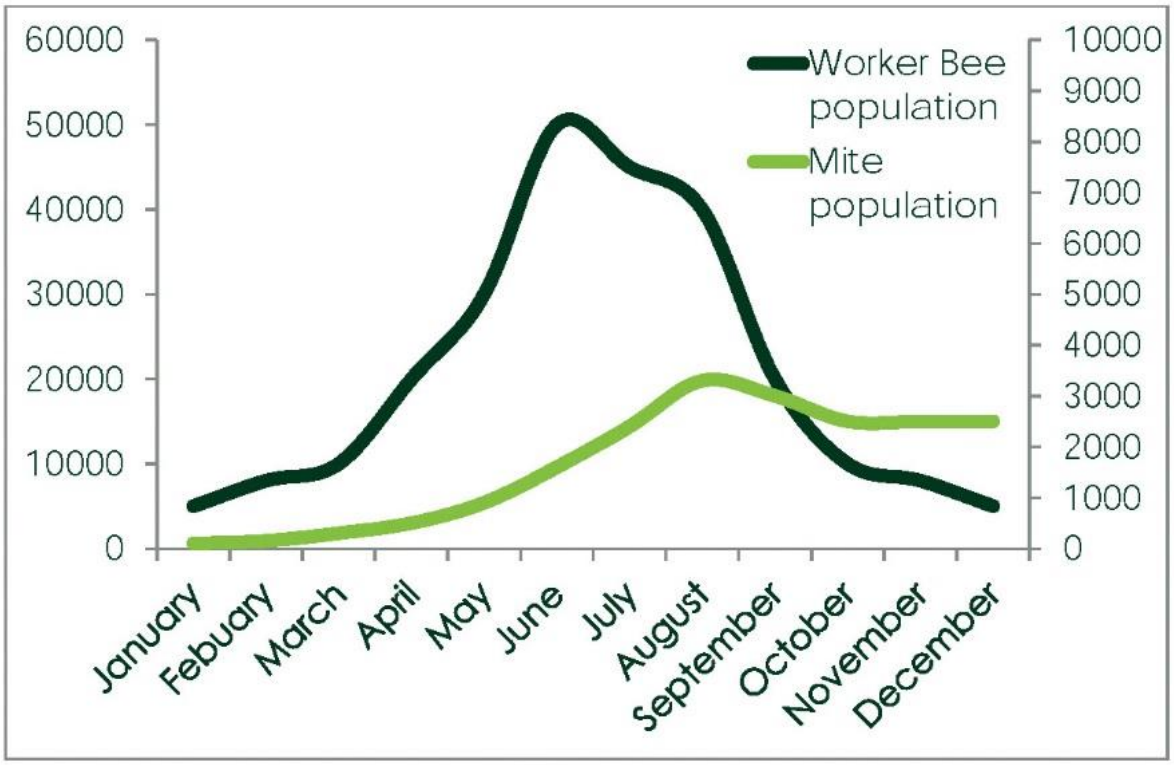
Life cycle of *Varoa*

An adult female *Varoe* is fed on a bee for 5-13 days and enters the brood cell from 24 to 60 hours before matching the cell with the brood. In winter, while there is no brood, she lives on a bee for 6 months. Average lifespan, when brood is present, is 27 days. She takes the first egg 60 hours after caped the cell, then every 30 hours thereafter. The first mite that is leaking is the male. Other mites are females who mate with a male and eat on a pupa. Adult and mature mites come out with bees, immature and males stay in a cell where they soon die. *Varoa* is transmitted from the bee on the bee on the frames with a brood and is mostly found on worker bees.



Life span of Varoa destructor

Population dynamics of varoa mites



In winter time Varoa is on adult bees. He enters in the brood in the spring, prefers drone brood. Each varoa seen on the bee comes in two more in the brood. Pop up the peak of the Varoe population is in July and August!

Sampling Methods

Method with powdered sugar A

Take about 400 bees in a jar of 720 gr with a mesh cap

Add 1 tablespoon of powdered sugar and stir strong (shake)

Sprinkle sugar on white paper

Count the varou

Release the bees

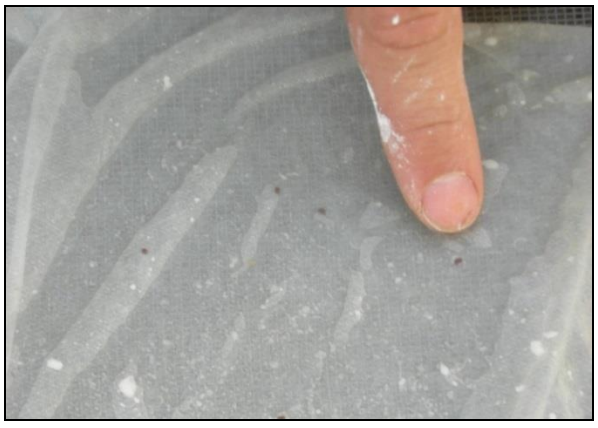
Method with powdered sugar B

Collect 300 bees (100mL beaker=300 bees)

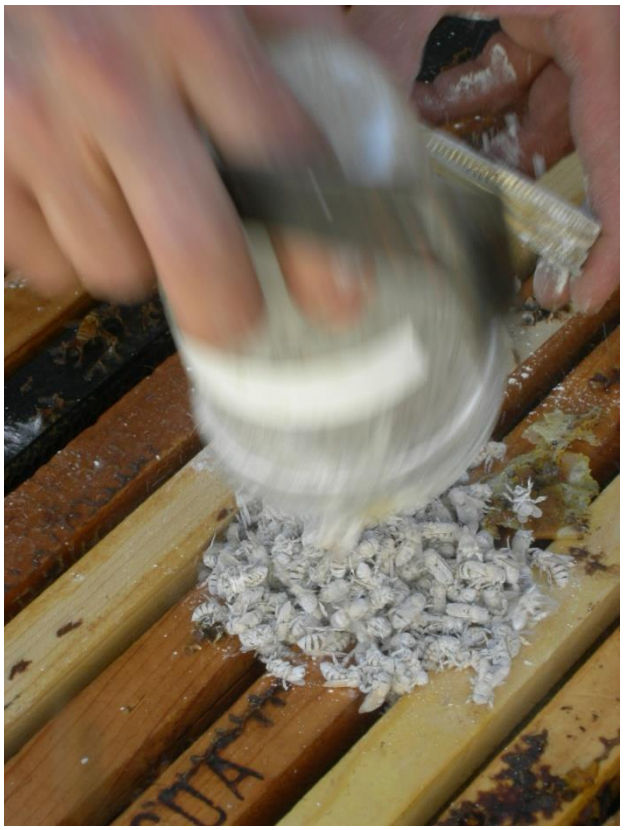
Collect 300 bees by brushing into a measured container

Add the 300 bees into jar with screened lid

MIXING OF SUGAR AND COUNTING OF VAROA



RETURN OF BEES IN HIVES



MONITORING OF INTENSITY OF INFECTION BY VAROA

METHOD WITH MOVABLE PLATE

Mesh platform with movable plate

There are commercial adhesive plates or a simple existing plate coated with petroleum jelly or some oily product

Place underneath the underbrush for 1 to 3 days

Count the naturally occluded mites

Calculate the daily drop of Varoe

FACTORS INTO THE QUANTITY OF VAROA

Period during the season

Size of the bee colony

Hygienic behavior of bees

The surface of the brood

The surface of the worker bee brood

If there is a little drone brood in a hive, the varoa will populate a bee brood

WHEN TO DO THE TESTING

Frequency of treatment:

1. If the intensity of invasion is low, the treatment goes twice a year.

2. If the varoe population is numerous, it is recommended to treat every two months (excluding the winter period).

Varoa destructor

ALTERNATIVE WAY TO DETERMINE LEVEL OF INFECTION OF THE VAROA

#Mites per 300 adult bees	Colony infestation	#Mites per 8 300 adult bee samples	Apiary infestation
1	1%	8	1%
2	1%	16	1%
3	2%	24	2%
4	3%	32	3%
5	3%	40	3%
6	4%	48	4%
7	5%	56	5%
8	5%	64	5%
9	6%	72	6%
10	7%	80	7%
11	7%	88	7%
12	8%	96	8%
13	9%	104	9%
14	9%	112	9%
15	10%	120	10%
16	11%	128	11%
17	11%	136	11%
18	12%	144	12%

Lee, K.V., G. Reuter and M. Spivak. 2010. Standardized sampling plan to detect Varroa density in colonies and apiaries. Amer. Bee Journal. 150: 1151-1155.

REVIEW OF IPM PRINCIPLE

IPM means managing the population of pests, not completely eradicating.

Understanding the life cycle of pests.

Monitoring is critical.

Knowledge of healing thresholds.

The time of treatment or treatment (when needed) is critical.

CHEMICAL TREATMENT AGAINST VAROE

Product Trade Name	Active Ingredient	Chemical Class
Apiguard	thymol	essential oil
Exomite Apis	thymol	essential oil
Apilife VAR	thymol, eucalyptol, menthol, camphor	essential oils
Apistan **	fluvalinate	synthetic pyrethroid
Apitol	cymiazole	iminophenyl thiazolidine derivative
Apivar **	amitraz	amadine
Bayvarol **	flumethrin	synthetic pyrethroid
Check-Mite+ **	Perizin coumaphos	organophosphate
Folbex	bromopropylate	chlorinated hydrocarbon
Sucrocide	sucrose octanoate	sugar esters
Hivestan	fenpyroximate	pyrazole (alkaloid)
generic	formic acid	organic acid
generic	lactic acid	organic acid
generic	oxalic acid	organic acid

*** No Longer Effective in some areas*

Compiled from Rosenkranz et al. 2010 and http://www.maf.govt.nz/biosecurity/pests-diseases/animals/varroa/guidelines/control-of-varroa-guide.pdf.

BIOLOGICAL TREATMENTS

MICROBIOLOGICAL AGENTS

Akaripathogenic fungi:

Beauveria bassiana

Metarhizium anisopliae var. anisopliae

Verticillium lecanii

Hirsutella thompsonii

Deficiency

It takes several days to kill your host

they can not adapt to the surroundings in a hive

Chandler et al., 2001, Shaw et al., 2002, James et al., 2006, Kanga et al., 2002, Kanga et al., 2006, Meikle et al., 2007 and Meikle et al., 2008.

VIRUSES, BACTERIA, NEMATODES, PROTOZOA, RICKETTSIAE

The use of pathogens can cause Varoe's flying infection in the hive.

Some strains of Bacillaceae and Micrococcaceae increase the mortality of Varoe in laboratory conditions. To date, the strain specific to Varoa has not been isolated.

(Aronson et al., 1986, Ball and Allen, 1988, Glinski and Jarosz, 1999; Tsagou et al., 2004, Van der Geest et al., 2000)

PREDATORS AND PARASITOIDS

Pseudoscorpies - they can feed on mites. The relationship with the bees has not been sufficiently explored. There are some studies with pseudoscorpies in the hive (Donovan and Paul, 2005, 2006, Gonzales et al., 2007, Van der Geest et al., 2000)

The conditions for breeding and inoculation of pseudoscorpions have not yet been sufficiently studied.

OTHER TREATMENTS

1. Bacteria Serratia marcescens, isolated from the A. cerana; excretes proteins that affect the degradation of the Varoe chitos

2. New oil application systems: orange oil is encapsulated through ceramic tiles

3. RNA interference (or RNAi), current research: deactivates certain genes that regulate gene expression

4. Attractiveness of the brood and the release and release of dissolved substances in the brood that are attractive to Varroa and the patterns of the trap; currently in the test

5. Chlorfenvinphos (organophosphate), effective but residues can cause problems

6. Azadirachtin (Neem) needs work on it, some products can be effective

7. Herbal monoterpenoids, some reported to be toxic to bees

8. Mineral oils from food are not effective

9. Sugar powder can be good only for samples, but not for full treatments

10. The lower plate on the hive is not effective (Ellis et al., 2001)

11. Smoke, various smelling materials can cause damage to the bees

12. Thermal treatments (oven racks) (Rosenkranz et al., 2010,)

13. Adjusting the size of the honeycomb cell, smaller cells do not reduce the number of Varoo (Berry et al., 2010)

14. Varoe castles and killing Varoe with special frames, eg Mite Zapper (Huang, 2001)

15. Propolis barriers

16. Antioxidants are currently being tested

CONCLUSION

Varoa is a mite that has been present at beehives worldwide for more than 40 years. If we summarize the efforts to combat this parasite, we can say that we have increased our knowledge of the distribution and spread of mites, its pathogenesis, the interaction between bees and varoe and the effective application of some preparations.

In most countries, the situation with Varoa is "stable": beekeepers have learned to live with Varoa in hives and most of our beekeepers do not know bee-honey without varoa.

Generally speaking, the original aim of extermination of the parasite was not realized. There is still no treatment against Varoa that meets all the criteria - "safe, efficient, and easy to apply". We also have not yet received a bee naturally resistant to Varroa. Instead, we are confronted with secondary diseases and damages in bee-colonies caused by the synergistic collaboration of Varoa and other pathogens or environmental factors.

WHAT IS NEED FOR FUTURE RESEARCH

Unique methodology for assessing the invasion of the varoa

Frequent sampling and testing of infection

Equalize treatment thresholds

New treatments without influence on quality of bee products in the hive

“Plant phenotyping, Soil and Plant Microbiome for Sustainable Agriculture”

Created by:

Assoc. Prof. Ing. Marek Zivcak, Ph.D.,

Prof. Ing. Marián Brestič, Ph.D.,

Ing. Marek Kovar, Ph.D.

Application of high-throughput phenotyping in crop studies

Currently, society has technology and resources to provide long-term food security for all, despite some risks and challenges. In the last few years, many approaches have been discussed and incorporated into the constantly improving new process of highly automated, non-destructive phenotyping of plants. Plant phenotyping is the extensive evaluation of multiplex plant features like architecture, cultivation, development, resistance, ecophysiology and harvest, as well as the analysis of particular significant specifications for quantity criterion which design the background for more complicated features. The detailed and specific phenotyping strategies are required for genome-wide association and permit high-resolution linkage mapping investigations, and also to practice models of genomic selection for plant advancement.

The improvement of correctness and throughput of phenotypic assessment at all biological levels – phenomics, metabolomics and genetics – are the main objectives of modern phenotyping. The phenotyping system minimizes labor and costs due to its:

automation,

improved data integration,

remote sensing,

experimental design.

The developed systems for phenotyping are important for representing the full set of genetic factors that play roles in the phenotypic variation of quantitative parameters for cells, tissues and also organs, stages of development, plant species, environments, and scientific appointments. Newly developed plant phenotyping platforms produce significantly more data than they did initially, and

they need special systems for data management, access and storage. New statistical tools are needed for enlarging the experimental design and for making greater use of ideas to promote integration of data together with deriving biologically significant signals from experimental and environmental noise.

The phenomics field covers two major challenges:

The features analysis of large quantity of genetic lines, and,

the replications of measurement of dynamic traits (i.e., traits whose phenotype changes during the vegetation period).

To characterize a genotype, it may be more valuable to determine the levels of a few key compounds in several organs and at several time-points rather than obtaining the full metabolic profile for just one sample. For phenotyping projects that engage several hundred samples, it can be useful to use more oriented, less costly methods for all samples with full-scale results. Preliminary work might indicate which traits are the main contributors to the phenotype of interest and lead to a focused analysis that is more productive and cost effective for phenotyping.

In most cases, in the field or in the laboratory platforms, these detector systems have been installed:

(semi)-automatic evaluation of morphometric parameters using RGB image analysis,

chlorophyll a fluorescence kinetic imaging,

hyperspectral or multispectral analysis of the light spectral reflectance

thermal (IR) imaging

environmental monitoring systems

soil status monitoring systems

In many indoor installations, systems are composed of regulated watering and nutrient regimes controlled by automatic weight systems and environmental controls in the imaging.

Control and programming of platform systems, as well as data analysis, are performed with sophisticated and user-friendly software packages. Such newly developed phenotyping systems have tools for estimating many photosynthetic parameters; RGB systems can estimate plant morphometry, IR-thermal cameras can evaluate stomatal conductance, and hyperspectral imaging systems can evaluate metabolomics of experimental plants in different stages of growth.

The first major automated phenotyping systems in controlled environment were built in Australia (Autraian Plant Phenotyping Facility in Adelaide and CSIRO in Cambera). However, in the last decade, the development of phenotyping platforms was concentrated mostly in Europe, including two main commercial developers of phenotyping systems – Lemnatec (Germany) and Photo System Instruments – PSI (Czech Republic). The most developed phenotyping platforms (up to 2018) are listed in Table 1.

Table 1. Automated and semi-automated high-throughput plant phenotyping platforms

Location / Producer	Platform	Features	URL
PSI	PlantScreen™	Conveyor phenotyping system in controlled environmental conditions with analysis of chlorophyll fluorescence, kinetic and thermal imaging, morphometric and RGB analysis, and hyperspectral and NIR imaging; uses an automated weighing and watering system.	http://www.psi.cz
LemnaTec	Scanalyzer^{3D}	Comprehensive non-destructive 2D-3D assessment of plant physiological traits in controlled environmental conditions.	http://www.lemnatec.com
INRA	Phenopsis	Specific platform for phenotyping *Arabidopsis* plant growth under controlled environmental conditions.	http://bioweb.supagro.inra.fr
INRA	Phenoscope	Automated phenotyping device to handle and monitor hundreds of individual pots.	https://phenoscope.versailles.inra.fr
INRA	Phenodyn	Temporal analyses of growth rate and transpiration of hundreds of monocot crop species.	https://www.phenome-fppn.fr
INRA	Phenoarch	Automated platform based on a LemnaTec system to analyze the genetic determinants of plant responses to environmental conditions.	https://www.phenome-fppn.fr
Phenospex	FieldScan	Phenotyping under field- or semi-field conditions that is designed to screen large populations.	http://phenospex.com

WPS	**WSP**	**Fully automated digital phenotyping system using high-throughput RGB sensors.**	**http://www.wps.eu**
Keygene	**PhenoFab^{R}**	**Greenhouse service operation that combines phenotyping technology with trait interpretation to exploit phenotypic variation.**	
Jülich Plant Phenotyping Centre	**Growscreen**	**Non-invasive methods designed to quantify shoot morphometrical and functional parameters and root architecture.**	**http://www.fz-juelich.de**
Wageningen UR	**PhenoBot**	**Autonomous mobile robot with camera promises to output direct registered depth and color image for morphometric analysis.**	**http://www.wageningenur.nl**
Wiwam	**Wiwam Conveyor**	**Integrated robotic system for phenotyping of larger plants with automated irrigation and measurement of a variety of plant growth parameters at regular time intervals.**	**http://wiwam.be**
Australian Plant Phenomics Facility	**PlantScan**	**Provides non-destructive analyses of plant morphology, structure and function by using high-resolution cameras with cutting-edge information technology.**	**http://www.plantphenomics.org**

Non-destructive analysis of growth and physiology of aboveground parts of plants by automated imaging

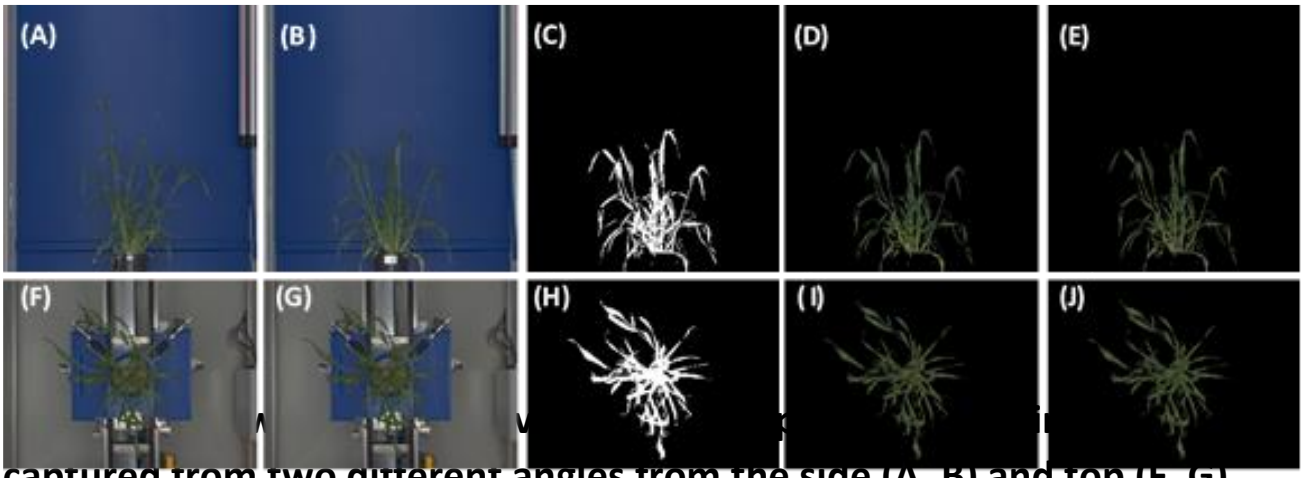
The system for automated non-invasive analysis of plant shoots may be based on different technical solutions or measured signals. In

following subchapters, we will introduce the most important sensors that have been used for studies of plant x environment responses or other studies.

Visible RGB imaging of plant abobveground biomass

In most of cases, the main observed trait in plant biology is the growth of plant shoots. In addition to numerous secondary traits describing the morphology of shoots, the primary and universal trait is the size (volume, fresh mass, dry mass) of biomass and the rate of the biomass formation. Standard way to asses the biomass is destructive, by a simple weighing of the fresh (FW) and dry (DW) masses. However, this can be done only in the end-point analyses. Similarly, leaf area and consequently the plant growth rate are usually determined by manual measurements of the dimensions of plant leaves. Such measurements are highly time consuming and thus cannot be used for large scale experiments. Therefore, plant phenotyping facilities prefer to evaluate the growth rate using imaging methods which employ digital cameras with subsequent software image analysis. This enables a faster and more precise determination of the leaf area and other parameters called projected area. In general, non-invasive techniques of shoot growth determination have proven very reliable, and high correlations between the digital area and the shoot fresh, or dry weights, respectively, were reported in Arabidopsis, tobacco and different crop species. Similarly, other common morphometric parameters such as stem length, number of tillers and inflorescence architecture can be assessed non-destructively and manually, but again the time requirements, limit the number of plants analysed. High-throughput approaches for analyses of these rather species-specific traits would be very valuable, however, with the exception of Arabidopsis, the range of accessible solutions has been rather limited.

The recent phenotyping platforms run the procedures to capture and automatically analyze the images of aboveground area. The images are taken from the top view and/or from the side view, in which the several views are analyzed taken when plants are rotating by a fixed angle. An example of top view and side wiw image is in Fig. 1.



captured from two different angles from the side (A, B) and top (F, G) was segmented and the background, including pot and substrate was automatically identified during image processing by the system (C, H), then removed, so the final image for the numerical analysis consists only from the plant parts on the black background (D, E, I, J). The image from the commercial PlantScreen® phenotyping system (PSI, Czech Republic), Slovak PlantScreen. SUA Nitra, Slovakia.

