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Beneficial fungal microbes as novel ecosustainable tools for forage crops

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ABSTRACT
Traditional approaches to protect agricultural crops are based on the use of chemical pesticides. Nevertheless, these products are costly, contaminate the environment and are harmful to animals and humans. Reduction or elimination of chemical applications in agriculture is extremely needed, and the application of biostimulants and biological control agents is an efficient alternative for an eco-sustainable agriculture. This study demonstrated that formulations based on fungi belonging to *Trichoderma* genus had beneficial effects on forage crops and significantly affected plant metabolome.

Section: EDITORIAL

Keywords: fungi; biocontrol; Trichoderma spp.; biostimulants; metabolomics; secondary metabolites

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1. INTRODUCTION

Formulations based on microbials and/or natural compounds are able to control pest and pathogens, improve crop yield, make plants more resilient to stress and represent a valid alternative to synthetic chemicals. Biostimulant is defined as any helpful agent to plants that is neither a nutrient, pesticide, or soil improver [1]. The use of "multipurpose" fungi as biostimulants and biological control agents (BCAs) is functional to achieve the long-term and difficult goals of Europe's new sustainable agriculture strategy for crop protection [2]. The BCAs are efficient microbes that reduce diseases caused by biotic and abiotic stresses, and that exhibit antagonistic activity [3].

The traditional methods to control agricultural crops from various disease-causing agents have been based on the use of synthetic Plant Protection Products (PPP).

Nevertheless, these chemicals are costly, pollute the environment, are harmful for animals and humans and repeated application of these formulations contribute to the development of resistant microbial strains. Thus, reduction or elimination of synthetic pesticides in agriculture is extremely needed, and the use of new tools based on beneficial microbes, is one of the most promising ways to achieve this aim.

Metabolomics is the study of low-molecular weight biochemicals involved in metabolism, belongs. to the category of the omics sciences, defined as "the systematic study of small molecule metabolic products in a given organism or biological sample, in a specific point of time" [4]. Metabolomics is a useful method for analysing variations in metabolite levels in response to different environmental stimuli [5]. Since metabolism plays important role in all biological processes, metabolomics studies are increasingly being used to better understand physiological

processes associated with economically important traits in livestock [6].

Fungi belonging to the *Trichoderma* genus are widely studied and used as biocontrol agents in agriculture to reduce the application of synthetic chemicals and increase crop yield. This genus includes ubiquitous fungi that show antagonism against several phytopathogens, through different mechanisms of action, for example myco-parasitism, antibiosis, competition and induced disease resistance (indirect effect). Selected *Trichoderma* strains are active ingredients of biostimulants, bioprotectants, biofertilizers, soil amendments and integrators, biodegraders, and bioremediators. Several strains demonstrated beneficial effects on plants by acting as growth promoters or antagonists against pathogens, and by adding nutrient resources. In this study, fungal formulations based on *Trichoderma harzianum* strain M10 and T22 and a secondary metabolite produced by *Trichoderma atroviride*, 6-pentyl-α-pyrone (6PP) were selected, since M10 and T22 demonstrated beneficial effects on crops [7]-[9], and since 6PP has a role in plant growth regulation and activation of plant defence responses [10]. Spores/6PP solutions at different concentrations were tested *in vitro* and *in vivo* (field trial) on forage crops (ryegrass, oats, rye, triticale and clover), with the aim to evaluate the effects on crops growth and on their metabolic profile.

2. MATERIALS AND METHODS

Trichoderma strain M10 was obtained from the fungal collection of the Plant Pathology Laboratories (University of Naples Federico II) as liquid suspension of spores in water from which it was possible to produce biomasses for all the experiments. T22 was supplied as commercial formulation, Trianum P® from Koppert and diluted at the concentrations used for the experiments. 6PP was purified from *T. atroviride* strain P1 liquid culture [10].