As an example, the RGB imaging system of PlantScreen® phenotyping system (PSI, Czech Republic) enables to calculate automatically this set of parameters using different modes of view. From the image captered from the top (top view), these parameters are calculated:

Area (pixel count / mm^2)

Perimeter (pixel count / mm)

Roundness

Compactness

Eccentricity

Rotation mass symmetry (RMS)

Slenderness of leaves

Color index

Leaf tracking and leaf analysis

Using the series of side-view images, the following parameters can be calculated:

Growth height (pixel count / mm)

Growth width (pixel count / mm)

Area (pixel count / mm^2)

Perimeter (pixel count / mm)

Compactbess

Number of leaves

Leaf movement

A more complex is the use of combination of top and side view, which enable to estimate these growth traits:

Total biomass volume or mass

Leaf movement

Relative growth rate

Chlorophyll fluorescence imaging

The technical development and release of commercially available devices have led to a wide expansion of practical applications of

chlorophyll fluorescence in plant biology, stimulating the progress in photosynthetic research and in crop science. The routines for distinguishing of different fluorescence quenching were developed, leading to important discoveries on the excess light energy dissipation and partitioning of the light energy between photochemical and non-photochemical processes. Although the different technical solutions enabled to measure chlorophyll fluorescence operating efficiencies, the most of the results have been obtained thanks to development of pulse amplitude modulation (PAM) fluorometers, using saturation pulse method.

Thus, since its introduction, now more than 30 years ago, the saturation pulse (SP) method employing the PAM technique has become a common way to assess the photosynthetic electron transport in plant tissues or other photosynthetically active samples. The parameters derived from the chlorophyll a fluorescence measurements based on the PAM method provides information about the fluxes of energy originating from the de-excitation of chlorophyll molecules in photosystem II (PSII) in plant chloroplasts, by non-invasive assessment of almost any plant. The parameters of chlorophyll fluorescence analysis can be calculated from quite a few fluorescence intensities, obtained either in dark-adapted or light-exposed samples. The principle of the modulation technique lies in the measurements of the rise of total fluorescence in response to a measuring pulse. Thanks to using the increment of fluorescence instead of the total values, the fluorescence parameters can be determined even under conditions of actinic light. Different light environments lead to different states of PSII de-excitation fluxes, which is reflected in changes of fluorescence intensities.

Figure 2 shows the principle of the fluorescence measurement using the saturation pulse method with quenching analysis.

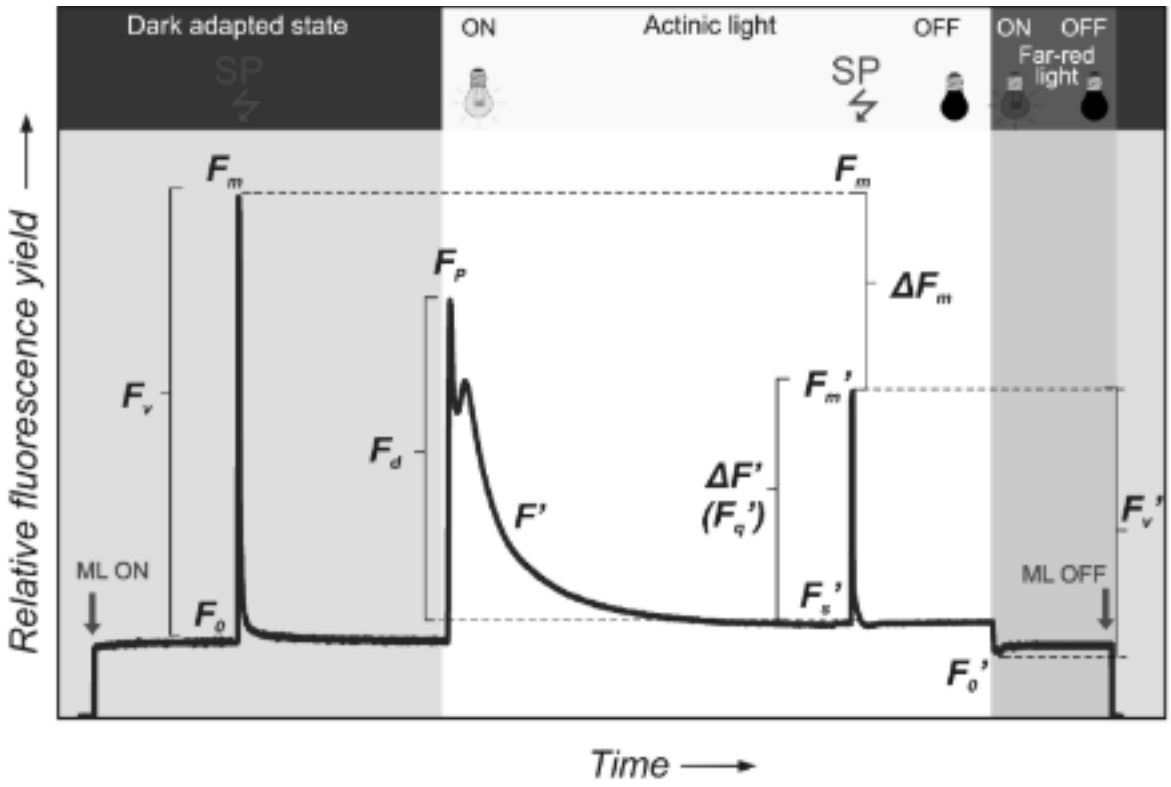


Fig. 1. The principle of the estimation of partitioning of absorbed light energy between the photochemical processes (photosynthesis) and non-photochemical processes (regulated or non-regulated conversion of excess light energy). The Figure of authors previously published by Kalaji et al. 2017. The explanation of the symbls is in the text below.

The measurement was done on dark-adapted sample (kept for 20 minutes in darkness before measurements). After modulated measuring light was turned on (ML ON), the fluorescence signal increased, reaching the value of minimum fluorescence in the dark-adapted sample, F0. Then, the short saturation pulse (SP, intensity 10,000 µmol m^{-2} s^{-1} for 1 second) was applied, which led to the fluorescence intensity increase to the maximum value, Fm, followed by the decrease back, almost on F0 level. In the next step, the non-saturating actinic light was turned on, leading to a steep increase of fluorescence signal (F'), reaching the maximum at given actinic light intensity (Fp) followed by the gradual decrease, reaching after few

minutes the steady-state value of actinic-light influenced fluorescence signal (Fs'). The same saturation pulse applied in the sample exposed to actinic light led to an increase of fluorescence signal (peak), reaching the maximum fluorescence in light-adapted state, Fm', followed by the fast decrease back to the Fs' level. In order to obtain minimum fluorescence of light adapted sample (F0'), a short period of far-red excitation was applied, leading to decrease of FS' signal to F0' value. Individual fluorescence intensities (F0, Fm, Fp, Fs', Fm', F0') represent the variables used for calculation of all fluorescence parameters (quantum yields, quenching parameters, etc.) derived from modulated chlorophyll fluorescence measurements.

Most of the instruments for the chlorophyll fluorescence analysis integrate the signal of the measured area. Anyway, advances in the technology of imaging detectors, LED light sources and processing of the data enabled to construct the systems for chlorophyll fluorescence imaging, which provided spatial resolution to the fluorescence records. These systems represent probably the most useful innovation of the technique of chlorophyll fluorescence, with universal applicability. Fluorescence imaging devices have been constructed for their use either at the microscopic level, at the plant, leaf and organ level or for remote sensing of chlorophyll fluorescence. Using the sensitive camera to image the fluorescence signal can be useful to observe the photosynthetic responses at sub-cellular levels, in plant cells, tissues, leaves or other plant organs, as well as at the whole plant level. In addition to integrated data, the images provide a precise visual information about photosynthetic performance at the different level of organization. The fluorescence imaging technique makes possible the observation of the spatial and temporal heterogeneities resulting of internal and/or environmental factors on photosynthesis over the large observed area. The conventional point measurements can just barely (or not at all) detect the

heterogeneities easily detectable using the chlorophyll fluorescence imaging.

Chlorophyll fluorescence imaging techniques can be used also for some special applications. The responses of stomata, especially the heterogeneity and dynamics of stomata opening (stomata patchiness) can be well identified. In addition to numerical assessment, the images can be created in false color palettes to encode the areas differing in stomata openness by different colors in a sufficient pixel resolution of the images. Such images correspond to some kind of the topological maps identifying the heterogeneity of the values of the measured parameters across the sample. Hence, imaging approach may help to avoid the imperfection typical for the point measurements of chlorophyll fluorescence that are responsible for many incorrect or imprecise results.

The imaging technique can be easily used for the analysis of different protocols, such an induction curve or light curve (see subchapter 5.4), including the calculation of quenching parameters and parameters of energy partitioning in dark- or light-adapted state. The visible spectra of PAR is commonly used for excitation of chlorophyll fluorescence, but application of other spectra (mostly UV-radiation) is also possible, providing, however, a specific type information, different from PAR excitation. Commonly, three types of light have to be used, i.e. pulse-modulated measuring light, actinic light for continuous light exposition and saturating light pulses. In similar to point measurements, the five key fluorescence levels are used to calculate fluorescence parameters for each measured pixels: F_0, F_0', Fm, Fm' and Fs'. Technical limitations in some of devices disable the direct F0' measurement by far red light; therefore the F0' have to be calculated using the formula. The commercially available fluorescence imaging devices provide the full operating possibilities, including programming

of any common (light curve, induction curve and recovery) or user-defined protocols. Typical set of fluorescence parameters, that can be achieved using automated chlorophyll fluorescence imaging is in Table 2.

The sensitivity of the chlorophyll fluorescence imaging to the stress effects was previously documented by many studies. Drought stress led to the heterogeneous distribution of values of chlorophyll fluorescence parameters on the leaf surface.

Table 2. The fluorescence parameters provided automatically by the FlourCam® imaging system as a part of automated phenotyping facility of SAU Nitra.

Fluorescence parameter	Definition
F, F´	Steady state fluorescence emission from dark- or light-adapted leaf, respectively
F_0, F_0´	Minimal chlorophyll fluorescence intensity measured in the dark- or light-adapted state, respectively
F_m, F_m´	Maximal chlorophyll fluorescence intensity measured in the dark- or light-adapted state, respectively
F_v, F_v´	Variable chlorophyll fluorescence (F_m-F_0) measured in the dark- or light-adapted state, respectively
F_q´	Difference in fluorescence between Fm' and F'
F_v/F_m	Maximum quantum yield of PSII photochemistry measured in the dark-adapted state
F_p	Peak fluorescence during the initial phase of the Kautsky effect
Rfd	Fluorescence decline ratio in steady-state (F_p – F')/F'
Φ_{PSII}	PS II operating efficiency; effective quantum yield of photochemical energy conversion in PSII (F_q'/F_m')
NPQ	Non-photochemical quenching (F_m/F_m') – 1
qL	Fraction of PSII centers that are 'open' based on the lake model of PSII (F_q'/F_v')(F_0'/F')
ETR	Electron transport rate

An example presented here (Figure 3) confirm these observations, showing relatively homogenous and drought insensitive responses of the Fv/Fm parameter, in contrast with high sensitivity and the heterogeneity of the values measured for ETR (Φ_{PSII}) in drought exposed lettuce plants.

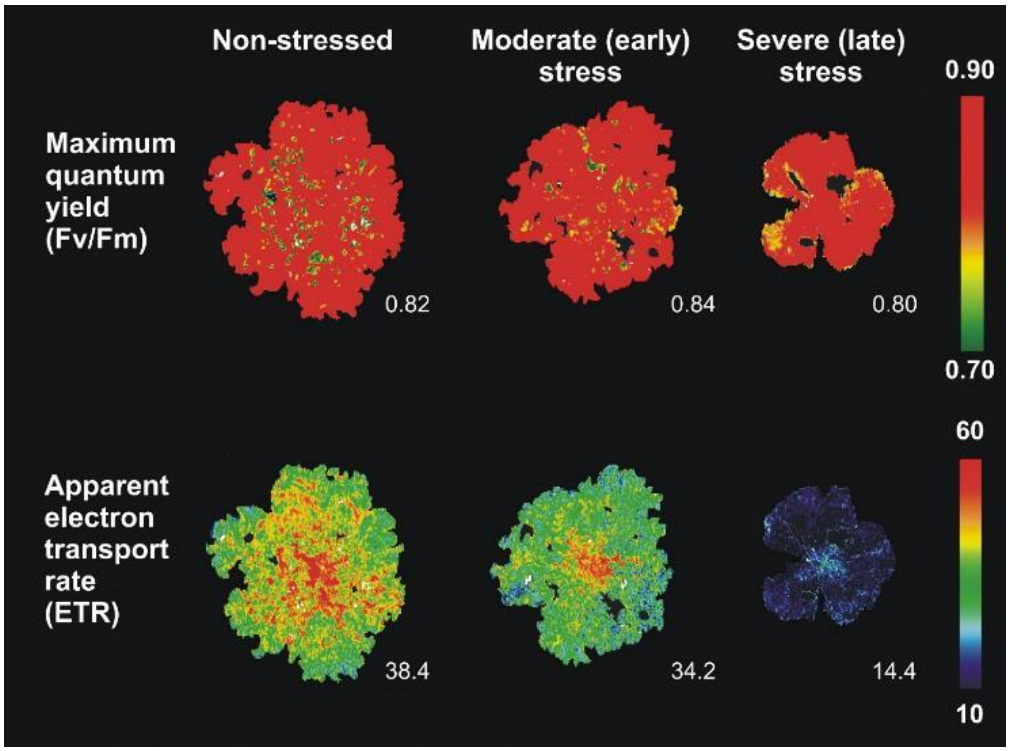


Fig. 3. Example of images of the maximum quantum yield of PSII photochemistry (Fv/Fv) and apparent electron transport rate (ETR_{PSII}) measured in lettuce plants (*Lactuca sativa* L.) grown under non-stressed conditions, moderate drought and severe long-lasting drought. It is obvious that Fv/Fm values were not significantly influenced by water deficit. Severe drought led to a significant decrease of ETR_{PSII}. The measurements were done on dark adapted plants (Fv/Fm) and in light exposed leaves (ETR_{PSII}). Data from the authors published by Kalaji et al. 2017.

The plant breeding represents one of desired future applications of fluorescence imaging, such as high-throughput screening of varieties resistant to biotic and abiotic constraints. Up to now, the evaluation of disease or stress resistance in breeding programs is done mostly using the visual scoring by skilled breeders; in addition to high time requirements, this approach can generate bias between experimental repeats and evaluations of different experts. To reduce the time requirements and to improve the objectivity, the high-throughput phenotyping tools are needed. A strong point of chlorophyll fluorescence imaging is the fact that it can be used to screen a large

number of plants in a short time. Moreover, it can be integrated in robots for automatic analyses).

In addition to previously mentioned, the imaging was used to show the effects of herbicides or the herbicide induced accumulation of reactive oxygen species (ROS) in plant tissues. Chlorophyll fluorescence imaging also identified heterogeneities caused by chilling stress, induction of photosynthesis, wounding, fungal diseases, viral infections, nutrient stress, senescence, drought, and ozone stress. Chlorophyll fluorescence imaging enables to study interactions between leaf structural properties and environmental conditions, directly related to photosynthetic assimilation.

The challenge using fluorescence imaging is to process all the data collected in a scientifically meaningful way. As an example of possible solution, the data can be analyzed by frequency distributions of parameters.

There are also several limitations of chlorophyll fluorescence imaging. For example, for reliable imaging measurements, it is critical that the whole sample area is illuminated homogeneously – this is, however, very difficult to achieve in larger plots. Moreover, the positions of leaves (leaf angles, distances of the leaf from the light sources) can cause a huge heterogeneity of illumination of the samples. Whereas the values of the maximum quantum yield of PSII photochemistry can be correct (except the parts of plants in which the incident light of saturation pulses will be below the saturating level), the values of efficient quantum yield of PSII photochemistry (Φ_{PSII}) and ETR_{PSII} can be partially overestimated in the positions with lower incident actinic light intensities. Despite some risks, the chlorophyll fluorescence imaging represents an emerging technique with a high potential for practical use.

Hyperspectral imaging of light reflectance

The development of new, more accessible cameras and sensors enabled to spread the use of hyperspectral imaging of light spectral reflectance from the remote sensing applications into plant phenotyping. The absorption of light by endogenous plant compounds is used for calculations of many indices which reflect the composition and function of a plant. A typical example is the normalized difference vegetation index (NDVI), which was originally develop to estimate plant chlorophyll content. Other example is the photochemical reflectance index (PRI), which can be used to estimate the photosynthetic efficiency. The absorption of a compounds (e.g., chlorophylls, carotenoids, anthocyanins, water, lignin, etc.) at a given wavelength can be used for direct estimation of the compound contents in the plant. For practical reasons, measurement of absorbance is replaced by measurements of reflectance. Depending on the measured wavelengths of reflected signal, various detectors are used. The most frequent are the VNIR (350–1200 nm) detectors, less abundand are the SWIR (short wavelength infrared region; 1400–3000 nm) detectors. Both wavebands are valuable for plant phenotyping. The reflectance signal can be detected at selected wavelengths or separated spectral bands (so-called multispectral detection). The whole spectral region can also be measured even for each pixel when cameras are applied and the hyperspectral imaging is carried out (Figure 4). Whereas the hyperspectral imaging in the VIS-NIR spectral region is used for evaluation of several indices as mentioned above, the SWIR spectral region is mainly used for the estimation of the plant's water or lignin content. Despite the many indices that have been defined so far, based on the reflectance measurements, it is difficult to assess them accurately. For this reason, critical revision of all of the reflectance indices is needed to evaluate which of them provide the required information in the best way.

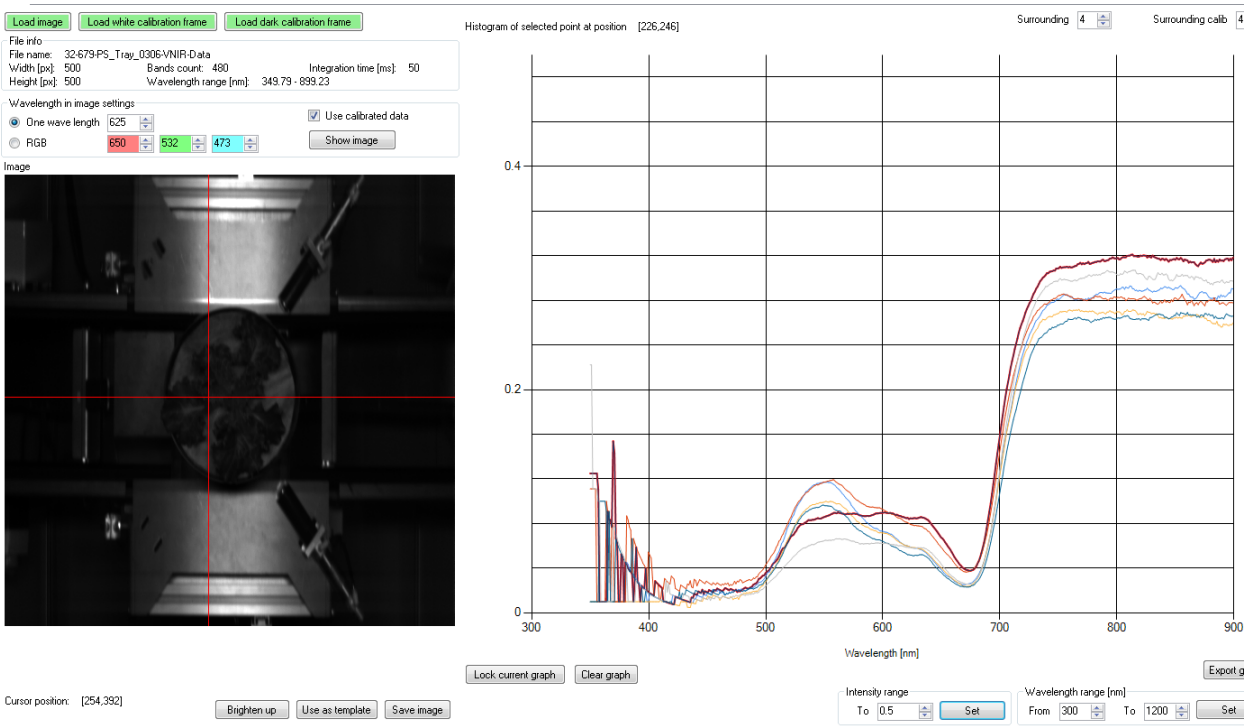


Fig. 4. Hyperspectral curves for selected pixels from the records of VNIR hyperspectral camera of SUA Nitra.

In the last years, hyperspectral imaging has been widely accepted as a non-destructive, rapid and safe method of qualitative analysis of plants. Spectral data can present much information about the object state. Usually, the dataset is very large and need to be analyzed by appropriate multivariate and machine-learning methods. The investigated spectral parameters of the leaf tissue were estimated non-destructively and interpreted with various methods, e.g. principal component analysis. The following ways of HIS applications are the most frequent:

Analyses of leaf pigments (chlorophyll, carotenoids, anthocyanins).

Based on previous, thepectral reflectance is also known as a fast method for determining nitrogen levels in plants. The general principle of spectral analysis involves reflectance values measured at

different wavelengths. Nitrogen content is predicted from linear dependence of reflectance and reference values of leaf nitrogen content.

Hyperspectral and fluorescent imaging may provide a means to directly and non-invasively detect and quantify secondary metabolites such as flavonoids and terpenoids.

Hyperspectral imaging has shown high effectiveness for assessing the fruit and vegetables quality and their safety regarding surface defects, contamination, index of starch, bruises, sugar content, freeze damage, firmness and bitter pits. Defect detection with HSI analysis is based on identifying the spectral trait wavelengths for the defect with next using these spectral parameters.

HIS was found to be useful for automatic detection of fungal infections

Classification models were established to detect insect damage The fruit analysis after hyperspectral imaging have been revealed the better detection ability compared to the standart investigation.

The moisture content and color of surface are also needed for estimation of the fruits and vegetables quality.

HSI has been used to classify crop seeds including maize, barley, rice, groat, oat,soybean and wheat seed.

HSI at the leaf level is proven to be specific relevant for the estimation and quantification of fungal invasion. For disease detection as a non-destructive diagnostic tool HSI has high potential.

Non-destructive analysis of growth and physiology of aboveground parts of plants by fast manually operated techniques

Although the automated systems represent the best choice for phenotyping, an alternative, cheaper way to assess the specific traits related to plant phenotype and physiological responses iare the

modern hand-held tools with low cost and low labour demand. We propose some of them, which are routinely performed at SUA Nitra.

Fluorescence excitation ratio method to asses flavonoid, anthocyanin, chlorophyll and nitrogen content in plants

Chlorophyll a fluorescence represents the reemission of light absorbed by photosynthetic pigments with emission spectra in red to far-red color (600-800 nm), with peaks at ~680 and ~730 nm. Although the emission of chlorophyll fluorescence is directly related to the photochemical activity running on the thylakoid membranes in the chloroplast (see chapter on Fuorescence Imaging), the fluorescence signal is strongly influenced also by optical properties of plant tissues not directly related to photochemical processes. It was shown, however, that adjustment leaf optical properties is not purposeless, but usually serve as protection of photosynthetic structures. Thus, in addition to others, an important defense mechanism against the deleterious effects of solar radiation involves synthesis of relatively stable compounds that serve as light screens and/or internal traps. Depending on concentration in cells and tissues, the protective compounds reduces the fraction of radiation absorbed by light-sensitive cell components, and thereby diminishes light-induced damage. Probably the most important position among compounds providing the passive photoprotection (screen) in plant are vascular flavonoids and anthocyanins.

The phenolic compounds, (flavonoids, hydroxycinnamic acids) have absorption maxima in the UV part of the spectrum. The flavonoids are located either in the epidermal vacuoles, cell walls, or dissolved in epicuticular wax. They have absorption maxima around 260 nm (isoflavones, flavanones), 320 nm (hydroxycinnamic acids), 260 nm and 340 nm (flavones) or 360 nm (flavonols), although the relative

importance of the different phenolic compounds as the UV-screen remains an open question.

Based on the strictly UV-absorbing properties, the effects of phenolic compounds on visible light-induced chlorophyll fluorescence is negligible, whereas their presence strongly suppress the chlorophyll fluorescence emission under UV excitation. This phenomenon has been successfully applied for estimation of transmittance of UV radiation by chlorophyll fluorescence. As the experiments confirmed that the phenolic compounds in the epidermis are responsible for most of the UV-absorption of the leaf, the ratio of visible light-excited to UV-excited chlorophyll fluorescence can serve as an indirect measure of content of the UV-absorbing phenolic compounds in leaves, as shown by the model (Fig. 5).

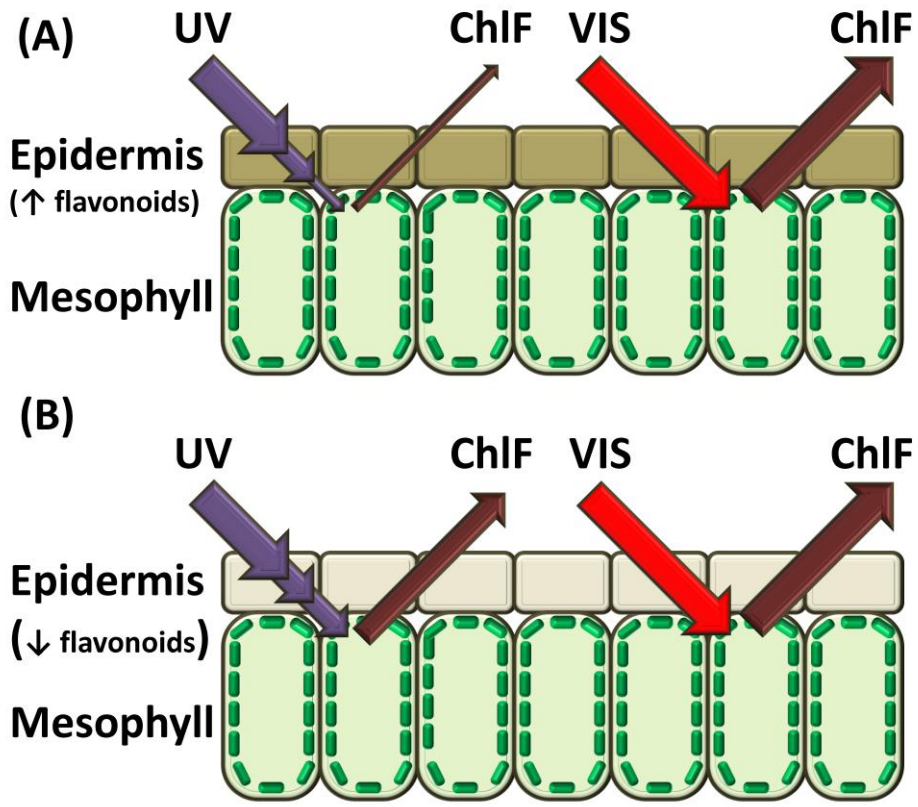


Fig. 5. A schematic drawing of the adaxial part of a leaf cross-section illustrating the principle of the chlorophyll fluorescence (ChlF) method

for assessment of content of UV-absorbing compounds in the sample with high (A) and low (B) flavonoid content. The thickness of the beams indicates relative intensity. The figure created by the authors, published in Sytar et al. (2016).

In parallel, anthocyanins are water-soluble vacuolar pigments of higher plants. They are responsible for red coloration of plant tissues, especially in fruits. Anyway, they can occur also in plant leaves. In many cases, significant accumulation of anthocyanins is induced as a result of environmental stresses such as low temperature, nitrogen and phosphorus deficiencies, UV-B stress, drought, pathogen infections, or due to toxic effects. Anthocyanins absorb strongly in the green region of the spectrum. The spectral band around 550 nm (green) was sensitive to anthocyanin content. Thus, similarly to flavonoids, the ratio of red (or blue) light-excited to green light-excited chlorophyll fluorescence can serve as an indirect measure of anthocyanin content in plant sample, as shown in the model (Fig. 6).

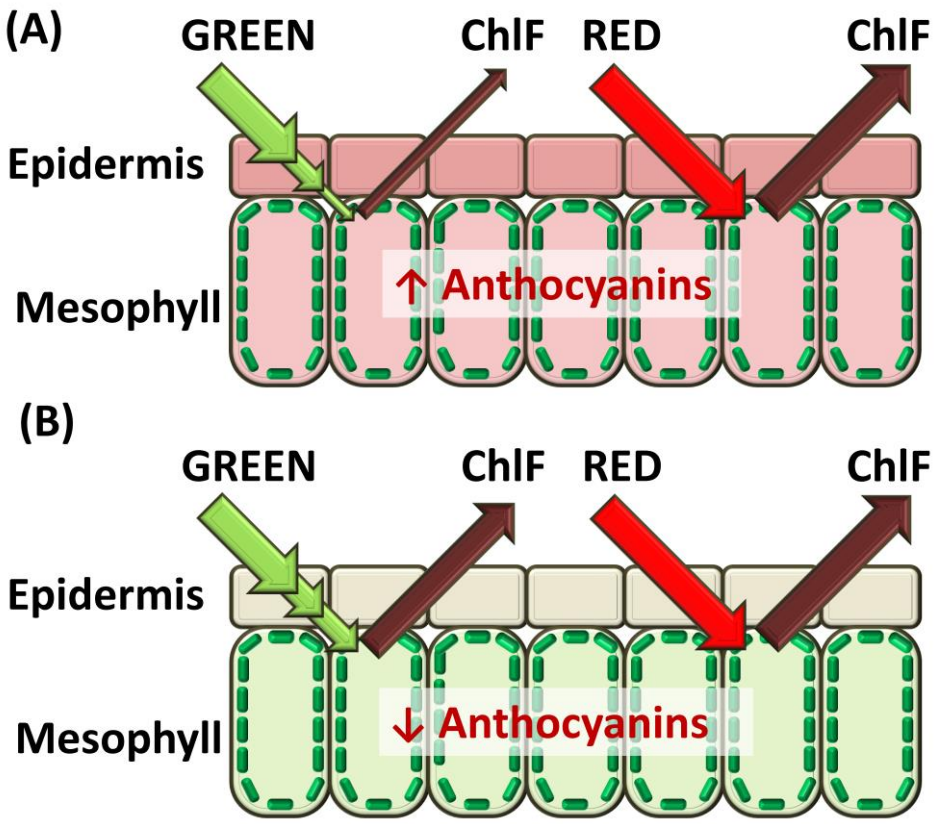


Fig. 6. A schematic drawing of the adaxial part of a plant sample cross-section illustrating the principle of the chlorophyll fluorescence (ChlF) method for assessment of content of anthocyanins using simultaneous green and red excitation, in samples with high (A) and low (B) anthocyanin content. The thickness of the beams indicates relative intensity. The figure created by the authors, published in Sytar et al. (2016).

In the previous decades, the numerous studies examined and confirmed possibility to use the chlorophyll fluorescence signal in the estimation of phenolics and anthocyanins. In addition to self-constructed devices or standard fluorometers combined with external

light sources and filters, which were used in the majority of studies, the factory-made special devices for this purpose were also introduced.

The research group of Z. Cerovic (France) developed several devices using the principle of multispectrally induced chlorophyll fluorescence described above. In principle, they introduced two types of devices: leaf clip-based instrument (commercially available under trademark Dualex, Force-A, France) as well as the non-contact type of instrument (under trademark Multiplex, Force-A, France).

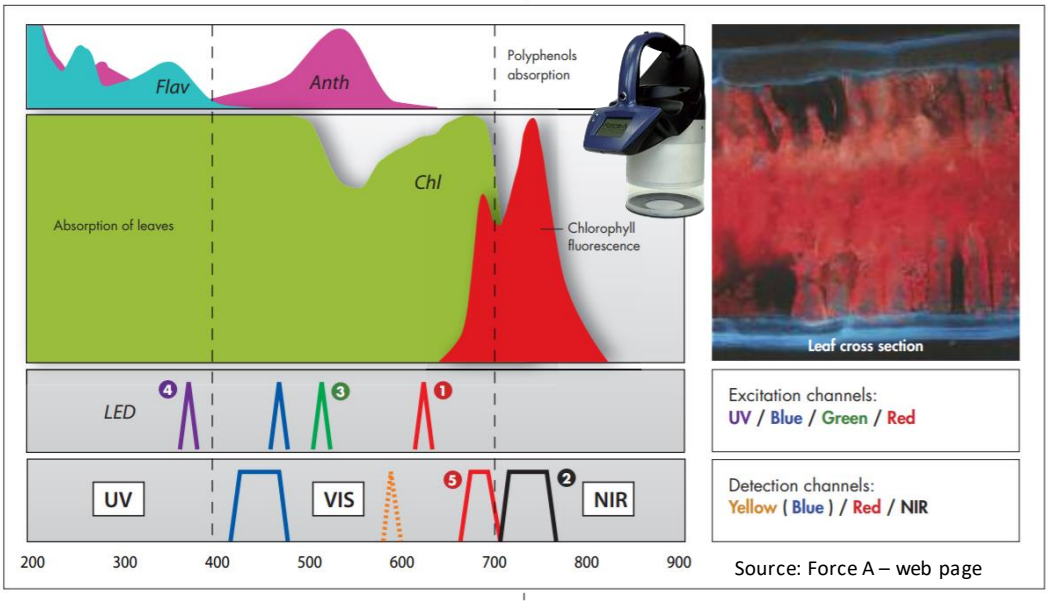


Fig. 7. A scheme of the wavelengths emitted by the LED-units of Multiplex-3 device (Force-A, France), and detected by 4 detector units of the device, which serve to estimation of flavonoid, anthocyanin, chlorophyll and nitrogen contents in leaves and/or fruits.

While the Dualex system measures only two signals (e.g. UV and VIS-light induced chlorophyll fluorescence), several kinds of Dualex are produced, specialized for estimation of UV-absorbing compounds (flavonoids), anthocyanins or chlorophylls. In contrary, the Multiplex

system measures simultaneously different fluorescence signals aft. r excitation under several spectral regions of light (UV, blue, green, red excitation); thus, this system enables estimate flavonoids, anthocyanns, chlorophylls and any other information from a single measurement. Thanks to the fact that the Multiplex system does not need any leaf clip, it can be used for measurements even in objects other than flat leaves, e.g. fruits, stems, flowers, etc. This makes this system especially useful for special applications, potentially also in automated systems (as it needs no direct contact with plants).

Moreover, the ratio of fluorescence signals measure in parallel at different wavelengths can serve to estimate the chlorophyll content and, in combination to FLAV signal, as an estimate of nitrogen content, as SFR decrease and FLAV increase in nitrogen deffiient conditions, hence, the ratio NBI =SFR/FLAV represent a good and reliable indirect estimate of the level of nitrogen nutrition.

In summary, the Multiplex-3 device (Force-A, France) measures:

Total flavonoid content determined as the FLAV index, derived from UV absorption properties of flavonoids.

Total anthocyanin content determined as the ANTH index, derived from green-light absorption properties of anthocyanins.

Total chlorophyll content determined as SFR index derived from red/far-red fluorescence,

Estimate of the level of nitrogen nutrition as NBI index (nitrogen balance index), Based on SFR and FLAV indices

Analysis of one-second measurements of fast fluorescence kinetics by the JIP-test

As the methods based on saturation pulse analysis are relatively time-consuming, a big effort has been applied to develop a more efficient way of measurements of photosynthetic performance and environmental effect. In the last decades, the exponential increase of the studies applying the fast fluorescence kinetics can be observed. Chlorophyll fluorescence induction represents a plot of measured fluorescence intensity as a function of time of continuous illumination (Fig. 4).

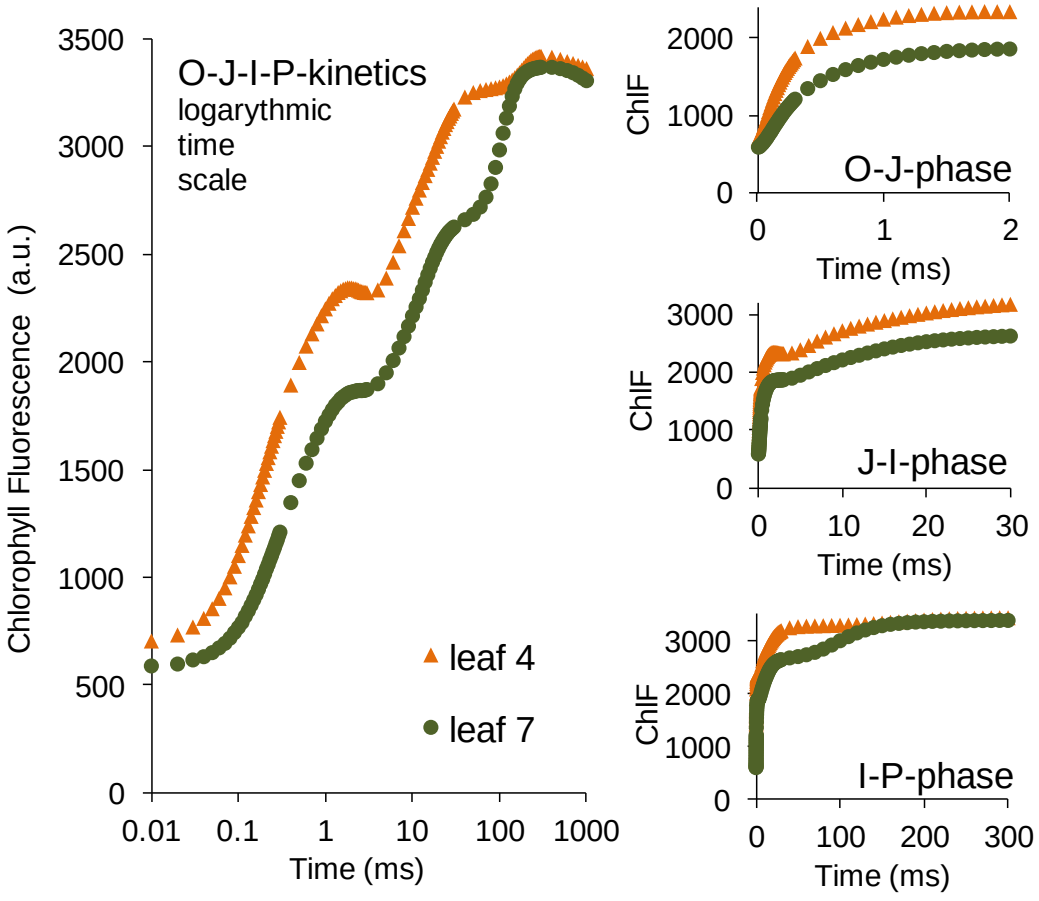


Fig. 8. Examples of O-J-I-P-curves recorded in two different leaves of barley (Hordeum vulgare L. cv. Kompakt) in post-anthesis stage. The leaves are numbered in the order in which they appeared. The leaf 7

represents a penultimate leaf (the second leaf from the top, well exposed to sun), the leaf 4 (an older leaf, below the others) was almost completely shaded inside the canopy. The main graph (left) shows the entire O-J-I-P-kinetics plotted on a logarithmic time scale. The small graphs (right) show individual phases plotted on a regular time scale: O-J phase in time 0 - 2 ms, J-I phase in time 2-30 ms, and I-P phase in time 30-300 ms. Data of the authors published in Zivcak et al. (2017).

Such a curve recorded under continuous light has a fast (less than one second) exponential phase, and a slow decay phase (duration of few min). The rise has a typical polyphasic shape, well evident when the curve is plotted on the logarithmic time scale, or if the individual steps are plotted separately, in different time resolution (Fig. 8). The shape of OJIP-transient is sometimes denoted as a 'fingerprint' of a sample of a given physiological status; any deviation of the curve indicate photochemical changes at the thylakoid membrane level. The analysis of OJIP curve taking the theoretical assumptions and probabilities derives different photosynthetic parameters for a dark adapted state of the photosynthetic systems. The nomenclature for 'OJIP' is as followed: O for origin or Fo level measured at 50 μs (or less) after illumination, J and I represent intermediate states measured after 2 ms and 30 ms, respectively, and P is the peak or Fp = Fm (maximal fluorescence). This is valid only if a sufficient light intensity is used. In heat-stressed samples, another peak arise between F0 and F_J at app. 300 μs, which is usually called K-step; therefore some authors call the fast chlorophyll fluorescence induction the OKJIP-curve or transient. The OJIP curve from F0 to Fm is correlated with the primary photochemical reactions of PS II and the fluorescence yield is controlled by a PS II acceptor quencher (the primary quinone acceptor, QA). Thus, the OJIP transient can be used for estimation of the photochemical quantum yield of PS II photochemistry, and electron

transport properties. The OJIP fluorescence curve analysis can be used to monitor the effect of various biotic and abiotic stresses, and photosynthetic mutations affecting the structure and function of the photosynthetic apparatus.

There are several groups of parameters derived from the fluorescence rise. In addition to the basic fluorescence values and fundamental parameters, such as Fo, Fm, Fv/Fm (similar to the saturation pulse method), there is also a group of parameters derived from the JIP-test, introduced by Strasser and coauthors. We can divide it into the fluorescence parameters derived from the data extracted from OJIP transient and the biophysical parameters calculated using the previous group of fluorescence parameters. In plant stress research there are several possible ways of interpreting the data. A multiparametric approach is based on the visualization of data e.g. by spider plots or pipeline models. On the other hand, the model offers the integrative parameters enabling simple assessment of the status and vitality of the photosynthetic apparatus, which are sensitive and created mostly for possible practical applications in pre-screening or selection in research and breeding programs.

From numerous JIP-test parameters, for practical applications in the crop research Performance Index (PI) was also introduced. This complex parameter integrates several independent structural and functional properties of the photochemistry, reflecting the functionality of both photosystems II and I and providing a quantitative information on the current state of plant performance under stress conditions.

However, the current level of knowledge does not entitle us to draw further conclusions about photosynthetic performance based on the fast chlorophyll fluorescence only. Even usefulness of the fast chlorophyll fluorescence for leaf photosynthetic performance testing

could be proven in the future, more probably, the method will remain the tool for assessment of the stress effects on the photosynthetic functions mostly.

In this respect, the availability of user-friendly portable fluorometers for high-frequency record of OJIP-transient and the useful software for the analysis of experimental data, make the JIP test derived from the fast chlorophyll fluorescence attractive even for users without a deep knowledge on photochemical processes at the thylakoid membrane level. As we have mentioned above, the small and portable devices allow efficient data records even in the field conditions. The chlorophyll fluorescence induction kinetics contains a valuable information about the photochemical efficiency of primary conversion of incident light energy, electron transport events, and related regulatory processes. These issues can be deciphered using advanced mathematical models based on the analysis of fluorescence curves, providing a large number of the fluorescence parameters. They cen be divided into:

They can be divided into:

Parameters directly derived from the fluorescence data (Fo, Fm, Fv, Area)

Specific quantum yields, i.e. energy fluxes per absorbed light spectra (TRo/ABS, DIo/ABS, ETo/ABS, ETo/ABS)

Energy fluxes per active reaction centre (ABS/RC; TRo/RC; ETo/RC, DIo/RC)

Energy fluxes per excited cross section: (ABS/CS; TRo/CS; ETo/CS, DIo/CS)

Density of reaction centres (RC/ABS; RC/CSo; RC/CSm)

Probabilities of electron transport between individual steps of electron transport chain

Performance indices and driving forces (PI_{ABS}, PI_{TOT}, df)

All parameters are precisely and simply defined and they can be used to characterize the status of PSII photochemistry, which reflects the effects of external factors and the status (vitality) of plants.

Analysis of the leaf chlorophyll content using chlorophyll meters

The chlorophyll content represent an important indicator of plant health status and basic information of the limitations of photosynthetic capacity. The main methods for determination (HPLC, spectrophotometric) are destructive. An alternative way is the use of chlorophyll content meters, which have been used successfully in many species to estimate foliar chlorophyll, and to allow the measuring of chlorophyll relative content on the same leaf over time. The readings from chlorophyll content meters can be also used to predict the nitrogen status in leaves. The meters/devices that calculate a chlorophyll content indices (e.g. SPAD value, CCI index) are based on measuring the reflectance, absorbance or fluorescence at particular wavelengths. The most common hand-held chlorophyll absorbance meters, of which several are commercially available, measure absorbance by the leaf of two different wavelengths of light: red and near-infrared. The red light is strongly absorbed by chlorophyll. The second is a 'reference wavelength' necessary to adjust for differences in tissue structure.

It was shown that all the chlorophyll meters available on the market are useful, butpreciseness are not always the same in all conditions; especially, the fluorescence-based instruments have some limits. The values of differet types of devices should not be combined and compared.

Based on the published book, book chapters and reviews:

Kalaji, M. H., Goltsev, V. N., Żuk-Gołaszewska, K., Zivcak, M., & Brestic, M. (2017). Chlorophyll fluorescence: understanding crop performance—basics and applications. CRC Press, Taylor and Francis, USA.

Sytar, O., Zivcak, M., & Brestic, M. (2016). Noninvasive methods to support metabolomic studies targeted at plant phenolics for food and medicinal use. In Plant Omics: Trends and Applications (pp. 407-443). Springer.

Zivcak, M., Olsovska, K., & Brestic, M. (2017). Photosynthetic Responses Under Harmful and Changing Environment: Practical Aspects in Crop Research. In Photosynthesis: Structures, Mechanisms, and Applications (pp. 203-248). Springer.

Sytar, O., Zivcak, M., Olsovska, K., & Brestic, M. (2018). Perspectives in High-Throughput Phenotyping of Qualitative Traits at the Whole-Plant Level. In Eco-friendly Agro-biological Techniques for Enhancing Crop Productivity (pp. 213-243). Springer

Brestic, M., & Zivcak, M. (2013). PSII fluorescence techniques for measurement of drought and high temperature stress signal in crop plants: protocols and applications. In Molecular stress physiology of plants (pp. 87-131). Springer, India.

Humplík, J. F., Lazár, D., Husičková, A., & Spíchal, L. (2015). Automated phenotyping of plant shoots using imaging methods for analysis of plant stress responses–a review. Plant methods, 11(1), 29. (text on RGB analysis)

Organic farming in Serbia

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Abstract

Organic farming methods combine scientific knowledge and modern technology with traditional farming practices in agriculture, aimed to produce food using substances and processes from nature. This means that organic farming tends to have a small environmental impact on nature as it encourages the responsible use of energy and natural resources, the maintenance of biodiversity, preservation of ecological balance, and in long term to enhance soil fertility and maintain water quality. Moreover, the principles of organic farming support high standards of animal welfare and require farmers to meet the specific behavioural needs of animals. The organic production is based on four principles of organic farming: the principle of health, principles of ecology, and principles of fairness and care too. In order for farmers to derive benefits from organic farming methods, consumers need to trust that the rules on organic production are being followed. Therefore, the EU and Serbia maintain the strict system of control and enforcement to guarantee that organics rules and regulations are being followed properly. There are five certification companies in Serbia, which follow European rules in certification. Most of the organic foods produced in Serbia are sold in the green market as a fresh product. Smaller amounts are sold in big supermarkets and exported. Fruits, crops, and vegetables are the most abundant organic fresh products. In 2019 the law for organic production was changed to meet European subsidiaries. Organic farming in Serbia started to develop in 2008, and in 2011 the European Law was implemented. The growing area increased up to 14000 ha in 2018. year and export value to 21.mil EUR. Organic agriculture in Serbia has big potential due to good soil quality, clean water and intensive education of young producers.