A forage mixture composed of four gramineous, ryegrass, oats, rye, triticale and one leguminous, clover, was used throughout all the experiments. The seeds were treated with T22 and M10, for both *in vitro* and *in vivo* (field trial) tests, while 6PP was used only for the *in vitro* experiments.

2.1. *In vitro* plant growth promotion

Two replicates for each type of seed were coated with 10 µl of a 10^7 spore/mL suspension of M10 and T22, and with 6PP at two concentrations (10^{-5} M and 10^{-6} M). Water was used as control. The number of seeds used in the experiments varied according to the size of the seed. Specifically, 200 seeds for each replicate were coated for ryegrass and clover, 50 seeds for oats, rye, and triticale. The experiment was conducted in dark condition and at room temperature; germination rate, steams and roots length were measured daily, while fresh/dry weights were measured at the end of the experimental time.

2.2. Field Trial

The experimental field ("San Salvatore 1988" farm, Giungano SA, Italy) was divided into randomized blocks (blocks: 400 m x 8 m). An amount of 20 kg of seeds were coated to cover 1 ha of land and coated with a 10^7 spore/mL suspension of *T. harzianum* M10 or T22 (plus a natural adhesive used to improve coating), and then sown using a precision planter. Untreated seeds (not coated with spore suspension) were used as control. A second treatment with 10^7 spore/mL suspension of M10 and T22 was applied after two months using a barrel tanker and precision bars with a length of 4 m each side. Plant samples were collected for evaluation of biometric parameters and for metabolomic analysis (GC-MS).

Plant growth promotion was estimated measuring the germination rate, stem and root length and dry and fresh weight.

2.3. Metabolomic analysis

Dry plant material was grounded using mortar and pestle, 60 mg of powder were extracted by addition of three volumes of 600 µl of three solvents (methanol, dichlorometane and n-hexane). After each addition of solvent, the suspension was vortexed for 30 s and centrifuged at 12000 rpm for 10 minutes. Each supernatant was recovered and dried under gentle nitrogen flow. The total extract was then derivatized with 1 mL of N,O bis(trimethylsily)trifluoroacetamide (BSTFA). The reaction was carried out in an ultrasonic bath for 30 minutes at room temperature.

2.4. GC-MS analysis

The trimethylsilyl derivatives were analysed using an Agilent 8890 GC instrument (Milan, Italy) linked to an Agilent 5977B Inert MS. In the GC oven, an HP-5MS capillary column ((5 %-phenyl)-methylpolysiloxane stationary phase) was employed for the separation. The oven GC temperature program listed below was used: initial temperature of 90 °C, rising at 10 °C/min to 300 °C, then holding at 300 °C for 10 minutes. The solvent delay time was set at 5 minutes. The GC injector was set to splitless mode at 250 °C and the carrier gas was helium at a flow rate of 1 mL/min.

Measurements were carried out in full scan mode (m/z 35-550) with electron im-pact (EI) ionization (70 eV). The temperatures of the EI ion source and the quadrupole mass filter were fixed at 230 and 150 °C, respectively.

2.5. Data processing and statistical analysis

Identification of metabolites was obtained by comparison of deconvoluted mass spectra with collected in NIST 20 library of known compounds (National Institute of Standards and Technology). Identification was also supported by calculation of retention index (RI) for each compound, using the standard C7-C40 n-alkane mixture and the Kovats equation.

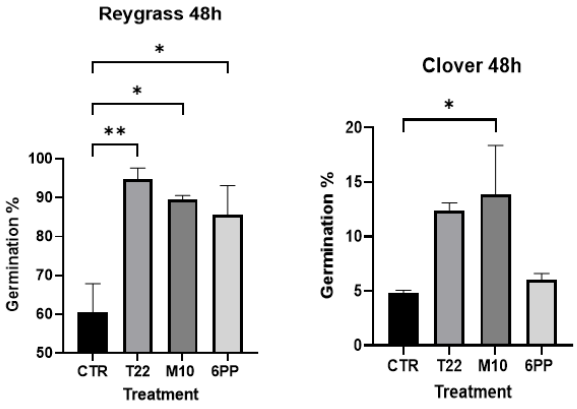

Figure 1. Germination percentage of treated seeds. Ryegrass (left) and clover (right) at 48 h. CTR: control (H2O) seeds; T22: Trichoderma harzianum T22 treated seeds; M10: T. harzianum M10 treated seeds; 6PP: Trichoderma metabolite 6PP treated seeds 10-5 M. Results are expressed as mean ± standard deviation; * for $p < 0.05$; ** for $p < 0.01$ according to Dunnett's test.

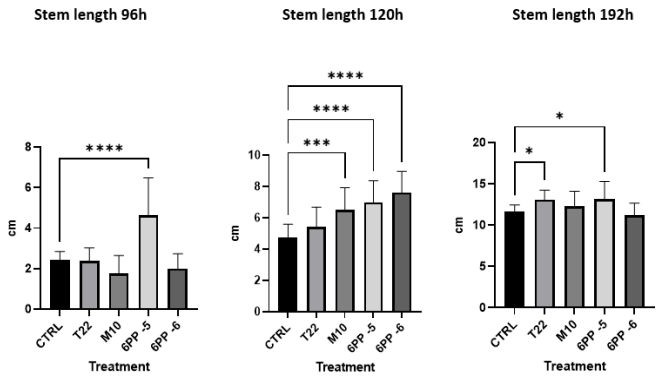

Figure 2. Oat stem length measured after 96h (left), 120h (centre) and 192h (right) for each treatment. CTR: control (H2O) seeds; T22: Trichoderma harzianum T22 treated seeds; M10: T. harzianum M10 treated seeds; 6PP: Trichoderma metabolite 6PP treated seeds. Results are expressed as mean ± standard deviation; * for $p < 0.05$; ** for $p < 001$; *** for $p < 0.001$; **** for $p < 0.0001$ according to Dunnett's test.

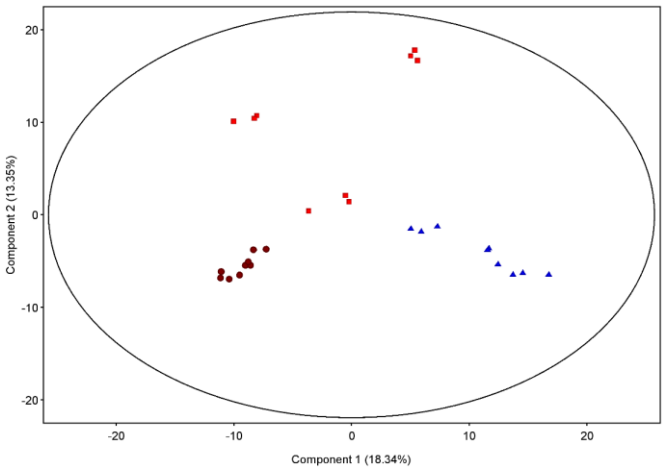

Figure 3. 2D principal component analysis (PCA) scores plot of the GC-MS dataset from plant organic extracts. Each treatment is depicted with a different colour and shape: Control (CTRL) is red squares; M10 is blue triangles; T22 is brown circles. PC1 accounts for 18.34% and PC2 accounts for 13.35% of total variance.

3. RESULTS AND DISCUSSION

In vitro germination assay showed that treatments with M10, T22, and 6PP enhanced germination rate in ryegrass after two days, whereas only M10 treatment improved germination rate in clover (Figure 1).

Plant growth promotion assay was monitored for seven consequential days to check the effects of treatments. Biometric parameters such as root length, germ length, fresh and dry weight were measured.