Introduction

IFOAM, (International Federation of Organic Agriculture Movement) was founded since 1972, and closely defines Organic Agriculture, its Principles and Scope, and provides Standards that draws the line between what is organic and not organic. This organization defines organic agriculture as:

"Organic Agriculture is a production system that sustains the health of soils, ecosystems, and people. It relies on ecological processes, biodiversity and cycles adapted to local conditions, rather than the use of inputs with adverse effects. Organic Agriculture combines tradition, innovation, and science to benefit the shared environment and promote fair relationships and good quality of life for all involved." (https://www.ifoam.bio/en/about-us)

Organic agriculture combines best practice and value chain through Health, Ecology, Society, Culture Accountability and Economy and many other parameters connected (Fig. 1.).

Figure1. Best practice parameters

They proposed four main principles (Fig.2.) as well as:

The Principle of Health: Organic Agriculture should sustain and enhance the health of soil, plant, animal, human and planet as one and indivisible. This principle points out that the health of individuals and communities cannot be separated from the health of ecosystems - healthy soils produce healthy crops that foster the health of animals and people.

The Principle of Fairness - This principle emphasizes that those involved in Organic Agriculture should conduct human relationships in a manner that ensures fairness at all levels and to all parties - farmers, workers, processors, distributors, traders, and consumers. Organic Agriculture should provide everyone involved with a good quality of life, and contribute to food sovereignty and reduction of poverty. It aims to produce a sufficient supply of good quality food and other products.

The Principle of Ecology Organic Agriculture should be based on living ecological systems and cycles, work with them, emulate them and help sustain them. Organic Agriculture should attain ecological balance through the design of farming systems, establishment of habitats and maintenance of genetic and agricultural diversity. Those who produce, process, trade, or consume organic products should protect and benefit the common environment including landscapes, climate, habitats, biodiversity, air, and water. For example, in the case of crops, this is the living soil; for animals, it is the farm ecosystem; for fish and marine organisms, the aquatic environment.

Principle of care and aging - Organic agriculture should be managed in a cautious and responsible manner in order to preserve the health and well-being of present and future generations and ecosystems.

Figure 2. Principles of Organic Agriculture

Organic Agriculture should attain ecological balance through the design of farming systems, establishment of habitats and maintenance of genetic and agricultural diversity. Organic farming, pastoral and

wild harvest systems should fit the cycles and ecological balances in nature. These cycles are universal but their operation is site-specific. Organic management must be adapted to local conditions, ecology, culture. Inputs should be reduced by reuse, recycling and efficient management of materials and energy in order to maintain and improve environmental quality and conserve resources.

The role of Organic Agriculture, whether in farming, processing, distribution, or consumption, is to sustain and enhance the health of ecosystems and organisms from the smallest in the soil to human beings. In particular, organic agriculture is intended to produce high quality, nutritious food that contributes to preventive health care and well-being. In view of this, it should avoid the use of fertilizers, pesticides, animal drugs and food additives that may have adverse health effects.

Advanced of organic agriculture

Sustainability over the long term.

Many changes observed in the environment are long term, occurring slowly over time. Organic Agriculture considers the medium and long-term effect of agricultural interventions on the agro-ecosystem. It aims to produce food while establishing an ecological balance to prevent negative changes on soil fertility or pest problems.

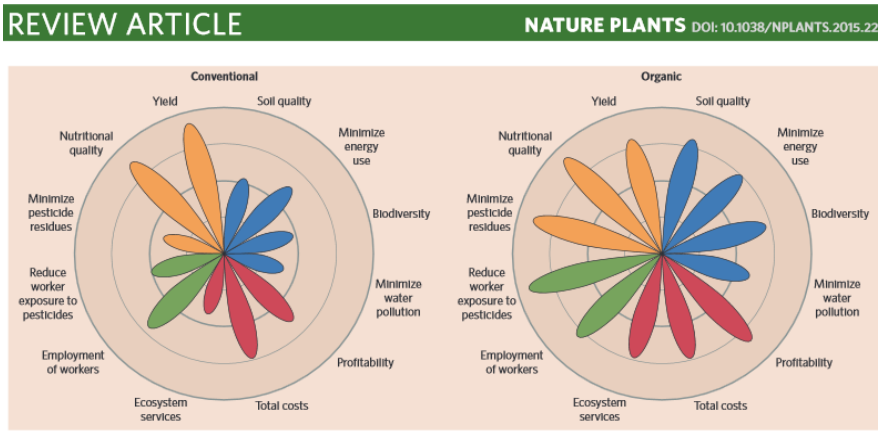


Figure 3. Assesment of organic farming relative to conventional farming in four major areas of sustainability.

John P. Reganold & Jonathan M. Wachter (2016): Organic agriculture in the twenty-first century. Nature Plants, (2),15221

Soil

Soil practices such as crop rotations, inter-cropping, symbiotic associations, cover crops, organic fertilizers, and minimum tillage are very important to organic practices. These practices encourage soil fauna and flora, improving soil formation and structure and creating long term stable systems. In turn, nutrient and energy cycling is increased and the retentive abilities of the soil for nutrients and water are enhanced, compensating for the non-use of mineral fertilizers. Such management techniques also play an important role in soil erosion control. The length of time that the soil is exposed to erosive forces is decreased, soil biodiversity is increased, and nutrient losses are reduced, helping to maintain and enhance soil productivity.

Farm-derived renewable resources usually compensate crop export of nutrients but it is sometimes necessary to supplement organic soils with potassium, phosphate, calcium, magnesium and trace elements from external sources. In every country, they have the list of allowed substances used as fertilizers in organic production (including Serbia, Ministry of Agriculture and Environment protection).

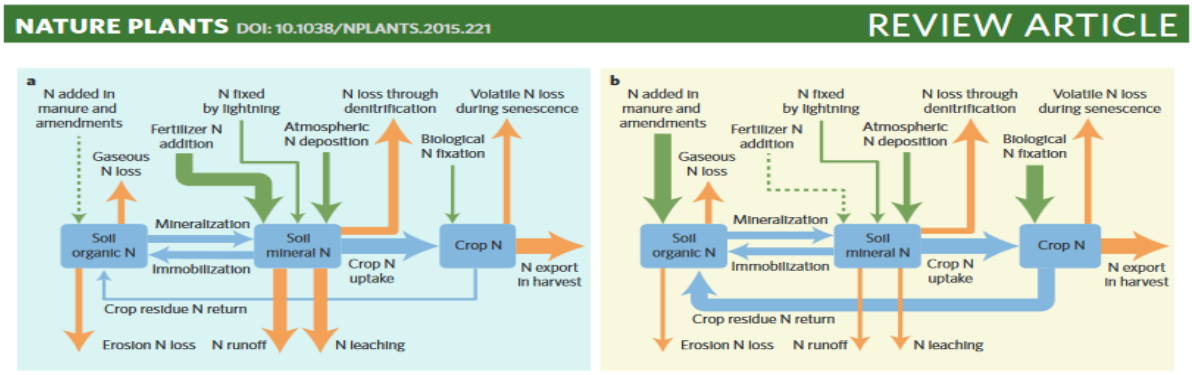


Figure 4. Hypothetical nitrogen stocks and flows of two contrasting cropping systems (a: conventional system; b: organic or integral system: arrows represent nitrogen inputs –green, losses-orange and transformations-blue)

John P. Reganold & Jonathan M. Wachter (2016): Organic agriculture in the twenty-first century. Nature Plants, (2),15221; Jovanovic LJ. (2014): Biological soil characteristic in organic agriculture production. In monograph: Production and Management in organic agriculture (Eds Jovanovic LJ, Educons University), 1-289

So, the contrast between organic and conventional production is more expressed in the organic system according to prolonged soil stability and quality, fewer erosions, etc.

Water.

Clean water is very important for all organisms in the world. In many agriculture areas, pollution of groundwater with synthetic fertilizers and pesticides is a major problem. High amount of fertilizers in surface water increases algae growth (eutrophication) and causes less amount of oxygen, which is dangerous for aquatic organisms.

As the use of mineral fertilizers is prohibited in Organic Agriculture, they are replaced by organic fertilizers (e.g. compost, animal manure, green manure) and through the use of greater biodiversity (in terms of species cultivated and permanent vegetation), enhancing soil structure and water retention. Organic systems with better nutrient retentive abilities greatly reduce the risk of groundwater pollution.

In some areas where pollution is a real problem (polluted underground waters, rivers, streams, low polluted industrial soil),

bioremediation and than conversion to organic agriculture is highly recommended as a restorative measure (e.g. by the Governments of France and Germany).

Air.

Organic agriculture reduces non-renewable energy use by decreasing agrochemical needs (these require high quantities of fossil fuel to be produced) in the long term. Organic agriculture contributes to mitigating the greenhouse effect and global warming through its ability to sequester carbon in the soil. Many management practices used by organic agriculture (e.g. minimum tillage, returning crop residues to the soil, the use of cover crops and rotations, and the greater integration of nitrogen-fixing legumes), increase the return of carbon to the soil, raising productivity and favoring carbon storage. Last 20 years the experiments showed that min 30% of C can be returned to the soil using waste biomass from fields.

Biodiversity

For organic farmers biodiversity at all levels is very important. At the gene level, autochthonous and adapted seeds and breeds are preferred for their greater resistance to diseases and their better adaption to local soil characteristics and climatic stress. At the species level, combinations of plants and animals optimize nutrient and energy cycling for agricultural production and are highly suggested. At the ecosystem level, the maintenance of natural areas within and around organic fields and the absence of chemical inputs create suitable habitats for wildlife. (M. Ricard: Summer courses, Amfissa July 2014, IFOAM)

The investigation showed that 23 species of rain earthworm in Germany are equally important for German productivity as well as the

German bank because the earthworm produces the healthy soil from which we all live. (Soil organisms 85 (3) 2013)

Genetically modified organisms

The use of GMOs within organic systems is not permitted during any stage of organic food production, processing or handling. As the potential impact of GMOs to both the environment and health is not entirely understood, organic agriculture is taking the precautionary approach and choosing to encourage natural biodiversity. (IFOAM)

The organic label, therefore, provides an assurance that GMOs have not been used intentionally in the production and processing of the organic products. This is something that cannot be guaranteed in conventional products, as labelling the presence of GMOs in food products has not yet come into force in most countries, in Serbia too.

Ecological services

The impact of organic agriculture on natural resources favours interactions within the agro-ecosystem that is vital for both agricultural production and nature conservation. Ecological services derived include soil forming and conditioning, soil stabilization, waste recycling, carbon sequestration, nutrients cycling, predation, pollination, and habitats. (IFOAM)

Organic agriculture in the world

From 1972. system of Organic Agriculture developed relatively quickly, adding new standards, high subsidiary, recognized by politicians; a lot of different projects, good promoted campaigns, economically increase and in many countries number of consumers increase too.

According to different reports given by the Research Institute of Organic Agriculture (FiBL) and IFOAM, the OA over the years dramatically increase in the number of countries (Tab.1.). As a good

example, organic production in France increases from 2014 (about 1,3 mil ha) to 2018 (1,7 mil ha). This is due, some big producers moved from integral production to organic production and good subsidiary politics. In the world, the total organic market in 2018 increases to 90 billion EUR.

Table 1. Organic agriculture: Key indicators and Top countries

Indicator	World	Top countries
Countries with organic activities[1]	2017: 181 countries	
Organic agricultural land	2017: 69.8 million hectares (1999: 11 million hectares)	Australia (35.6 million hectares) Argentina (3.4 million hectares) China (3.0 million hectares)
Organic share of total agricultural land	2017: 1.4 %	Liechtenstein (37.7 %) Samoa (37.6 %) Austria (24.0 %)
Wild collection and further non-agricultural areas	2017: 42.4 million hectares (1999: 4.1 million hectares)	Finland (11.6 million hectares) Zambia (6.0 million hectares) Tanzania (2.4 million hectares)
Producers	2017: 2.9 million producers (1999: 200'000 producers)	India (835'000) Uganda (210'352) Mexico (210'000)
Organic market	2017: 97 billion US dollars*[2] (more than 92 billion euros) (2000: 17.9 billion US dollars)	US (45.2 billion US dollars; 40 billion euros) Germany (11.3 billion US dollars; 10 billion euros) France (8.9 billion US dollars; 7.9 billion euros)
Per capita consumption	2017: 12.8 US dollars (10.8 euros)	Switzerland (325 US dollars; 288 euros) Denmark (315 US dollars; 278 euros) Sweden (268 US dollars; 237 euros)
Number of countries with organic regulations	2017: 93 countries	
Number of affiliates of IFOAM – Organics International	2018: 726 affiliates from 110 countries	Germany - 76 affiliates India - 47 affiliates China - 45 affiliates United States - 43 affiliates

Source: FiBL survey 2019, based on national data sources and data from certifiers
**Global market: Ecovia Intelligence (formerly Organic Monitor) 2019*

FiBL

Organic agriculture in Serbia (history)

Organic agriculture is not just a production method, but also a new way of living of modern man and new hope for people lived in low populated villages. The increase of organic plant and animal products will make a lot of jobs for young people who live in villages and even some chance for the young man who's lived in towns to return to villages. Export of such products is the only chance for our farmers, due to the social and economic situation. In Serbia, they have a lot of

educational institutions, but it is necessary to develop the network of extension services that will spread the knowledge.

The Serbian Ministry of Agriculture and Environmental Protection recognized the huge potential of organic production and the importance it might have for the future economic development of the country. Therefore, as a result of the successful cooperation with the Ministry and other public and private partners significant progress has been achieved so far: The National Action Plan for organic production was developed and fully integrated into the National Strategy for Rural Development, the law on organic production is mostly in line with the EU requirements, a database was created containing relevant numbers on organic production and producers, an integrated control system was implemented, including an EU approved domestic certification body (for 2019 they have 5 certification body) was established and organic production know-how has been transferred successfully from Western Europe to Serbia. The first steps in organic production development were taken back in 1990 when NGO Terras (Subotica) established a promotional network of producers, farmers, advisors, technical and academic staff involved in organic food production. Twenty-five years later and taking advantage of the support of many national and international institutions, ministries, Universities, and many enthusiasts, the organic sector in Serbia has attained a respectable level. From 2008 the number of organic producers sharp increase up to 2018 (Fig. 2.). Up to 2018, they have 436 certificated producers and 7400 workers were included in the production chain.

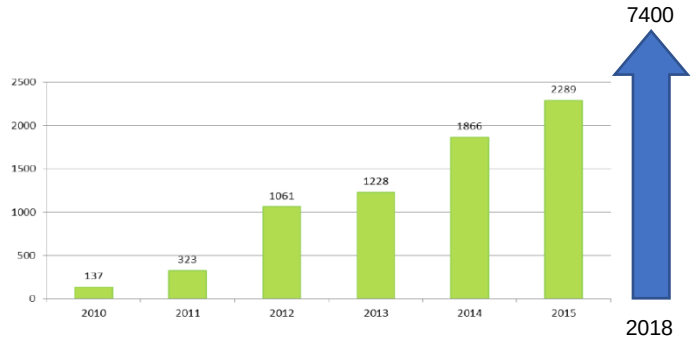
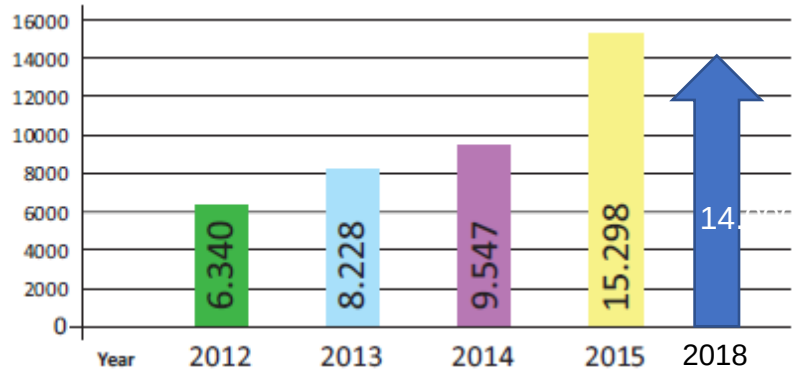


Figure 2. Organic producers in Serbia (number)

As a problem in organic production they have no enough seasonal workers because of manual work common in OA. Most of young people live the villages and try to find better job both in big towns or left to abroad, mainly in Western Europe. Even so much household (630000), in organic production only 15000 ha were certified (Fig.3).



Graphic 3. Area certified for organic production (ha)

In Serbia the organic production stagnated (Fig.3.) from the 2015 due less subsidiaries were done and stagnation with exports, or some low which is not positive for organic producer in crop agriculture. It's because of no low for producers who want to produce final products (jam, etc.) with higher values.

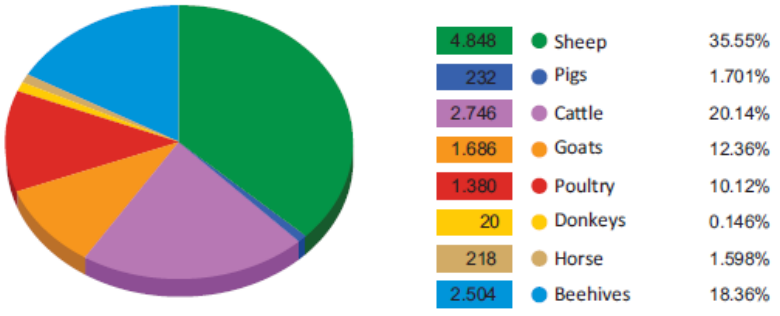
Main products are consisting of fruits and field crops, with the constant growth of cereals and oilseeds production (Tab. 2.).

Table. 2. Area by plant structure in Serbia in 2015.(ha)

Category	Areas in conversion (ha)	Areas in organic status (ha)	Total (ha)
Cereals	2.069	2.183	4.252
Industrial plants	1.216	1.458	2.674
Vegetables	45,6	124,9	170,5
Fodder	397,6	1.042	1.440
Fruit	1.291	1.604	2.895
Medicinal and aromatic plants	2,7	68,3	71
Other	1.845	50,4	1.895
Total arable land	**6.867**	**6.531**	**13.398**
Pastures/meadows	803	1.097	1.900
TOTAL	**7.669,5**	**7.628,5**	**15.298**

Source: MAEP

Last few years in Serbia started certification of organic livestock producers (Graf.4.). Every farmer keeps livestock in extensive conditions and this provides excellent organic fertilizer, manure from farm which could be used for organic crop production.



Graphic 4. Organic animal production (%) in 2015

Typical regional products are dairy products like cheese made of cow and sheep milk. Regional species are autochthonous breeds like Busha

and Pramenka that can be used for meat production as well.

The main market was the green market as a fresh product. Some of the organic products are exported, mostly to the EU. Most of processed organic products from Serbia are exported as frozen or dried organic fruits.The European IPARD program is one of chance for the organic sector in Serbia. Through investment support from IPARD, both farmers and processors (small number) can start to increase production efficiency and gradually strengthen the countries role in the European organic industry, using Serbian advantages in uncontaminated soil and water, as well as prominent R&D and educational institutions (5 Universities, Research Institutes, general education, NVO etc.). Moreover, 6 Centres for organic production were established in Serbia (Leskovac, Svilajnac, Valjevo, Uzice, and Negotin; in province Vojvodina: Selenca and Vojvodina cluster for organic agriculture too). Most of the local municipalities start initiatives and money support for the development of organic production (very good examples are Novi Sad municipality: enough money support, green market). Green markets are organized in Belgrade (3 specific markets only for organic producers). In Serbian Parliaments, the Action plan for the development of organic production for the period 2015 - 2020 year is adopted. (Ivana Simic (2018): Organic Agriculture at a Glance 2017, (eds, Zagorka Markovic), National Association Serbia Organica, 1-59)

In Serbia they have a 620000 small household; most of them have average 4.2 ha per household. This is a very good condition for developing organic agriculture. According to structure, size and product type in Serbia farms are divided into three categories. First type represent: *small*, mainly family households where plant and animal production is often integrated (about 80%); second type make

***specialized farms*, for example; specialized for organic field crops or organic vegetable production and similar which is not recommended from the aspect of organic principles, or business (about 10%). The third type of organic production represents *big farms* that combine plant and animal production on a bigger surface, often dealing with processing as well, which makes the most favourable model (EcoAgri, Bela Crkva – crop production, Global Seed, Čurug, the biggest cow farm in EU in, Midi organic, Kopaonik, organic fruit production and processing). (Ivana Simic (2018): Organic Agriculture at a Glance 2017, (eds, Zagorka Markovic), National Association Serbia Organica, 1-59)**

Main problems in the region are lack of certified processing premises where the original organic product would be made and offered as such to the market, majority of farmers are forced to sell raw material and /or original products as conventional. Of relevance for the organic sector are companies operating in the fruit and vegetable sectors. Cold storages dominate in the organic sector because cold storage can be obtained easier compared to whole technology line. More than 40 food processing companies also process organic products, virtually all of them processing conventional produce while operating an organic line additionally. Some of the primary producers also process their own products. According to data of the Serbian Chamber of Commerce and Industry 520 processing facilities have been registered with the exploitation rate of 80%. Based on the data of the MAFWM from 2011 Serbia has 363 cold storages, fruit, vegetables and mushroom storages with an overall capacity of 550.000 tonnes. Capacity exploitation rate is 75%.

Domestic market of organic products is still underdeveloped despite significant positive changes in recent years. Awareness of consumers started to rise as well, but mostly in the bigger urban environment where organic products are mostly present. This way they have

become available to most consumers who could have bought organic produce only in several retail shops or some green markets. Online shopping becomes available as well. The first specialized market of organic products started to work in mid-2011 in New Belgrade as a result of cooperation between Public Company City markets and Serbia Organica as a pilot project.

Export to EU

Demand for organically grown produce exists in many countries and Serbia has excellent eco-climatic and technical conditions to cultivate, in addition to berries and fruits that are traditionally grown, also organic cereals and oilseeds that are in high demand. According to Customs Administration Serbian organic products mainly exported to the EU countries (Germany, the Netherlands, Belgium, Austria, and Poland); with overall export in 2015 more of 13 million EUR, what makes 70% of the total value. In 2018 the total exported value increases to 21 million EU. Most of the export is fresh and frozen fruits (Tab. 2.). In 2015 raspberry was top exported product of fruits with 10, 9 mil EUR, frozen blackberry 3,2 mil EUR, and fresh organic apple 1.7 mil EUR. Of processed fruits apple concentrates amounted 1,0 mil EUR, dry fruits 750.000 EUR and sour cherry, quince and blackberry puree amounted 232.000 EUR.

Table 2. Export of organic products according to categories in 2015.

No.	PRODUCT CATEGORY	Value (€)	Share (%)
1	Fresh or frozen fruits	17.082.205	87,27%
2	Fruit products (concentrate, puree, dried)	2.115.178	10,81%
3	Fresh and frozen mushrooms	172.239	0,88%
4	Herbs (pepper)	62.570	0,32%
5	Vegetables products	55.315	0,28%
6	Medicinal and aromatic plants	32.200	0,16%
7	Vinegar	20.027	0,10%
8	Cereals products	16.381	0,08%
9	Juices	9.789	0,05%
10	Industrial plant (poppy)	4.240	0,02%
11	Butters	3.072	0,02%
12	Oils	173	0,00%
	TOTAL	**19.573.389**	**100,00%**

Source: Customs Administration

Agro ecological conditions in Serbia are particularly favourable for the production of organic berries; however, raspberry production is dominant over other fruits, with the production of the "Arilje" raspberry comprising 30% of the total global production of raspberries and Serbia being a world leader in the production of this type of fruit.

Advantages of organic agriculture in Serbia

54% of the population lives from the agriculture

About 630,000 households/3 -4 ha per household

2 cows per household

Mostly traditional/conventional agriculture

Climate agro ecological condition

No GMO

Good education system

Problems:

Only 0.4 % of the agricultural area is organic

Cheap workers and agriculture production

Lack of labor in agriculture

Small subsidies in organic agriculture

References:

Serbian Ministry of agriculture and Environmental Protection

IFOAM, https://www.ifoam.bio/en/about-us

FiBL

Custom Administration, Serbia

Ivana Simic (2018): Organic Agriculture at a Glance 2017, (eds, Zagorka Markovic), National Association Serbia Organica, 1-59.

Soil organisms 85 (3) 2013

M. Ricard: Summer courses, Amfissa July 2014, IFOAM)

TRAINING MATERIAL

Education of young scientist in ecologically friendly agriculture through WB6-W4 networking

First workshop

Sremska Kamenica, Educons Univerisity

Created by IGOR VUKELIC, MSc, dr Danka Radic and dr Gordana Racic

Chemical Soil Analysis - Determination of pH of Soil

Soil pH is an indication of the acidity or alkalinity of the soil. pH is defined as the negative logarithm of base 10 of the hydrogen ion H+ concentration ($pH = -\log[H^+]$). According to the pH values, the soil can be divided into acidic, neutral and alkaline. Determination of the pH of the soil or soil reaction is obligatory and the most frequent analysis in soil testing.

As the soil reaction is one of the most important environmental factors, it has a direct and immediate effect on the plants and soil microorganisms, which is reflected in the nutritional condition, the solubility of certain metals and the toxicity of pollutants. Plants - microbe interactions are an integral part of our terrestrial ecosystem. There are several ways for how plants interact with microbes: mutualism, commensalism, competition, and parasitism. This suggests that the pH value of the soil have a direct impact on the growth and development of microorganisms /plants and therefore it indirectly influences on growth and development of plants / microorganisms.

Microorganisms have a greater or smaller range of pH values in which they can grow and multiply (Table 1.) depending on the type and the reaction of the environment.

Table 1.: Minimum, maximum and optimum pH for growth of certain prokaryotes

Organism	Minimum pH	Optimum pH	Maximum pH
Thiobacillus thiooxidans	0,5	2,0-2,8	4,0-6,0
Sulfolobus acidocaldarius	1,0	2,0-3,0	5,0
Bacillus acidocaldarius	2,0	4,0	6,0
Zymomonas lindneri	3,5	5,5-6,0	7,5
Lactobacillus acidophilus	4,0-4,6	5,8-6,6	6,8
Staphylococcus aureus	4,2	7,0-7,5	9,3
Escherichia coli	4,4	6,0-7,0	9,0
Clostridium sporogenes	5,0-5,8	6,0-7,6	8,5-9,0
Erwinia caratovora	5,6	7,1	9,3
Pseudomonas aeruginosa	5,6	6,6-7,0	8,0
Thiobacillus novellus	5,7	7,0	9,0
Streptococcus pneumoniae	6,5	7,8	8,3
Nitrobacter sp.	6,6	7,6-8,6	10,0

pH value has a special significance for studying the fertility of soil and fertilization, as well as for growing certain cultures. The acidity of soil directly affects the growth of plants. Most plants grow best on neutral soil, ie, on a soil whose pH value ranges from 6.5 to 7.5. The optimal soil pH limit values for individual plant species based on a large number of literature data are shown in Table 2. (Kastori and Milosevic, 2011).

Table 2.: The optimal soil pH values for individual plant species

Plant species	pH value	Plant species	pH value
Fruits			
Apple	5,7-7,6	Pear	6,2-7,5
Sweet cherry	6,2-8,2	Tart cherry	6,2-8,2
Plum	6,2-8,2	Apricot	6,5-8,3
Peach	8,8-8,3	Walnut	6,2-8,2
Raspberry	6,5-7,2	Chestnut	6,5-7,2
Grape vine	6,0-8,5	Grape vine (grafted)	5,5-8,0
Vegetables			
Tomato	6,8-7,2	Paprika	6,0-7,6
Eggplant	5,5-6,8	Watermelon	6,7-7,5
Cucumber	6,5-7,5	Peas	7,5-8,0
Cabbage	6,5-7,8	Kohlrabi	6,5-7,5
Broccoli	6,5-7,5	Savoy cabbage	6,5-7,5
Sweet corn	5,5-7,2	Salad	5,8-7,6
Spinach	6,5-7,5	Onion	6,5-7,5
Garlic	5,5-6,8	Carrot	5,5-8,2
Celery	6,3-7,5	Beet	6,0-7,0
Asparagus	6,8-7,2	Radish	5,2-6,7
Lean	6,0-7,0	Parsnip	~ 6,5
Potato	5,0-6,0	Broad bean	5,5-5,6
Herbaceous plants			
Wheat	5,5-7,5	Soya	6,0-7,5
Barley	6,0-7,5	Bean	6,0-7,5
Rice	5,0-6,5	Sunflower	6,0-7,5
Oat	5,0-7,5	Sugar beet	6,5-8,0
Cannabis	5,0-6,5	Tobacco	5,5-7,5
Cotton	5,0-6,5	Flax	4,0-7,0
Rye	4,0-7,0	Sorghum	6,0-8,3

Fodder crops			
Lucerne	**6,2-7,8**	**Savoy cabbage**	**5,0-7,0**
Dutch clover	**5,6-7,0**	**Wild carrot**	**6,5-7,0**
Red clover	**6,0-7,0**	**Alsike clover**	**5,5-7,5**
White turnip	**6,5-7,5**	**Turnip rape**	**6,0-6,5**
Perennial woody plants			
Aspen	**7,5-8,0**	**Willow**	**7,0-8,0**
Spruce	**oko 4,5**	**Scots pine**	**~4,5**
Creeping pine	**oko 4,5**	**Juniper**	**4,5-5,5**
European wild pear	**8,0-10,5**	**American ash**	**8,0-10,5**
European oak	**8,0-10,5**	**Fir**	**5,5-6,5**

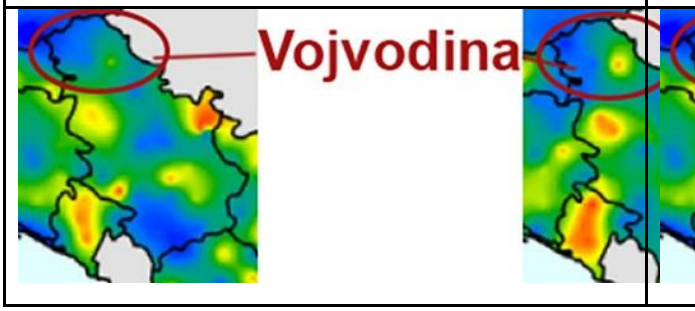
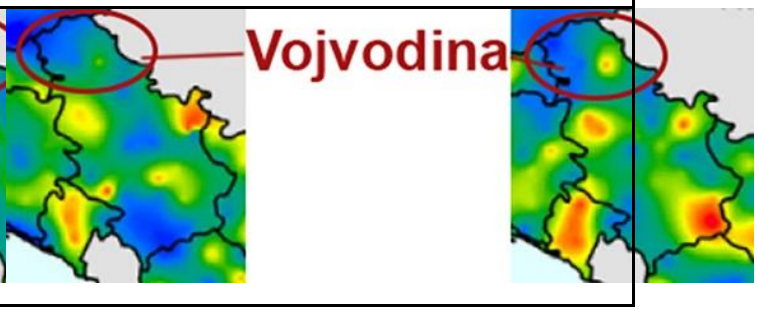
The soil's pH in Europe and Serbia

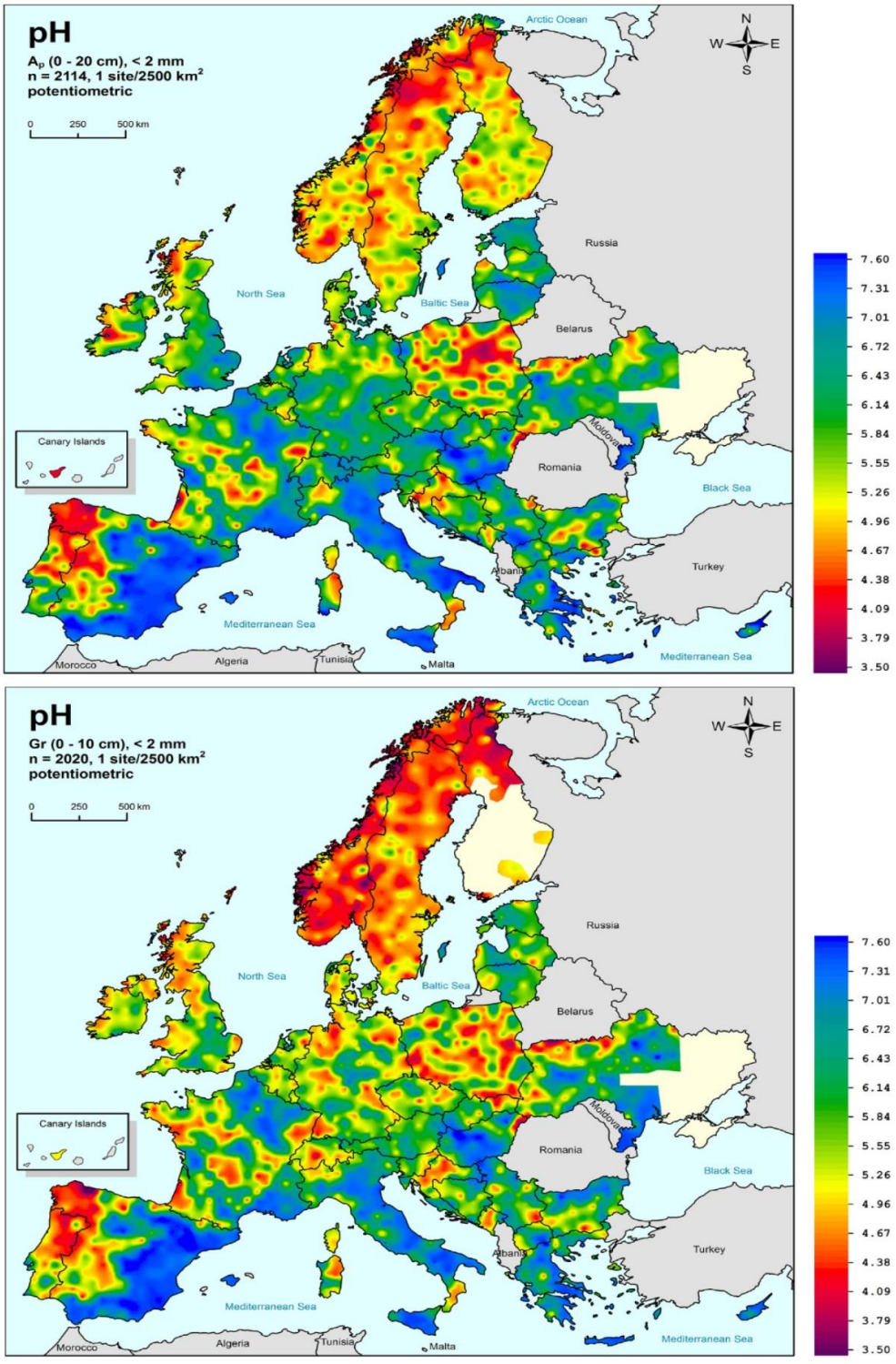
Median European pH value is 5.8 for the agricultural (Ap) and 5.5 for the grazing land soil sample (Gr) (picture 1.) (Fabiana, 2014). Based on pH soil value (Table 3.), Europe is separated into two main pH zones. In the first one dominated by acidic soils in Northern Europe which have lower pH values (Ap-5.2, Gr-4.8), while southern Europe has higher pH values (Ap-6.3, Gr- 5.9). The main factors which are controlling pH soil in Europe are climate (precipitation and temperature) and geology (crystal-line bedrock) (Fabiana 2014).

Most of the Serbian agricultural soil is acidic. The class of highly acidic to acidic soils is equivalent to 43% (1,197,000ha) of the total area surveyed in Serbia, which have increased levels of exchange acidity. 35% are in the group of weakly acidic to neutral soils, while 20% are acidic to weakly acidic soil (Licina et al, 2011). Unlike Serbia, province of Vojvodina is dominated by alkaline soil. The largest part of alkaline and saline soils of Serbia is located in the province of Vojvodina

(Picture 2.). pH value and composition of the soil make province of Vojvodina one of the best quality of soil in Europe.

Table 3.: The U.S. Department of Agriculture Natural Resources Conservation Service classifies soil pH ranges as follows:

Denomination	pH range
Ultra acidic	< 3.5
Extremely acidic	3.5–4.4
Very strongly acidic	4.5–5.0
Strongly acidic	5.1–5.5
Moderately acidic	5.6–6.0
Slightly acidic	6.1–6.5
Neutral	6.6–7.3
Slightly alkaline	7.4–7.8
Moderately alkaline	7.9–8.4
Strongly alkaline	8.5–9.0
	



Picture 1.: European maps of pH (CaCl2) from agricultural (Ap) and grazing land soils (Gr) (Fabiana, 2014).

Picture 2.: Maps of pH (CaCl2) in Serbia and province of Vojvodina for soil samples from agricultural (Ap) on left and grazing land soils (Gr) on right side (Fabiana, 2014).

PRACTICE 1

In the soil, two basic types of acidity are distinguished:

1. active (acidity of the soil solution-liquid phase of the soil) and

2. potential reserve / exchange (acidity of the soil adsorptive complex-solid soil phase)

1. The active acidity is understood to mean the concentration of free hydrogen ions (H+), in the soil solution or in the aqueous extract in the soil. This means that the hydrogen ion concentration is determined in the aqueous suspension and it is expressed as pH.

2. Potential reserve / exchange acidity depends on the concentration of H+ ions adsorbed on the surface of the colloidal particles of the soil solution. Since all adsorbed hydrogen ions on the surface of colloidal particles are not bound together with the same strength, some of them pass into the soil solution easier than others. This is the reason why there are two different types of potential acidity:

a) substitution and

b) hydrolytic.

a) The substitution (potential) acidity of the soil is represented by H+ ions that are more labile to the colloidal particles and determined by the action of a neutral salt solution on the soil most often with KCl. It is marked with pH in KCl.

b) Hydrolytic potential acidity of the soil is represented by H+ ions that are more strongly related to colloidal particles and determined by the action of a solution of a stronger base salt on the soil.

The active and substitution acidity is determined by electrometric-potentiometric and/or colorimetric.

-pH-meters is using for potentiometric determinations, it allows the measurement of voltage, which depends on the activity of hydrogen ions.

-Colorimetrically, the pH is determined by dint of universal indicator, which changes color at different levels of acidity, passing from non-ionized to ionized form or vice versa.

With the increase of pH value, the number of H ions in the solution is decreased. This leads to accelerated ionization from the reactive groups as well as the charge of the soil solution...

Most of the soil colloids, whether minerals or humus origin, have a negative charge. Colloids with a negative charge are called acidoids and with positive charge basoids. Depending on the pH, some behave amphoteric, so they are called ampholytes.

Soil reaction is a stable value and it is important for the understanding of soil properties for the purpose of cultivating certain plants, studying the fertility of the soil, fertilization... It should be determined again after the performed meliorative operations (deep processing, calcification, etc.). However, due to the intensive application of fertilizers after every 8-10 years, the pH of the soil should be determined again.

Soil pH determination in water

Reagents

pH standard solution (pH = 7,00 and pH = 4,01) for instrument calibration,

destillled H2O

Equipment

technical balance

H meter

glasses 100 ml

glass sticks

funnel

graduated cylinder 25 ml...

DETERMINATION PROCEDURE

- Preparation of soil suspension for determination of pH in aqueous solution

On a technical balance measure 10 g of air-dry soil, transfer to a glass of volume about 100 ml and add 25 ml of distilled water (the pH of the water should be 6.6-6.8). Mix with a glass stick for approximately 5 minutes, let it clear and determine pH with the pH meter.

- Work with a pH meter

Electrometric methods are the fastest and most accurate since the chemical reaction of the soil can be determined to the accuracy of 0.01 pH number.

The room must be free of corrosive gases, moderate relative humidity, without significant oscillations in temperature. Turn on pH meter at least 10 minutes before starting the measurement. Before starting the measurement, the apparatus should be calibrated using standard solutions (pH 10.00, pH 7.00 and pH 4.01) (Picture 1.). During measurement, the electrode should be completely immersed in the suspension. The electrode is removed from the suspension only after switching off the appliance. Between two measurements, wash the electrodes with distilled water and carefully wipe with filter paper. After completing the measurement, keep the electrode according to the manufacturer's instructions.

Picture 3.: pH meter (www.kisker-biotech.com)

QUANTITATIVE DETERMINATION OF CARBONATES IN SOIL

Carbonates are salts of carbonic acid and belong to the mineral part of the soil. The carbonates in the soil affect the physical and chemical properties of the soil, and therefore its productive ability. CaCO3 soil aggregate structure is stimulating the growth of fungal hyphae and plant roots, earthworm activity and absorption of above-ground crop residues (Palmu and Hedlund, 2016). The carbonate content is important for the movement of nutrients in the soil and for determining the types of crops and quantities of fertilizers. In pedological research, determining the amount of carbonate is necessary. These determinations are of great importance during the selection of soils for the cultivation of fruits.

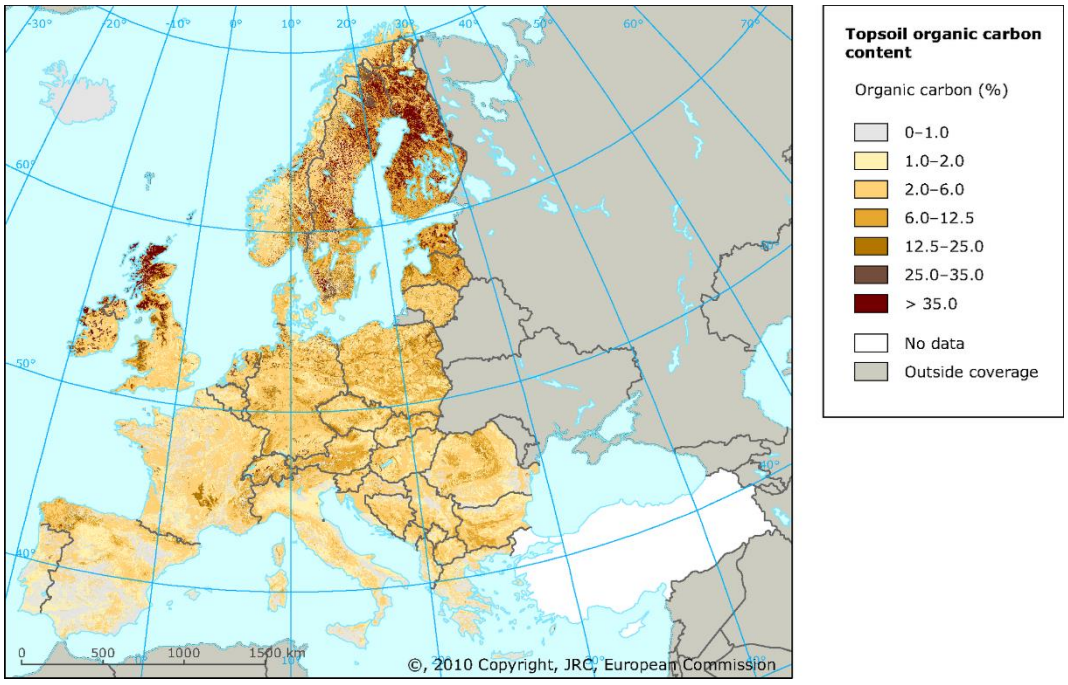
In soil carbonates are mostly present as salts of calcium and magnesium, whereas in saline soil they can be found in sodium carbonate. Carbonate content in soil is important, mainly because it can affect growth of some plant species.

Carbonates often limit the growth of the plant, causing the deficiency of some necessary microelements (Zn, Fe...). Also, the over content of $CaCO_3$ have a negative effect on the accessibility of potassium to crop production because it reduces its activity in the soil. For crop production is preferable soil with the lowest content of calcium carbonate (Nachtergaele et al, 2009).

The content of carbonates in Europe and Serbia

In Northern and Central Europe young soils are dominant. Northern European soil has higher organic carbon matter content compared to soil in the south (Jones et al, 2012) while in southern Europe dominated carbonate-rich soils (Fabiana, 2014). Mediterranean basin is characterized by poorly developed soil with large amounts of $CaCO_3$.

Picture 4.: Map of organic carbon content in Europe



According to Miljkovic 1996, chernozem in the province of Vojvodina is in the initial phase of carbonate flushing in the form of bicarbonate. The process of migration of CaCO3 in the soil is very slow and lasting for hundreds of years because of distinguished climate with relatively little precipitation. So, the content of $CaCO_3$ in the soil does not

change during the season. The fertility of this soil type in the province of Vojvodina is high and it is considered as the best soil for plant production.

PRACTICE 2

Determination of carbonate in the soil can be:

a) qualitative

b) quantitative

- gravimetric (the mass of CO_2 generated by the destruction of $CaCO_3$ is determined)

- volumetric (the amount of CO_2 generated by the destruction of $CaCO_3$ is determined)

Table 4.: Correlation of the qualitative test and the content of carbonates in the soil

Intensity of foaming	content of carbonates		
	mark	description	% $CaCO_3$
Does not have	-	does not have	0
Hardly noticeable	Trace	very small	0-2
Poor	+	small	2-4
Medium	++	moderate	4-7
Strong	+++	high	7-10
Very strong	++++	very high	> 10

Quantitative determination is based on determining the exact content of carbonates in the soil. In volumetric determinations, the volume of CO_2 generated by the $CaCO_3$ depletion in the soil sample is determined while the mass of CO_2 generated by the destruction of $CaCO_3$ from the sample is determined in gravimetric conditions.

In pedological research, the volumetric method is more often used, which is performed by a special apparatus - calcimeter. There are several types of calcimers that have been named according to their constructors: Scheibler, Bernard, Pason, and others.

Working principle

The determination of carbonates is based on the measurement of the volume generated by CO_2 by decomposition in hydrochloric acid.

The destruction proceeds according to this equation:

$$CaCO_3 + 2\,HCl \longrightarrow CaCl_2 + H_2O + CO_2\uparrow$$

Volatile CO_2 is caught in a graduated tube of calcimeter, where volume is measured and based on data calculated % $CaCO_3$.

Reagents:

To determine the carbonate, a solution of hydrochloric acid (1:3), about 10% (1 part HCl and 3 parts of distilled H_2O).

Equipment:

- Scheibler calcimeter

- barometer

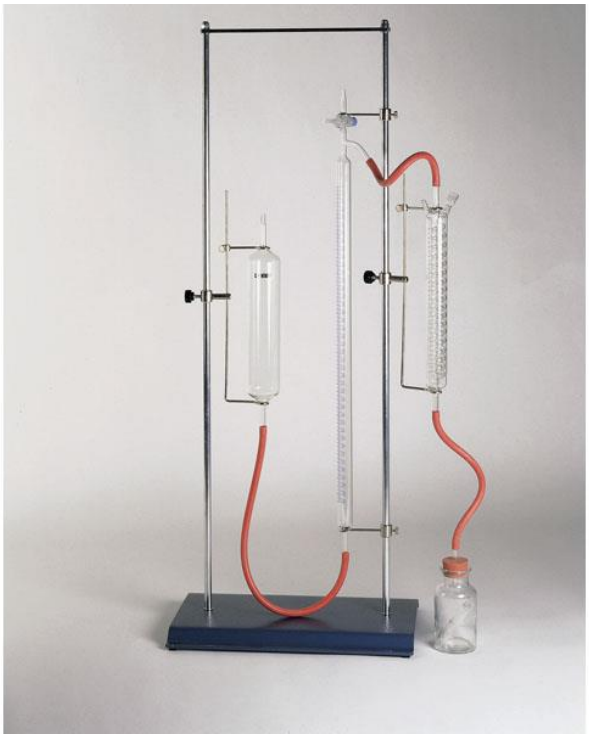
- thermometer

- glass containers, samplers for sample measurement

The principle of this determination is to obtain the volume of CO_2 under certain pressure and temperature conditions. The found CO_2 volume is converted to mass using special plates, then converted to carbonates by multiplying with factor 2.273 and finally expressed in %.