Despite all these parameters were monitored for each seed, only measurements of the stem length of oat were found to be statistically significant. In particular, the statistical analysis revealed 6PP 10^{-5} M promoted the development of oat stems when compared to control after 96 hours. All treatments significantly improved oat stem length at 120 hours, while only treatments with 6PP 10^{-5} M and T22 were significant at 192 hours when compared with control (Figure 2).

Treated ryegrass, rye, triticale, and clover did not show significant results compared with control samples (*Trichoderma* treated vs not treated - data not shown).

Microbial and metabolite treatments have positively influenced the germination percentage in the ryegrass and clover and increased the growth of oat seedlings. These findings are consistent with earlier studies that demonstrated how selected fungal and bacterial strains have positive effects on diverse fodder crops [6], [10], [11], [12].

For both biometric parameters and metabolomic analysis, a representative sample of the forage mixture (ryegrass, oats, rye, triticale, clover) was collected for each treatment.

Statistical analysis revealed that biometric parameters (dry/fresh weight and stem length) were not significantly different among treatments (data not shown). Moreover, no symptoms of natural diseases were detected.

Concerning the analysis of metabolic profiles, GC-MS datasets, such as molecular weights, retention times, and intensity values, were aligned and then statistically evaluated. The data were first submitted to Principal Component Analysis (PCA, Figure 3) and shown as a hierarchical cluster (Figure 4), to evaluate the formation of clusters within samples belonging to the same group. PCA scores plot (Figure 3) shows that metabolic profiles changed based on the specific treatment and a clear separation of samples in the primary component of variance was

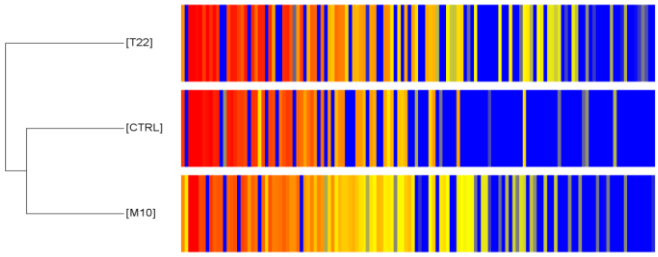

Figure 4. Hierarchical clustering heatmap of differential metabolic profiles of plants extracts. Red colour indicates higher abundance (> 0), blue colours lower (< 0), yellow a neutral change from the overall average abundance. Statistical significance was tested by one-way ANOVA ($p < 0.05$). CTRL: control (H_2O treated seeds); T22: seeds treated with Trichoderma harzianum T22; M10: seed treated with Trichoderma harzianum M10; 6PP: seeds treated with Trichoderma metabolite 6PP.

registered. A minimum spread for M10 (blue) and T22 (brown) is noted, while for the control (red) there is a spread with three subclasses of samples. In Figure 4, the hierarchical cluster obtained from metabolomic profiles of the three treatments is reported. By categorizing the data according to the treatment, hierarchical cluster analysis characterizes similarities and differences in the metabolic profiles of the sample groups (control, M10, and T22). This graph represents the normalized abundance of each compound that is present or absent in each treatment. Each line is a component with colour range from red, more abundant, to blue, less abundant. Despite having a substantial amount in common, the clustering clearly demonstrated a different accumulation of molecules in plants after applying either M10 or T22. This suggests that metabolic profile was significantly affected by the treatments in a unique way.

Specifically, the cluster shows that plants metabolic profiles of plants treated with M10 is more similar to control that to T22 treatment.