- Scheibler calcimeter -

Scheibler calcimeter (Picture 5.) consists of two cylindrical tubes, glass bottles for leveling (filling the burette) and a reaction vessel. A glass tube that is graduated is called burette and is filled with a 22% NaCl solution acidified with sulfuric acid and colored in a pink with methyl orange. The burette is used to measure the volume of CO_2 emissions. It is, on the one hand, connected to the tube with a glass tube and through it with a container (small Erlenmeyer) for the sample, and on the other side with a leveling vessel.



Picture 5.: Scheibler calcimeter (www.controls-group.com)

PROCEDURE

Before beginning the gas volume measurement, the calcimeter must be filled with NaCl solution to zero. This is achieved by lifting the leveling vessel with an open tap so that all the air is pushed out of the burette. Then close the tap and leave the leveling bottle on the work table. Weigh 1-5 g of soil (depending on the amount of carbonate) and transfer it to the filler, and then carefully insert it into a small Erlenmeyer flask and overflow with hydrochloric acid (1:3). The flask must be closed immediately with a rubber stopper and carefully shaken so that the HCl is poured over the test sample. Carbon dioxide develops immediately, passes into the undeveloped tube, and from it through an open tap in the burette, which reads the volume of the developed gas in ml. When determining, it is necessary to read the barometric pressure and the temperature at which CO_2 is determined.

CALCULATION

For carbonates determination use the formula:

$$\% \ CaCO_3 = \frac{m(CO_2) \cdot 2{,}274 \cdot 100}{m_s}$$

V **(CO_2) – the volume of CO_2**

m **– the mass of 1 ml CO_2 determined for exact temperature and pressure following the table**

2,274 – coefficient for transforming CO_2 u $CaCO_3$ (M_{CaCO3}/ M_{CO2}) =(100/44)

m_s **– soil sample mass**

From the table, on the basis of the known pressure and temperature data for which the determination is made, the apparent mass is 1 ml of CO2. From the volume of the developed CO2, the mass of CO2 that was found in CaCO3 from the sample is obtained. Based on this, the% CaCO3 content in the soil is calculated.

Table 1. Table for calculation of CO_2 mass

t°C	Pressure in mb (millibar)								
	996	998.5	1001.5	1005.5	1008	1010.5	1013.5	1017	1020
25	1810	1816	1823	1829	1836	1842	1847	1852	1856
24	1816	1822	1829	1835	1842	1848	1853	1858	1862
23	1822	1828	1835	1841	1848	1854	1859	1864	1868
22	1828	1834	1841	1847	1854	1860	1865	1870	1875

21	1835	1841	1848	1854	1861	1867	1872	1877	1882
20	1841	1847	1854	1860	1867	1873	1878	1883	1888
19	1847	1853	1860	1866	1873	1879	1884	1889	1894
18	1853	1859	1866	1872	1879	1885	1890	1895	1900
17	1860	1866	1873	1879	1886	1892	1897	1902	1917
16	1866	1872	1879	1886	1892	1898	1903	1908	1913
15	1872	1877	1886	1892	1899	1905	1910	1915	1920

ANNEX:

The limit values:

> 5% $CaCO_3$ - strong and long-lasting spraying (measured 1 g)

3-4% $CaCO_3$ - clear and short-term spraying (measured 2 g)

2-1% $CaCO_3$ - short-term spraying (5 g is measured)

<1% $CaCO_3$ - free of foam (not working)

Example of calculation:

m_s = 3,00g

V (CO_2) = 12,5 cm^3

p= 1010,5 mbar

t= 20 ^{0}C

Based on the known pressure and temperature, data from the table (CO_2 mass) is read. This value is multiplied by the measured volume of CO_2 from the calcimeter, obtained mass m (CO_2) should be added to the expression.

Data from the table: p= 1010,5 mbar t= 20 ^{0}C read mass 1,873 mg CO_2

1 cm^3 CO_2 : 1,873 mg CO_2 = 12,5 cm^3 CO_2 : m (CO_2)

m (CO_2) = 1,873 mg x 12,5 cm^3 / 1cm^3 = 23,41 mg = 0,023 g

% $CaCO_3$ = m (CO_2) x 2,274 x 100 / m_s

% $CaCO_3$ = 0,023 g x 2,274 x 100 / 3,00 g = 1,74

The results:

V_{CO2} = 48 cm^3

p= 1005,5 mbar

t= 20 ^{0}C

m_s = 3, 15 g

DETERMINATION OF HUMUS CONTENT IN SOIL

General Introduction

"Chemically, humus consists of certain constituents of the original plant material resistant to further decomposition; of substances undergoing decomposition; of complexes resulting from decomposition, either by processes of hydrolysis or by oxidation and reduction; and of various compounds synthesized by microorganisms." **(Waksman, 1936).**

Humus contains: proteins, lipids, carbohydrates, protein derivates, phenol-aromatics, and cyclic nitrogen compounds, and some still unknown compounds. Humus **is a very important soil component and it has the main role in nutrition of soil biota.**

The structure of soil, water, physical and absorbing properties, and fermentation activities are depending on humus content.

The general character of humus is that of a black or brown, loose, porous and absorbent substance.

Humus cannot be decomposed readily because of its interactions with soil mineral phases and is chemically too complex to be used by most organisms.

Humic substances are very complex and large, with high molecular weights and quite different from simple organic molecules.

Humus is important for soil microorganisms and plants, certain bacteria and fungi produce it by plant material decomposition while other microorganisms help plants roots to absorb necessary nutrients.

<u>Humus consists of:</u>

Humin- is the fraction of humus that is not soluble in water at any pH and that cannot be extracted with a strong base, such as sodium hydroxide (NaOH);

Humic acids- are the fraction of humus soluble in water, except for conditions more acid than pH 2.

One of the most striking characteristics of humic substances is their ability to interact with metal ions, oxides, hydroxides, mineral and organic compounds. Common colors are dark brown to black;

Fulvic acids - are the fraction of humus that is soluble in water under all pH conditions. Their color is commonly light yellow to yellow-brown. They are produced in the earlier stages of humus formation.

Humic and fulvic acids enhance plant growth and are capable of improving seed germination, root initiation, uptake of plant nutrients and can serve as sources of N, P and S. But, the significance of the various types of humic substances to plant growth is yet to be established.

Functions of humus:

Improves texture and the structure of the soil;

Increases porosity of the soil-has more air voids than typical soils;

Increases the water holding capacity of the soil;

Modifies the chemical content of the soil by returning chemicals to the soil in a highly usable form, particularly of P and Ca;

Increases the nitrogen content of the soil;

Stimulates variability of soil biota;

Improves salinity management – humates "buffer" plants from excess sodium;

Improves soil N status – for example, urea transformation lasts 60–80 days longer;

Increases soil C levels;

Reduces erosion-disperses the force of raindrops.

Transformation of humus

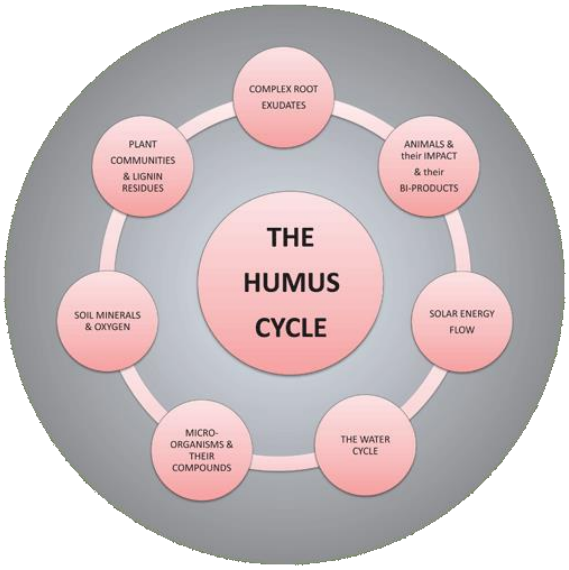
Humification is a process, which implies changes in the stoichiometry and chemical complexity of the original macromolecular materials leading to humic substances with enhanced resistance to mineralization and biodegradation. Humus cycle connects soils and organic sediments and also natural water and air.

The microorganisms influence the cycle of humus in nature.

The microorganisms have crucial role in humus formation from plant and animal residues.

The microorganisms continuously transform humus, under favorable conditions, and decompose it completely or "*mineralize*."

Their own cells substance contributes directly as a source of humus.



The humus cycle

Humus status in soils in Serbia

Map of Serbia

Monitoring

Continuous soil monitoring system not established

Macro project:

"*Control of soil fertility and determination of harmful and hazardous substances in soils on the territory of the Republic of Serbia*, 1992. "

Research:

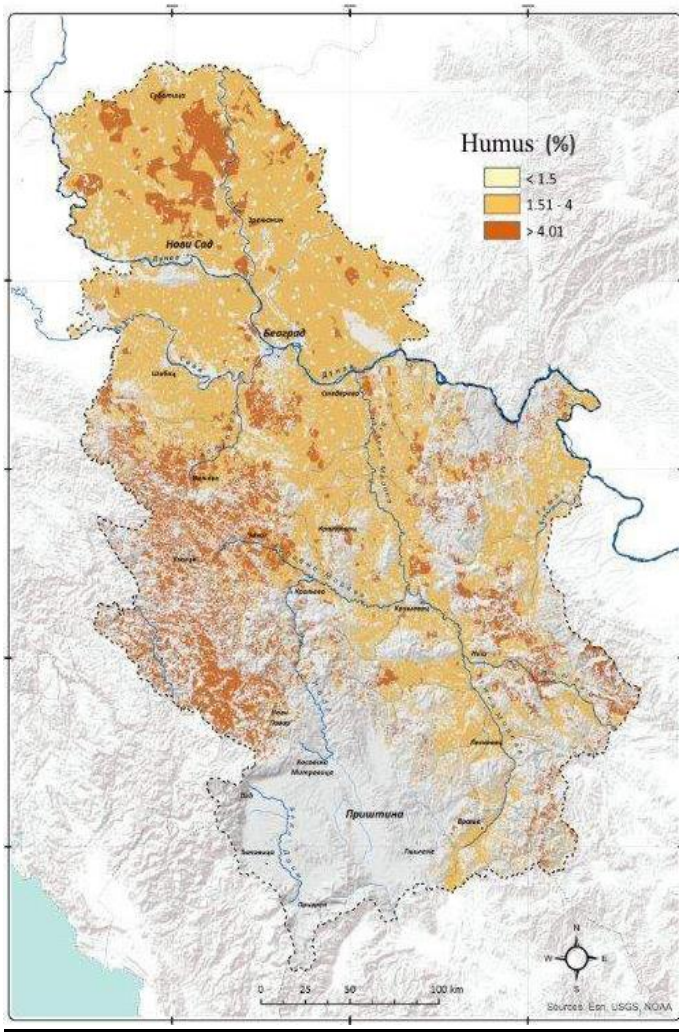
Humus loss – causes and effects;

Humus preservation and increase;

Humus quality;

Humus effect on: soil properties;

Humus effect on: plant yield.

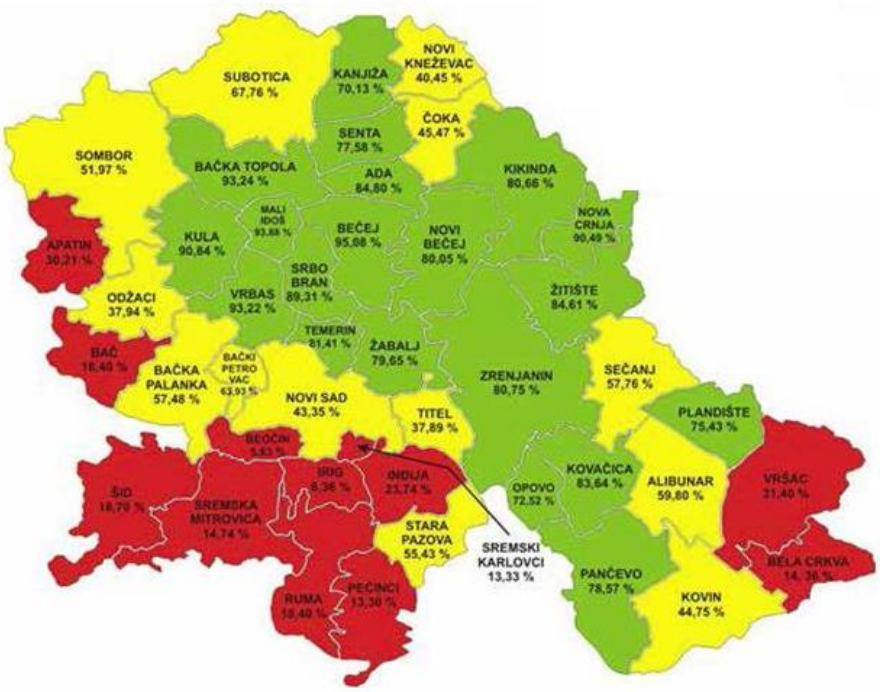


Organic matter in agricultural soil in the range of 0-30 cm (%).

Source: ***Republic of Serbia Ministry of Agriculture and Environmental Protection, 2016.***

According to the analysis of 96.011 soil data from the State Soil Fertility control program, the average rate of soil organic matter expressed as humus content in the top 30 cm of the agricultural soils is 3.4 %. Only 4.3 % of samples have the rate of soil organic matter beyond 6 % (*Republic of Serbia Ministry of Agriculture and Environmental Protection, 2016*).

Humus status in Vojvodina soils



Humus status in Vojvodina soils. Green color - favorable humus content; Yellow color - medium favorable humus content; Red color - unfavorable humus content. Source: *Republic of Serbia Ministry of Agriculture and Environmental Protection*, 2016.

An analysis of over 184.899 samples of plowland in AP Vojvodina indicated that most samples belong to the category of low humus soils with the organic matter content ranging from 1% to 3%.

Organic matter content in Vojvodina soils is under strong anthropogenic influence.

In order to increase the humus content by only 1%, it takes a period of 50 to 100 years!

How to increase of humus content?

Humus content in soil is the basic requirement for soil fertility and sustainable agriculture.

It is important to ensure:

Soil monitoring system;

Adequate supply of crop residue and organic matter;

Responsible fertilization of crops to increase plant biomass;

Reduced soil cultivation;

The introduction of organic fertilizers into the soil (manure, compost, peat);

Sowing plants which grow and create a large amount of organic matter (oilseed rape, red clover);

Increase in microbiological activity;

Crop rotation, tillage and fertilization.

<u>Future outlook:</u>

To improve humus quality;

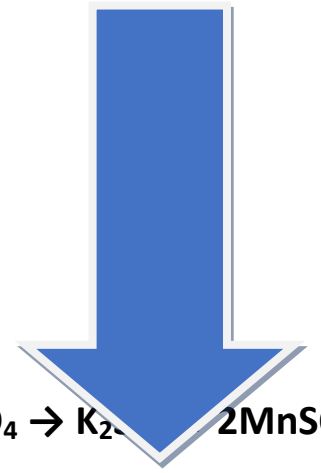
To intense research of: new approaches to increase soil humus and humus influence on soil fertility...

PROTOCOL FOR THE DETERMINATION OF TOTAL HUMUS

The most commonly used is the permanganate method, *according to Kocman*.

The principle of the method is based on the oxidation of organic soil substances using 0.02 mol/dm^3 of the $KMnO_4$ solution, whereby the carbon from the humus is oxidized and converted to carbon dioxide CO_2. After completion of the reaction, the excess $KMnO_4$, which is not consumed for oxidation, is titrated with 0.05 mol/dm^3 oxalic acid solution ($H_2C_2O_4$) in the acid medium until the pink color disappears.

Determination principle is based on the following equation:



$2KMnO_4 + 5H_2C_2O_4 + 3H_2SO_4 \rightarrow K_2$ $2MnSO_4 + 8H_2O + 10CO_2$

Reagents:

0.02 mol/dm^3 $KMnO_4$

H_2SO_4 (1:4)

0.05 mol/dm^3 $C_2H_2O_4$ x $2H_20$

Equipment:

- Analytical balance;

- Heater;

- Erlenmeyer flask 100 ml;

- Burette 25 ml;

- Pipette 10 ml.

Erlenmeyer flask, analytical balance and burette.

Measure 300-500 mg of air-dry soil from the prepared soil sample. Transfer the soil sample to the Erlenmeyer flask and add 130 cm^3 of distilled water, 20 cm^3 H_2SO_4 (1: 4) and 50 cm^3 0.02 mol/dm^3 $KMnO_4$. Prior to heating, place a funnel on the Erlenmeyer, for preventing the release of drops of the suspension during boiling. Heat the suspension in the erlenmeyer for 15 min.

The liberated oxygen oxidizes carbon from organic substances in the soil to carbon dioxide (CO_2) evaporating from the Erlenmeyer. The $KMnO_4$ decomposition will last as long as there is organic carbon in the soil sample. After 15 minutes of boiling, remove the erlenmeyer from the burner and titrate the solution immediately with 0.05 mol/dm^3 oxalic acid solution ($C_2H_2O_4$), until the pink solution is discolored. Since the process of discoloration is gradual, it is necessary to add oxalic acid in excess. The amount of these acids is re-tritated with $KMnO_4$ solution from another burette until a pink color of the solution indicates the ending point of the titration.

RESULTS

$$\text{\% of humus} = \frac{V(KMnO_4) \cdot 0.514 \cdot 1.72}{} \cdot 100$$

m_{sample}

V ($KMnO_4$) – volume of spent 0.02 mol/dm^3 $KMnO_4$ for oxidation of organic matter

0.514 mg - constant that explains that every cm^3 of 0.02 mol/dm^3 $KMnO_4$ oxidates 0.514 mg of carbon from humus to CO_2

1.72 is coefficient for transforming C in humus

m_{sample} – mass (mg) of the sample (300-500 mg)

Humus soil status	Humus content in %
Low humic	>2
Medium humic	2-4
Humic	4-10
High humic	10-15

Example:

0.1 M $KMnO_4$	**50 cm^3 • 0.9985 (factor)**	**49.9250 cm^3**
0.1 M $KMnO_4$	**1.5 cm^3• 0.9985 (factor)**	**+ 1.4977 cm^3**
		51.4227 cm^3
0.1 M $C_2H_2O_4$	**32 cm^3 • 1.0025 (factor)**	**-32.08 cm^3**

Difference *V*($KMnO_4$) = 19.3427 cm^3

It means that for organic matter oxidation of 300 mg of soil, 19.30 cm^3 0.02 mol/dm^3 $KMnO_4$ solution is used:

$$\textit{\% of}\ \text{humus} = \frac{19.30 \bullet 0.514 \bullet 1.72}{300} \bullet 100 = 5.60\ \%$$

Recommended books and book chapters

Bogdanović, D., & Ubavić, M. (1995). Agrohemija. Institute of Field and Vegetable Crops, Novi Sad.

Lavelle, P. & Spain, A. (2001). Soil ecology. Dordrecht, The Netherlands, Kluwer Academic Publishers.

Guggenberger, G. (2005). Humification and mineralization in soils, in *Microorganisms in Soils: Roles in Genesis and Functions*, eds. F. Buscot and A. Varma (Berlin: Springer), 85-106.

Recommended articles

Nešić, Lj., Belić, M., Ćirić, V., Sekulić, P., & Vasin, J. (2013). Content of clay and humus in some soils of Vojvodina. Proc.Intl.Conf. "Ecology in the Service of Sustainable Development", 26-28 September 2013, Fruška Gora, Andrevlje, Serbia, p. 70-75.

Vancampenhout, K., Wouters, K., De Vos, B., Buurman, P., Swennen, R., Deckers, J. (2009). Differences in chemical composition of soil organic matter in natural ecosystems from different climatic regions – A pyrolysis–GC/MS study. Soil Biology & Biochemistry, 41, 568-579.

Piccolo, A. (2002). The supramolecular structure of humic substances. A novel understanding of humus chemistry and implications in soil science. Adv. Agron. 75, 57-134.

Heavy metal content in soil

HEAVY METALS – GENERAL INTRODUCTION

Heavy metals are defined as the toxic metals and metalloids having density of more than 5g/cc and atomic weight more than of iron. Other definition is that they have a relatively high density compared to water. According to this, 76 trace elements such as Fe, Mn, Cu, Zn, and Ni [which exhibit both deficiencies and toxicity] and other metals Pb, Cd, Cr, Hg, Se, As have been referred as the heavy metals.

Although they are mostly connected with toxic effects some of them such as cobalt (Co), copper (Cu), chromium (Cr), iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo), nickel (Ni), selenium (Se) and zinc (Zn) are essential nutrients that are required for various biochemical and physiological functions. Inadequate supply of these micro-nutrients results in a variety of deficiency diseases or syndromes (WHO/FAO/IAEA). The essential heavy metals exert biochemical and physiological functions in plants and animals. They are important constituents of several key enzymes and play important roles in various oxidation-reduction reactions.

HEAVY METALS IN SOIL

Understanding of heavy metal presence and availability in soil is of great importance to environmental health, livestock production, soil and water quality, food production and ecotoxicology (Alloway, 2012).

Soil is a complex system composed of organic and inorganic material. It contains full range of heavy metals from periodic table, although their concentrations vary. There are two types of metal concentrations in soil, total and available. Total metal content includes all forms of the element in soil (ions bound in the crystal structure of primary and secondary minerals, metals adsorbed on the surface of secondary minerals, such as clays, oxides and carbonates, element bound in organic matter, free ions and soluble organic and inorganic complexes present in soil solution). Available metal content present fraction of total metal concentration which is potentially available to the plants (Alloway, 2012).

Metal toxicity to plants grown on particular soil is dependent on total metal concentration, adsorptive capacity of the soil and physico-chemical factors (such as pH, redox potential).

SOURCES OF HEAVY METALS IN SOIL

Soil contaminated with heavy metals has become a serious worldwide problem due to natural and anthropogenic sources. Heavy metals are non-degradable and thus persist indefinitely in the environment (Ma et al., 2016).

Natural sources are parental rocks and metallic minerals, while the anthropogenic sources are from different sources, mostly from agriculture (fertilizers, animal manures, pesticides, etc), metallurgy (mining, melting, metal finishing, etc) and energy production (leaded gasoline, battery manufacture, power plants, etc.). They can be released in all three aggregate states: gaseous (aerosol), particulate, aqueous (or) solid form depending on the industry. Also, pollution with heavy metals may be from both, point (or) diffuse sources. Point sources emission of large metal industries can cause large-scale contamination of land.

Metals that are most commonly found at contaminated sites are: lead (Pb), chromium (Cr), arsenic (As), zinc (Zn), cadmium (Cd), copper (Cu), mercury (Hg), and nickel (Ni). Metals found in soils usually do not undergo microbial or chemical degradation, and their total concentration in soils persists for a long time. However, changes in their chemical forms (speciation) and bioavailability are possible. Figure 1 represents that out of different pollutants heavy metal pollution is mostly affecting soil and groundwater in Europe.

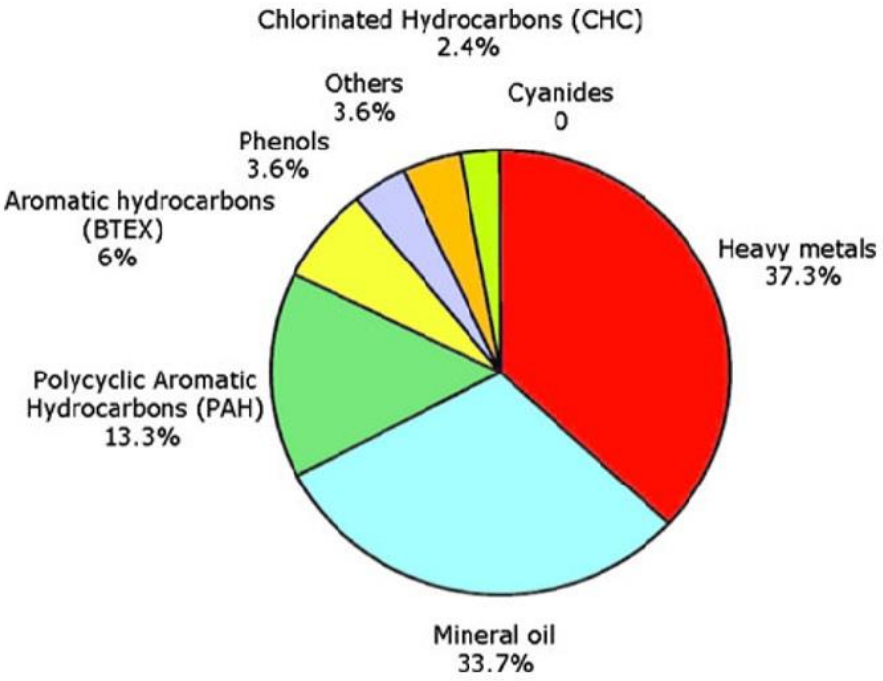
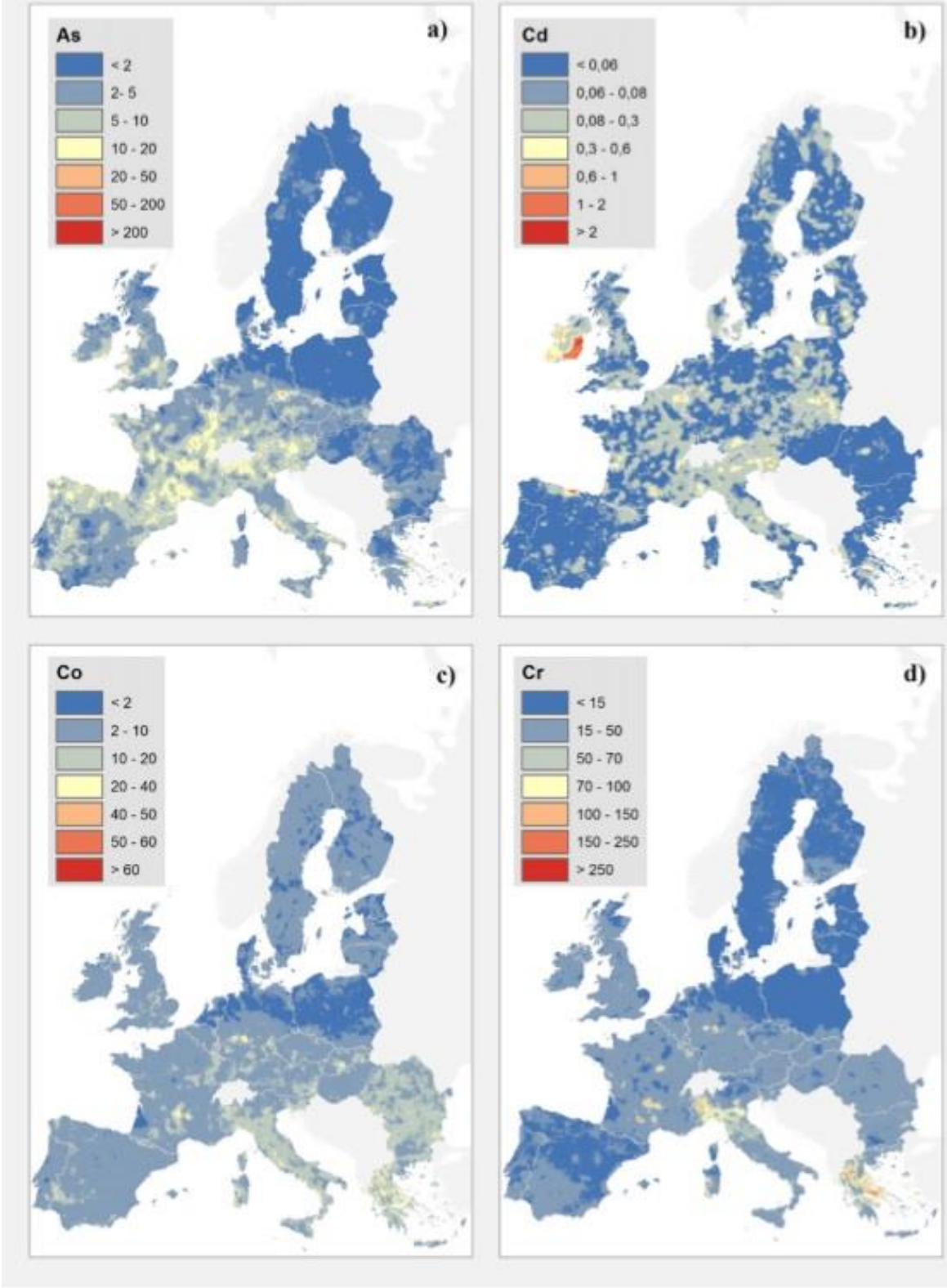


Figure 1. Contaminants affecting soil and groundwater in Europe (the ranking of contaminants is calculated on the basis of the frequency a specific contaminant is reported to be the most important in the investigated sites)

SITUATION IN EUROPE

As previously mentioned heavy metal pollution is highly affecting soils of Europe. In order to get detailed information on metal pollution as well as detailed information on soil characteristics in Europe The LUCAS Topsoil Survey of the European Union has been developed (Tóth et al., 2013**). The LUCASTopsoil Survey provides a unique opportunity for an appraisal of the situation of heavy metal levels in the soils of the European Union. Based on the data from the LUCAS samples a series of maps where produced which predict the concentrations of different metals present in soils of the EU. Although concentrations of most of these elements are under the corresponding threshold values, one or more of the elements exceed the applied threshold concentration on 1.2Mkm^2, which is 28.3% of the total surface area of the EU. Detailed assessment and monitoring is needed in the Western Central Europe, Central Italy, Greece and South-East Ireland. These maps are still not able to separate natural from anthropogenic pollution some tendencies and hotspots were identified. On Figure 2, concentrations of some heavy metals were presented on a map.**


a)
As
< 2
2- 5
5 - 10
10 - 20
20 - 50
50 - 200
> 200
b)
Cd
< 0,06
0,06 - 0,08
0,08 - 0,3
0,3 - 0,6
0,6 - 1
1 - 2
> 2
c)
Co
< 2
2 - 10
10 - 20
20 - 40
40 - 50
50 - 60
> 60
d)
Cr
< 15
15 - 50
50 - 70
70 - 100
100 - 150
150 - 250
> 250

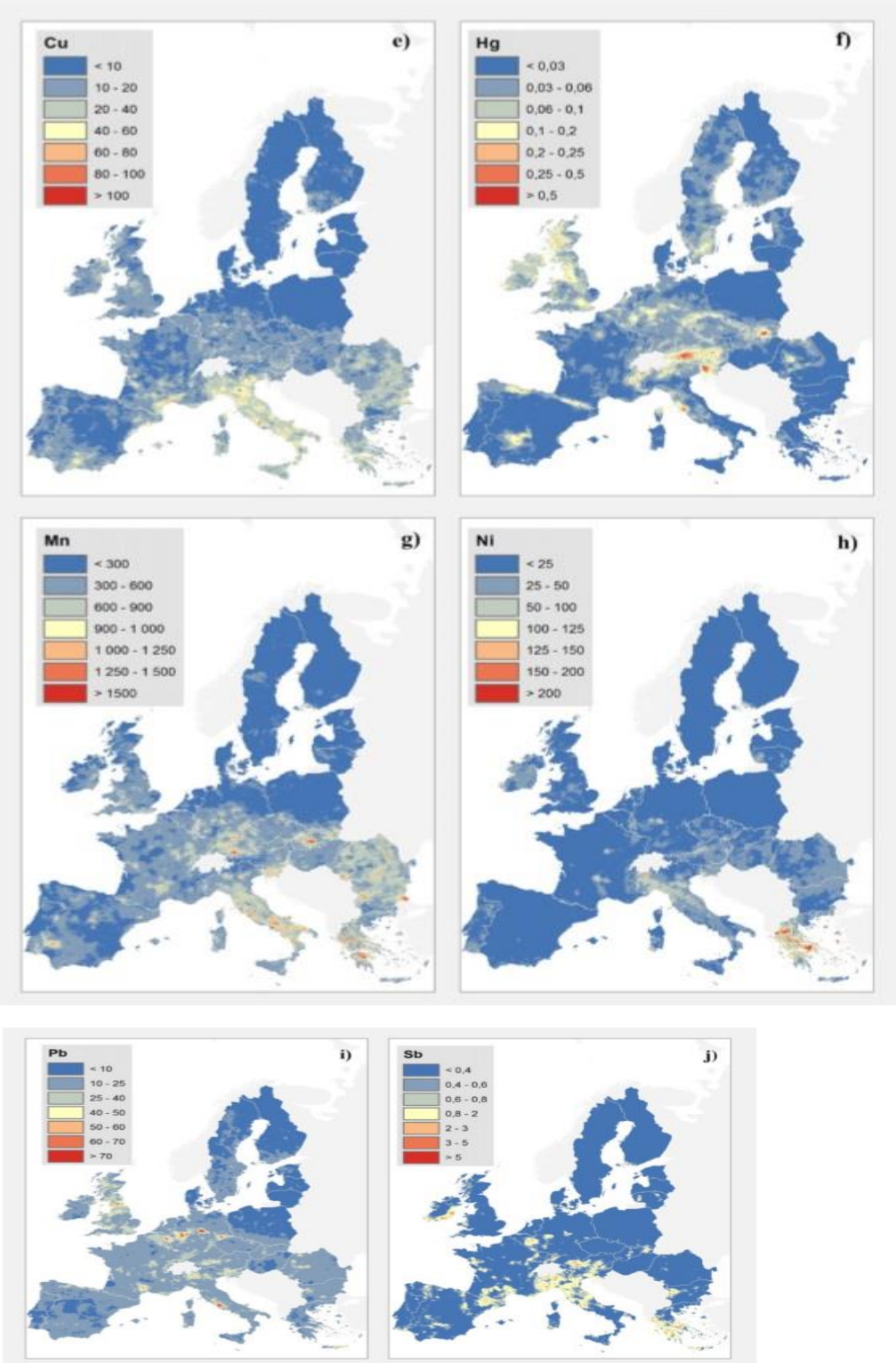


Figure 2. Concentration of some heavy metals in topsoil of European union

CONTAMINATION WITH HEAVY METALS IN SERBIA

Analysis of heavy metal content in Serbia has been performed for agricultural soils in the area of cities and municipalities affected by the floods in 2014 and 2016.

In total there were 150 tested samples. The locations from which the samples were taken are located in the river basins: Kolubara, Drina,

Sava, Western Morava, Ibar and the Great Morava.

Of the total number of samples tested, the maximum allowed concentration – MDK according to the serbian legislative, was exceeded for Ni in 104 samples, while the values Cr exceeded 33 samples and Pb in 16 samples. MDK is not exceeded for Zn and Cd in all 150 soil samples.

DETERMINATION

In order to determine concentrations of total and available concentrations of heavy metals in soils multielement analysis are being used:

ICP-AES - an analytical method using inductively coupled plasma atomic emission spectrometry. This method provides a rapid and precise means of monitoring up to 50 elements simultaneously for minor- and trace- levels.

ICP-MS - an analytical method using Inductively Coupled Plasma Mass Spectroscopy. It is a type of mass spectrometry which is capable of detecting metals and several non-metals at concentrations as low as one part in 10^{15} (part per quadrillion, ppq) on non-interfered low-background isotopes.

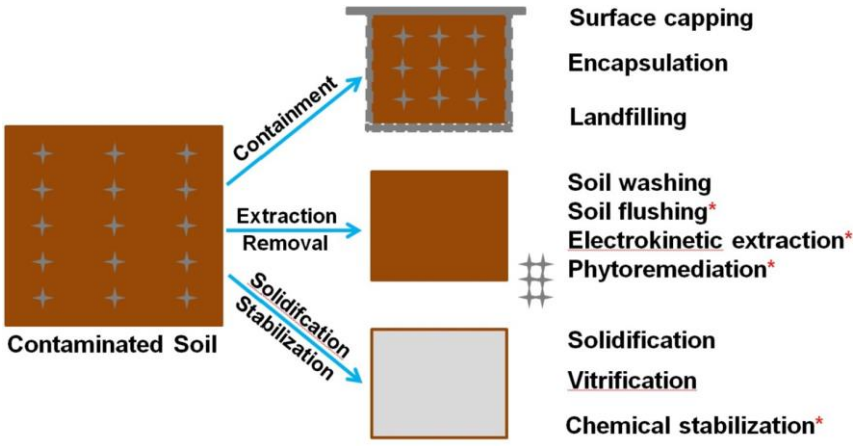
XRF - X-ray fluorescence spectrometry. It is a non-destructive analytical technique used to identify and determine the concentrations of elements present in solid, powdered and liquid samples.

REMEDIATION TEHNIQUES

It is estimated that there is around 2.5 million ha of soil contaminated with heavy metals such as As, Cd, Cr, Hg, Pb, Co, Cu, Ni, Zn, and Se, globally. Their concentrations are mostly higher than the geo-baseline or regulatory levels. In order to clean up contaminated sites two types of remediation techniques are developed: in-situ and ex-situ. These remediation techniques are based on physical, chemical, biological, electrical, and thermal remedy processes (Van Liedekerke et al., 2014).

Remediation techniques for heavy metal removal are described on Figure 3 and they include:

surface capping, encapsulation, landfilling, soil flushing, soil washing, electrokinetic extraction, stabilization, solidification, vitrification, phytoremediation and bioremediation (Figure 3).



Remediation techniques for heavy metal-contaminated soils
* Under development

(Liu et al., 2018)

In-situ soil remediation **is more cost-effective than ex-situ treatment, and contaminant removal/extraction is more favorable than immobilization and containment. Among the available soil remediation techniques all have been practice at full field scales except electrokinetic extraction, chemical stabilization, and**

phytoremediation which are at the development stage. Comprehensive assessment indicates that chemical stabilization serves as a temporary soil remediation technique, phytoremediation needs improvement in efficiency, surface capping and landfilling are applicable to small, serious-contamination sites, while solidification and vitrification are the last remediation option.

The cost and duration of soil remediation are technique-dependent and site-specific. However, before selecting feasible techniques for a soil remediation project treatability studies are necessary, with considerations of the type and degree of contamination, remediation goals, site characteristics, cost effectiveness, implementation time, and public acceptability (Liu et al., 2018).

LITERATURE

Alloway, B. J. (Ed.). (2012). ***Heavy metals in soils: trace metals and metalloids in soils and their bioavailability*** **(Vol. 22). Springer Science & Business Media.**

Liu, L., Li, W., Song, W., & Guo, M. (2018). Remediation techniques for heavy metal-contaminated soils: principles and applicability. ***Science of the Total Environment***, ***633***, **206-219.**

Ma, Y., Oliveira, R. S., Freitas, H., & Zhang, C. (2016). Biochemical and molecular mechanisms of plant-microbe-metal interactions: relevance for phytoremediation. ***Frontiers in plant science***, ***7***, **918.**

Tóth, G., Jones, A., & Montanarella, L. (Eds.). (2013). ***LUCAS Topsoil Survey: methodology, data and results***. **Publications Office.**

Van Liedekerke, M., Prokop, G., Rabl-Berger, S., Kibblewhite, M., & Louwagie, G. (2014). Progress in the management of contaminated sites in Europe. ***Reference Report by the Joint Research Centre of the European Commission***, ***1***(1), 4-6.

WEB PAGES:

(PDF) Heavy metal pollution in soil**. Available from: https://www.researchgate.net/publication/289299230_Heavy_metal_pollution_in_soil [accessed Apr 09 2019].**

http://www.sepa.gov.rs/download/Izvestaj_2017.pdf

PRACTICE 5

METAL DETERMINATION IN SOIL-ICP/OES METHOD

Reagents

ccHNO_3

hydrogen peroxide solution

Equipment

analytical balance

Teflon cuvette

ICP-OES system Thermo iCAP 6500 Duo

Microwave Milestone Ethos U

Procedure

Preparation of a soil sample to determine the content of total heavy metals involves their digestion and conversion into a solution. The samples were prepared according to the EPA 3051 method (http://www.caslab.com/EPA-Methods/PDF/EPA-Method-3051.pdf**.**

Measure 0.4 g of soil sample on the analytical balance, with an accuracy of 0.0001 g.

Weighed sample quantitatively transfer to Teflon kivets, and add 7 ml of ccHNO_3 and 2 ml of hydrogen peroxide solution to the cuvettes, seal them with protective teflon and aluminum rupture discs and finally close with teflon seals. The entire sample preparation process should take place in the fume hood.

After the incineration, centrifuge and filter the samples into normal vessels of 25 ml and fill up with ultra-pure water (conductivity 0.055 µS / cm at 25 ^{o}C, ie resistance 18.2 MΩcm at 25 ^{o}C).

Blank samples prepare as a mixture of concentrated acid and oxidizing agent, used for sample digestion, digested in the same way as samples.

WORKSHOP 2, UNIVERSITY OF SZEGED

Training 1: „Screening for potential bacterial biocontrol agents”

Trainer: Mónika Vörös

Training 2: „Screening for potential fungal biocontrol agents”

Trainer: Bettina Bóka

Task 1. Isolation of *Bacillus* and *Trichoderma* strains from soil samples

Materials: 8 Petri dishes, soil sample, 0.9% NaCl solution, 1 beaker, 5 test tubes, pipettes, vortex, water bath, glass spreader

T1 media:

0.5% glucose

0.3% yeast extract

2 % agar

after autoclaving:

nystatin stock solution, 3 mg/ml in DMSO

carbendazim stock solution, 3 mg/ml in DMSO

T2 media:

1% glucose

0.5% peptone

0.1% KH_2PO_4

0.05% $MgSO_4 \times 7H_2O$

2% agar

after autoclaving (for 100 ml):

0.1 ml 0.2% dichloran in ethanol

0.05 ml 5% Rose Bengal

0.01 % streptomycin

Method:

***Bacillus* isolation:**

Prepare soil solution from 5 g soil and 50 ml 0.9% NaCl. Make a series of ten-fold dilution in 4 steps. Incubate this series at 90 °C for 15 minutes. Streak 50-50 µl of soil solution on 1-1 Petri dishes on T1 media. Incubate these plates for a week at room temperature.

Trichoderma isolation:

Scatter 0.5-1 g soil sample on the surface of the T2 media. Incubate these 3 plates for a week at room temperature.

Evaluation:

Count the _Bacillus_ and _Trichoderma_ colony forming units (CFU). Calculate the colony numbers of the most concentrated _Bacillus_ sample.

Task 2. Separate and clean the colonies

Materials: 2 Petri dishes, inoculation loop

T1 media:

0.5% glucose

0.3% yeast extract,

2 % agar

after autoclaving:

nystatin stock solution, 3 mg/ml in DMSO

carbendazim stock solution, 3 mg/ml in DMSO

T2 media:

1% glucose

0.5% peptone

0.1% KH_2PO_4

0.05% $MgSO_4*7H_2O$

2% agar

after autoclaving (for 100 ml):

0.1 ml 0.2% dichloran in ethanol

0.05 ml 5% Rose Bengal

0.01 % streptomycin

Method:

Pick 1 separate *Bacillus* and 1 separate *Trichoderma* colony from the isolation plates. Inoculate the *Trichoderma* colony again to the *Trichoderma* selective media. Spread the bacterial colony with inoculation loop (Fig. 1). Draw zig-zag line on the media surface, then sterilize the inoculation loop and draw another zig-zag line from the first zig-zag line. Finally sterilize the inoculation loop again and draw a third zig-zag line from the second zig-zag line.

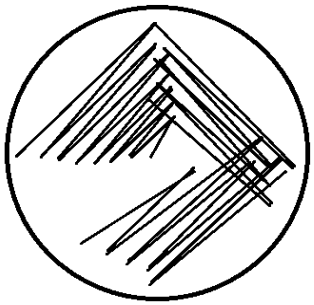


Fig. 1.

Evaluation: Pick one separate colony and repeat this process 3 times.

Task 3: DNA isolation

DNA isolation from *Trichoderma* strains

Materials: E.Z.N.A.® Fungal DNA Mini Kit, microcentrifuge capable of at least 10,000 × g, nuclease-free 1.5 mL or 2 mL microcentrifuge tubes, water bath, pestles for grinding tissue, sterile deionized water, isopropanol, 100% ethanol

E.Z.N.A.® Fungal DNA Mini Kit Protocol - Fresh or Frozen Specimens

This protocol is suitable for most fresh or frozen tissue samples. However, due to the tremendous variation in water and polysaccharide content of various fungi, sample size should be limited to ≤200 mg.

To prepare samples, collect tissue in a 1.5 or 2 mL microcentrifuge tube and freeze by dipping in liquid nitrogen with a pair of tweezers to fill the tube. Grind the tissue using disposable Kontes pellet pestles. Alternatively, one can allow liquid nitrogen to evaporate and then store samples at 70°C for later use. For critical work such as PCR and cloning, pestles are best used a single time then soaked in a dilute bleach solution immediately after use until clean. Disposable pestles may be autoclaved several times.

Before starting: heat the sterile deionized water and Elution Buffer to 65°C, prepare an ice bucket.

1. **Prepare 100 mg tissue in a 1.5 or 2 mL microcentrifuge tube.**
2. **Add 600 µL FG1 Buffer. Vortex vigorously to mix. Make sure to disperse all clumps. Note: Process in sets of four to six tubes: grind, add FG1 Buffer, then proceed to Step 3 before starting another set. Do not exceed 200 mg tissue.**
3. **Incubate at 65°C for 10 minutes. Mix sample twice during incubation by inverting tube.**
4. **Add 140 µL FG2 Buffer. Vortex to mix thoroughly.**
5. **Let sit on ice for 5 minutes.**
6. **Centrifuge at 10,000 x g for 10 minutes.**

7. Transfer supernatant to a new microcentrifuge tube, making sure not to disturb the pellet or transfer any debris.

8. Add 0.7 volumes isopropanol. Vortex to precipitate DNA.

Note: In most cases 600 µL supernatant can easily be removed. This will require 420 µL isopropanol. Note that depending on the sample, the volume of supernatant may vary. After transferring to a fresh tube, measure the volume and add the correct amount of isopropanol. This step will remove much of the polysaccharide content and improves spin-column performance by increasing DNA binding capacity (and hence yield) in the steps that follow. No incubation is required after addition of isopropanol.

9. Immediately centrifuge at 10,000 x g for 2 minutes. Longer centrifugation does not improve yields.

10. Aspirate and discard the supernatant, making sure not to dislodge the DNA pellet.

11. Invert the microcentrifuge tube on a paper towel for 1 minute to allow residual liquid to drain. It is not necessary to dry the DNA pellet.

12. Add 300 µL sterile deionized water heated to 65°C. Vortex to resuspend the pellet.

Note: A brief incubation at 65°C may be necessary to effectively dissolve the DNA.

13. Add 4 µL RNase A. Vortex to mix thoroughly.

14. Add 150 µL FG3 Buffer and 300 µL 100% ethanol. Vortex to mix thoroughly.

Note: A precipitate may form upon addition of ethanol; it will not interfere with DNA isolation.