Metabolomic analysis revealed more than a hundred compounds, as a result of the data processing. Among them, several differently accumulated compounds were highlighted by univariate statistical analysis (ANOVA, $p < 0.05$ and fold change > 2.0). The different abundances of each compound depend on the treatment used and are correlated with the

Table 1. Identified metabolites that are down (↓) and up-regulated (↑) in M10 and T22 treatments versus control (CTR)

	Regulation	
Compound	**M10 vs CTR**	**T22 vs CTR**
(E)-3,7,11,15-Tetramethylhexadec-2-enoic acid	↓	↓
(R, S*)-3,4-Dihydroxybutanoic Acid	↓	↓
11-Octadecenoic acid	↓	↑
1-Octacosanol	↓	↓
1-Tetracosanol	↓	↓
1-Triacontanol	↓	↓
9H-Purin-6-ol	↑	↑
Butanoic acid	↑	↓
Callitrisic acid	↑	↑
Campesterol	↓	↑
Cholesterol	↑	↑
D-Allofuranose,	↑	↑
Diethylene glycole	↑	↓
Dodecanoic acid	↓	↓
Ergosterol	↑	↑
Erythritol	↑	↓
Glyceryl-glycoside	↑	↓
D-Allofuranose	↑	↑
Diethylene glycole	↑	↓
Isoferulic acid	↑	↓
L-5-Oxoproline	↓	↓
L-Glutamic acid	↓	↑
Lignoceric acid	↓	↓
L-Isoleucine	↓	↑
L-Threonic acid	↓	↑
Pantothenic acid	↑	↑
Pentadecanoic acid	↓	↓
Phosphoric acid monopropyl ester	↓	↓
Pyrimidine	↑	↑
Serine	↓	↑
Shikimic acid	↑	↓
β-Sitosterol	↓	↑
Octacosanoic acid	↓	↑
Tricosanoic acid	↓	↓

functional role in plant. All the differential identified metabolites are reported in Table 1; these compounds belong to several classes, including organic acids, terpenes, sterols, and vitamins, among others.

Plants possess several classes of sterols, including campesterol, which is one of the most abundant. This metabolite is a precursor of oxidized steroids, known as brassinosteroids, that serve as growth hormones for the regulation of leaf development and senescence [13]. In this study, campesterol was up-regulated in T22, while down-regulated in M10 treatment.

Callitrisic acid is a diterpene with antiviral properties. Terpenes have a variety of significant roles in the plant, including disease resistance related to the antibacterial and antifungal activities [14]. Both M10 and T22 treated plants accumulated this metabolite compared to control plants. Shikimic acid is a natural organic acid used by the plants to limit pathogen development, enhance anti-microbial chemical synthesis, and improve the synthesis of lipids, proteins, and carbohydrates. This metabolite was up-regulated in M10 and down-regulated in T22; M10-based treatment may increase the plant's defence capabilities more than T22. Shikimic acid is precursor of L-tyrosine and L-phenylalanine, and plants use them to suppress pathogen growth and promote the production of antimicrobial chemicals [15]. To increase crop protection under stress conditions plants, modify the enzymes involved in their defence responses. Pantothenic acid, also known as vitamin B5, is involved in coenzyme A (CoA) and acyl carrier protein (ACP) synthesis. This compound, in the form of CoA, regulate over 70 enzymatic pathways, such as fatty acid oxidation, carbohydrate metabolism, amino acid catabolism, heme synthesis, acetylcholine synthesis, pyruvate degradation, and phase II detoxification acetylation. Pantothenic acid was also found to help the development of green plants [16]. Both T22 and M10-based treatments induced an increase of this metabolite, compared to control.

4. CONCLUSIONS

The methods developed in this study produced significative results and provided information for the use of beneficial fungi as treatments to boost the growth of forage crops. Understanding how the microbial-based treatments, at specific concentrations, boost plant development and modify the plant metabolome is important to improve the immune response to abiotic, biotic stress and to enhance plants' nutritional value. However, it is planned to carry out at least two more microbial treatments on crops in the field before the next harvesting. Subsequently, further metabolomic analysis will be performed, first on plants and then on biological samples of buffalo calves, following the administration of the forage obtained from the produced crops.

The use of selected microbes and their effector metabolites, that are able to enhance plant fitness and help them withstand stresses, could help to implement more environmentally friendly production systems, which could lead to an improvement in nutritional parameters and an increase crop yield while minimizing the damaging effects of synthetic chemicals to the environment.

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