15. Insert a HiBind® DNA Mini Column into a 2 mL Collection Tube.

Optional Protocol for Column Equilibration: 1. Add 100 µL 3M NaOH to the HiBind® DNA Mini Column. 2. Let sit for 4 minutes. 3. Centrifuge at maximum speed for 60 seconds. 4. Discard the filtrate and reuse the Collection Tube.

16. Transfer the entire sample (including any precipitate that may have formed) to the HiBind® DNA Mini Column.

17. Centrifuge at 10,000 x g for 1 minute.

18. Discard the filtrate and the Collection Tube.

19. Transfer the HiBind® DNA Mini Column to a new 2 mL Collection Tube.

20. Add 750 µL DNA Wash Buffer. Note: DNA Wash Buffer must be diluted with 100% ethanol prior to use.

21. Centrifuge at 10,000 x g for 1 minute.

22. Discard the filtrate and reuse the Collection Tube.

23. Repeat Steps 20-22 for a second DNA Wash Buffer wash step.

24. Centrifuge the empty HiBind® DNA Mini Column at maximum speed for 2 minutes to dry the membrane. Note: It is critical to remove any trace of ethanol that may otherwise interfere with downstream applications.

25. Transfer the HiBind® DNA Mini Column to a nuclease-free 1.5 or 2 mL microcentrifuge tube.

26. Add 100 µL Elution Buffer (or sterile deionized water) heated to 65°C.

Note: Smaller elution volumes will significantly increase DNA concentration but decrease yield. Elution volumes greater than 200 µL are not recommended.

27. Let sit for 3 to 5 minutes.

28. Centrifuge at 10,000 x g for 1 minute.

29. Repeat Steps 26-28 for a second elution step.

Note: Any combination of the following steps can be used to help increase DNA yield. After adding the Elution Buffer, incubate the column for 5 minutes. Increase the elution volume. Repeat the elution step with fresh Elution Buffer (this may increase the yield, but decrease the concentration). Repeat the elution step using the eluate from the first elution (this may increase yield while maintaining elution volume).

30. Store DNA at -20°C.

DNA isolation from *Bacillus* strains

Materials: microcentrifuge capable of 13,000 x g, centrifuge to pellet culture capable of 4,000 × g, nuclease-free 1.5 mL microcentrifuge tubes, water bath, shaking water bath, vortexer, 100% ethanol, 100% isopropanol

E.Z.N.A.® Bacterial DNA Kit - Centrifugation Protocol

Before starting: Set a water bath to 37°C. Set a water bath to 55°C. Set an incubator or water bath to 65°C. Heat Elution Buffer to 65°C.

1. Culture bacteria in LB media to log-phase. (Overnight culture can be used in many cases.)

2. Centrifuge no more than 3 mL culture or 1 x 10^9 cells at 4,000 × g for 10 minutes at room temperature.

3. Aspirate and discard the media.

4. Add 100 µL TE Buffer. Vortex to completely resuspend the pellet.

5. Add 10 µL Lysozyme.

Note: Lysozyme must be resuspended with Elution Buffer before use.

6. Incubate at 37°C for 10 minutes.

Note: The amount of enzyme required and/or the length of incubation may need to be modified depending on the bacterial strain used. Complete digestion of the cell wall is essential for efficient lysis. Longer incubation time may yield better results.

Optional: Follow the short protocol below for bacteria difficult to lyse.

1. Add 25 mg Glass Beads S to a 1.5 mL microcentrifuge tube.

2. Add sample to the Glass Beads S.

3. Vortex at maximum speed for 5 minutes.

4. Let sample stand to allow the beads to settle.

5. Transfer supernatant to a new 1.5 mL microcentrifuge tube.

7. Add 100 µL TL Buffer and 20 µL Proteinase K Solution. Vortex to mix thoroughly.

8. Incubate at 55°C in a shaking water bath.

Note: Usually no more than 1 hour is required for bacterial lysis. If a shaking water bath is not available, incubate the samples and shake or briefly vortex every 20-30 minutes.

9. Add 5 µL RNase A. Invert tube several times to mix.

10. Let sit at room temperature for 5 minutes.

11. Centrifuge at 10,000 × g for 2 minutes to pellet any undigested material.

12. Transfer the supernatant to a new 1.5 mL microcentrifuge tube. Do not disturb the pellet.

13. Add 220 µL BL Buffer. Vortex to mix thoroughly.

14. Incubate at 65˚C for 10 minutes.

Note: A wispy precipitate may form upon addition of BL Buffer; it does not interfere with DNA recovery.

15. Add 220 µL 100% ethanol. Vortex for 20 seconds at maximum speed to mix thoroughly. Note: If any precipitate can be seen at this point, break the precipitate by pipetting up and down 10 times.

16. Insert a HiBind® DNA Mini Column into a 2 mL Collection Tube.

17. Transfer the entire sample to the HiBind® DNA Mini Column, including any precipitate that may have formed.

18. Centrifuge at 10,000 × g for 1 minute.

19. Discard the filtrate and the collection tube.

20. Insert the HiBind® DNA Mini Column into a new 2 mL Collection Tube.

21. Add 500 µL HBC Buffer.

Note: HBC Buffer must be diluted with 100% isopropanol before use. Please see Page 4 for instructions.

22. Centrifuge at 10,000 × g for 1 minute.

23. Discard the filtrate and reuse the collection tube.

24. Add 700 µL DNA Wash Buffer.

Note: DNA Wash Buffer must be diluted with 100% ethanol before use.

25. Centrifuge at 10,000 × g for 1 minute.

26. Discard the filtrate and reuse the collection tube.

27. Repeat Steps 24-26 for a second DNA Wash Buffer wash step.

28. Centrifuge the empty HiBind® DNA Mini Column at maximum speed (≥13,000 × g) for 2 minutes to dry the column.

Note: This step is critical for removal of trace ethanol that may interfere with downstream applications.

29. Insert the HiBind® DNA Mini Column into a new nuclease-free 1.5 mL microcentrifuge tube.

30. Add 50-100 µL Elution Buffer heated 65°C.

Note: Make sure to add the Elution Buffer to the center of the HiBind® matrix. Each 50-100 µL elution typically yields 60-70% of the DNA bound to the HiBind® matrix. Two elutions generally yield ~90%. However, increasing elution volume reduces the concentration of the final product. To obtain DNA at higher concentrations, elution can be carried out using 50 µL Elution Buffer (which slightly reduces overall DNA yield). Volumes lower than 50 µL greatly reduce yields.

31. Let sit for 3 to 5 minutes at room temperature. Note: Yields may be increased by incubating the column. at 65°C rather, than at room temperature.

32. Centrifuge at 10,000 × g for 1 minute to elute the DNA.

33. Repeat Steps 30-32 for a second elution step.

34. Store eluted DNA at -20°C.

Task 4: Identification of *Bacillus* and *Trichoderma* strains

Materials: PCR tubes, pipettes, dd H_2O, Dream *Taq* polymerase, Dream *Taq* puffer, dNTP mix, primers, PCR machine, agarose, Tris–Acetate–EDTA buffer, ethidium-bromide, electrophoresis equipment

Method:

PCR was carried out in a final volume of 50 µl containing 5 µl of Dream Taq polymerase 10× buffer, 200 µM for each of the dNTPs, 10 pM primers, 100 ng of template DNA in distilled water and 1 U of Dream Taq DNA polymerase.

For *Bacillus*, use *gyrA* gene primers: *gyrA*-F 5'-CAGTCAGGAAATGCGTACGTCCTT-3', gyrA-R 5'-CAAGGTAATGCTCCAGGCATTGCT-3'. The amplification starts with an initial denaturation at 94℃ for 2 min, followed by 30 cycles of denaturation at 94℃ for 30 sec, annealing at 50℃ for 45 sec, and elongation at 72℃ for 1min, and a final extension step at 72℃ for 10 min.

For *Trichoderma* use ITS 4 and ITS 5 primers: ITS4 5'-TCCTCCGCTTATTGATATGC-3′ and ITS5 5'-GGAAGTAAAAGTCGTAACAAGG-3′. The amplification starts with an initial denaturation 94℃ -5 min, followed by 30 cycles of denaturation at 94℃ for 30 sec , annealing at 50℃ for 40 sec, and elongation at 72℃ for 1 min, and a final extension step at 72℃ for 3 min.

After PCR, separate the PCR products by electrophoresis (1% agarose gel in Tris–Acetate–EDTA buffer, containing 0,02% ethidium bromide) and investigate under UV light. After sequencing, analyze the sequence with NCBI BLAST search.

Training 3: „Enzymological characterization of potential biocontrol agents”

Trainer: Dr. Miklós Takó

Microorganisms:

1 *Bacillus* and 1 *Trichoderma* strains from the following microorganism set:

Bacillus velezensis*, *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Trichoderma harzianum*, *Trichoderma asperellum*, *Trichoderma simmonsii

Task 1: Measurement of cellulase activities

Materials:

Inductive broth, 7 100-ml Erlenmeyer flasks, 21 Eppendorf tubes, 96-well microtiter plate, centrifuge, water bath, pipettes, spectrophotometer (with microplate reader function), 2 mg/ml carboxymethyl cellulose solution in distilled water, dinitrosalyclic acid reagent, 2 mg/ml 4-nitrophenyl β-D-cellobioside solution in distilled

water, 2 mg/ml 4-nitrophenyl β-D-glucopyranoside solution in distilled water, 10% sodium carbonate solution

Inductive Broth:

1% mannitol

2% wheat bran

0.5% KH_2PO_4

0.2% $NaNO_3$

0.1% $MgSO_4$

Inoculation:

Measure 20 ml inductive broth per 100-ml Erlenmeyer flask and inoculate them with 10 µl from *Bacillus* or *Trichoderma* suspension; 1 strain/1 flask. Leave one tube as background control (microorganism-free medium).

Incubate the tubes for 4 days at 30℃.

Sample preparation:

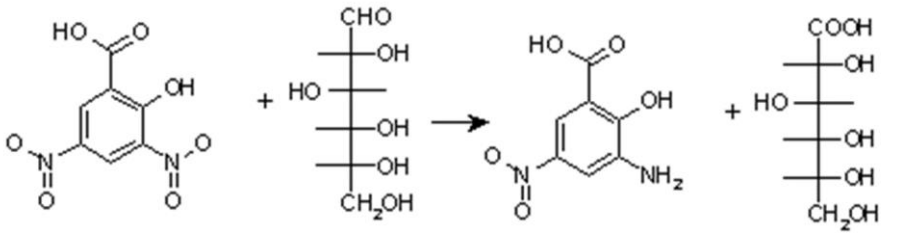
After the incubation, pipette 1-1 ml from the broths into Eppendorf tubes. Centrifuge them at 10000 rpm for 5 minutes and transfer the supernatant to a new Eppendorf tube. This clear supernatant will be used for enzyme activity measurements.

A) Carboxymethylcellulase activity (endoglucanase, CMCase)

Method:

Mix 200 µl supernatant with 200 µl 2 mg/ml carboxymethyl cellulose solution in a new Eppendorf tube. After 30 min of incubation at 30℃, add 600 µl dinitrosalyclic acid reagent to each tube and put them into hot water bath for 15 minutes. Detect the color shift from yellow to brown. To quantify the results, measure the samples at 570 nm.

Background of the reaction:



3,5-Dinitrosalicylic acid is reduced to 3-amino-5-nitrosalicylic acid in the presence of reducing sugars (here glucose and cellobiose).

Evaluation:

Visualize the CMC degrading potential of your isolates using bar graph.

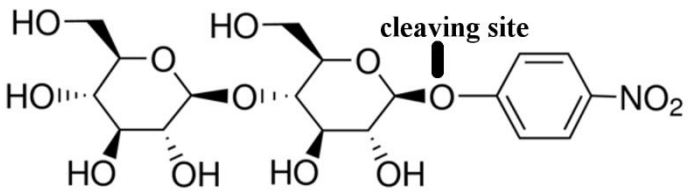
Calculation: OD corresponds to enzyme activity = OD of the sample - OD of the background control.

<u>B) Cellobiohydrolase activity</u>

Method:

Prepare 10-times dilution from the supernatant. Mix 50 µl of diluted supernatant with 50 µl 2 mg/ml 4-nitrophenyl β-D-cellobioside solution in 96-well microtiter plate. After 30 min of incubation at 30℃, add 100 µl 10% sodium carbonate solution to each tube and monitor the 4-nitrophenol release (yellow color) at 405 nm using microplate reader.

Background of the reaction:



cellobiose 4-nitrophenol

Evaluation:

Visualize the cellobiohydrolase activity (absorbance values) of your isolates using bar graph.

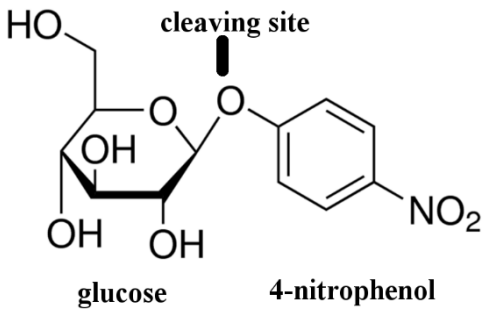
Calculation: OD corresponds to enzyme activity = OD of the sample - OD of the background control.

C) Beta-glucosidase activity

Method:

Prepare 10-times dilution from the supernatant. Mix 50 µl of diluted supernatant with 50 µl 2 mg/ml 4-nitrophenyl β-D-glucopyranoside solution in 96-well microtiter plate. After 30 min of incubation at 30℃, add 100 µl 10% sodium carbonate solution to each tube and monitor the 4-nitrophenol release (yellow color) at 405 nm using microplate reader.

Background of the reaction:



Evaluation:

Visualize the beta-glucosidase activity (absorbance values) of your isolates using bar graph.

Calculation: OD corresponds to enzyme activity = OD of the sample - OD of the background control.

Task 2: Measurement of xylanase activity

Materials:

The clear supernatant prepared after submerged fermentation on wheat, 7 Eppendorf tubes, 96-well microtiter plate, water bath, pipettes, spectrophotometer (with microplate reader function), dinitrosalyclic acid reagent, 2 mg/ml xylan solution

Method:

Mix 200 µl supernatant with 200 µl 2 mg/ml xylan solution in a new Eppendorf tube. After 30 min of incubation at 30℃, add 600 µl dinitrosalyclic acid reagent to each tube and put them into hot water bath for 15 minutes. Detect the color shift from yellow to brown. To quantify the results, measure the samples at 570 nm.

Background of the reaction:

See Task 1, part A. Reducing sugars are the xylose and xylobiose.

Evaluation:

Visualize the xylanase activity of your isolates using bar graph.

Calculation: OD corresponds to enzyme activity = OD of the sample - OD of the background control.

Task 3: Measurement of lipase activity

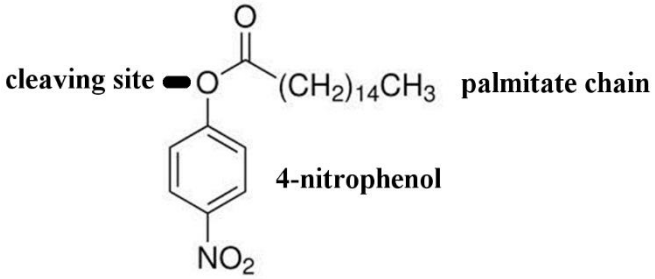
Materials:

The clear supernatant prepared after submerged fermentation on wheat bran, 1 Eppendorf tube, 96-well microtiter plate, pipettes, spectrophotometer (with microplate reader function), 4 mg/ml (3 mM) 4-nitrophenyl palmitate stock solution in dimethyl sulfoxide, 100 mM phosphate buffer (pH = 6.8), 10% sodium carbonate solution

Method:

Prepare 10-times dilution from the supernatant. Pipette 500 µl of sodium phosphate buffer to 500 µl 4-nitrophenyl palmitate stock solution. Then, mix 50 µl of buffered substrate with 50 µl of diluted supernatant in a 96-well microtiter plate. After 30 min of incubation at 30℃, add 100 µl 10% sodium carbonate solution to each tube and monitor the 4-nitrophenol release (yellow color) at 405 nm using microplate reader.

Background of the reaction:



Evaluation:

Visualize the lipase activity of your isolates using bar graph.

Calculation: OD corresponds to enzyme activity = OD of the sample - OD of the background control.

Task 4: Measurement of chitinase (exochitinase) activity

Materials:

Inductive broth, 7 half tubes, 14 Eppendorf tubes, 96-well microtiter plate, centrifuge, pipettes, spectrophotometer (with microplate reader function), 4 mg/ml (11 mM) 4-nitrophenyl N-acetyl-β-D-glucosaminide stock solution in dimethyl sulfoxide, 100 mM phosphate buffer (pH = 6.8), 10% sodium carbonate solution

Inductive Broth:

1% mannitol

0.5% colloidal chitin

0.5% KH_2PO_4

0.2% $NaNO_3$

0.1% $MgSO_4$

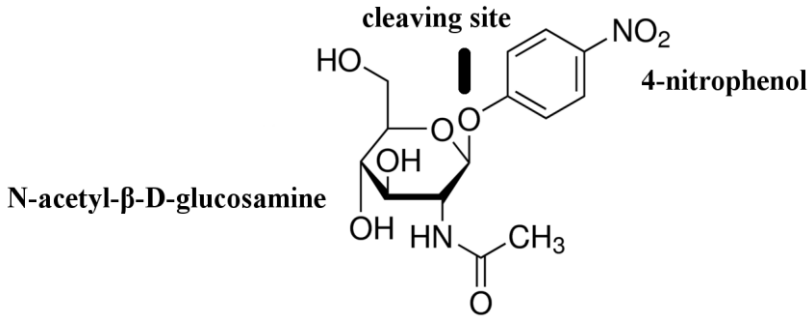
Inoculation and sample preparation:

Follow the protocol described under Task 1.

Method:

Pipette 500 µl of sodium phosphate buffer to 500 µl 4-nitrophenyl N-acetyl-β-D-glucosaminide stock solution. Then, add 50 µl of buffered substrate to 50 µl supernatant in 96-well microtiter plate. After 30 min of incubation at 30℃, add 100 µl 10% sodium carbonate solution to each tube and monitor the 4-nitrophenol release (yellow color) at 405 nm using microplate reader.

Background of the reaction:



Evaluation:

Visualize the exochitinase activity of your isolates using bar graph.

Calculation: OD corresponds to enzyme activity = OD of the sample - OD of the background control.

Task 5: Measurement of protease (chymotrypsin) activity

Materials:

Inductive broth, 1 mg/ml Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide stock solution in dimethyl sulfoxide, tube shaker, Eppendorf tubes, centrifuge, pipette, 96-well microtiter plate, spectrophotometer (with microplate reader function)

Inductive Broth:

1% mannitol

1% peptone from casein

0.1% yeast extract

0.3% NaCl

0.1% K_2HPO_4

0.1% $MgSO_4$

0.01% $MnSO_4$

0.03% $CaCl_2$

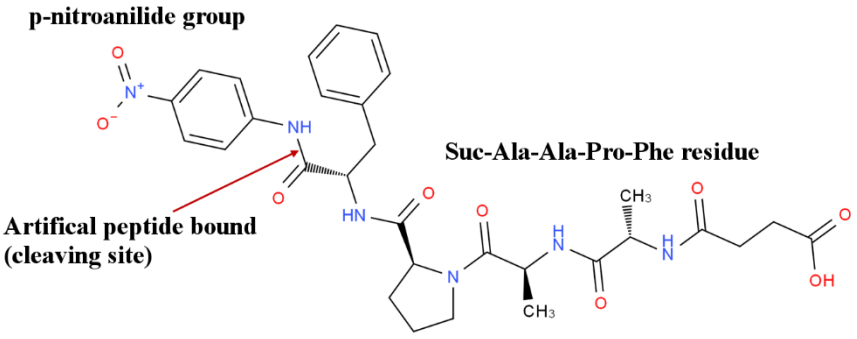
Inoculation and sample preparation:

Follow the protocol described under Task 1.

Method:

Prepare 10-times dilution from the supernatant. Pipette 100 µl of diluted supernatant and 50 µl of chymotrysin substrate stock solution into a well of a microtiter plate. After 30 min of incubation at 30℃, measure the plate at 405 nm using microplate reader.

Background of the reaction:



Evaluation:

Visualize the chymotrypsin-type protease production of your isolates using bar graph.

Calculation: OD corresponds to enzyme activity = OD of the sample - OD of the background control.

Training 4: „Advanced PCR-techniques for strain monitoring and biodiversity studies” **Trainer: Dr. Sándor Kocsubé**

Background of UP-PCR:

Conventional PCR reactions are designed to amplify a specific region of a genome. The primer pairs used in these reactions are designed based on the previous knowledge of the sequence of the target region. In contrast to gene specific PCR reactions, UP- and RAPD PCR (Random Amplified Polimorphic DNA) reactions are often carried out by using one primer instead of primer pairs. UP-PCR similarly to RAPD PCR is a fingerprinting method, which can be used to differentiate genetically very similar but not clonal isolates of a species without the knowledge on the exact sequences of their genome. The two techniques are basically the same, however RAPD PCR is generally less reproducible because of the shorter primers (10 nt, called decamers) and the low annealing temperature during amplification. UP primers are generally longer (on average 16 nt in length) and target intergenic regions with high variability of the genome, therefore it is useful for the detection of intraspecific variation. The results of the reaction can be visualized by standard gel electrophoresis. Moreover, it can be used to obtain binary matrices based on the presence and absence of the amplification products. These matrices can serve as an input for phylogenetic analysis of closely related species. Other application of UP-PCR is the development of sequence-characterized amplification region (SCAR) markers to differentiate between isolates belonging to the same species, if the UP reaction yields a unique fragment which is present only in the anding pattern of the isolate of interest. By sequencing the UP-PCR derived discrete fragment, isolate-specific

SCAR primers can be developed. These primers can be used for monitoring the presence of a specific isolate (e.g. a good biocontrol strain) in the environment.

Materials for the PCR reaction:

Pipettes (2-20 µl, 20-200 µl), tips (200 µl), microcentrifuge tubes (1,5 ml), PCR tubes (250 µl), bidistilled water, DreamTaq (Thermo Fisher Scienific) DNA polymerase, thermal cycler (PCRmax), dNTPs (1mM each), primer (0,2 µM)

Materials for visualizing the results of UP-PCR:

TAE buffer (40 mM Tris-acetic acid (pH 7,6); 1 mM Na2EDTA) 2% Agarose gel (SeaKem LE), 6× DNA Loading Dye (Thermo Fisher Scienific), GR Green DNA Stain (Excellgen), horizontal gel electrophoresis unit (Cleaver), Dark Reader blue transilluminator (Clare Chemical)

Primers used in UP-PCR

Name	Length	Seequence 5'-3'
L45	17	GTAAAACGACGGCCAGT
3-2	16	TAAGGGCGGTGCCAGT
AA2M2	16	CTGCGACCCAGAGCGG
AS15inv	17	CATTGCTGGCGAATCGG
L15/AS19	15	GAGGGTGGCGGCTAG

Task 1: Set up and run the PCR

The components of the PCR reaction mixture:

Component	20 µl reaction	Final Concentration
10X Dream*Taq* Reaction Buffer	2 µl	1X
1 mM dNTPs	4 µl 16	200 µM
1 µM Primer	4 µl 16	0.2 µM
Diluted template DNA	1 µl	<1,000 ng
DreamTaq DNA Polymerase	0,2 µl	1 units
Nuclease-free water	to 20 µl	

Steps of the PCR protocol:

Step	Temperature	Time	Number of cycles
Initial denaturation	95℃	2 minutes	1
Denaturation	95℃	20 seconds	35
Annealing	58℃	20 seconds	
Extension	72℃	40 seconds	
Final extension	72℃	2 minutes	

Task 2: Run the samples in an agarose gel and analyse the resulting banding patterns.