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Selection of redox dye and inoculum conditions for the optimisation of respirometric indices in *Verticillium* and *Trichoderma***

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Abstract. Understanding which carbon or nitrogen sources the competing fungal species prefer is pivotal for specific biotechnological applications. However, this is not straightforward, as each strain sometimes behaves differently under the experimental conditions adopted. To analyse the trophic overlap of two species, it is necessary to refine diagnostic techniques and exclude variables that may interfere with the measurements. A protocol for establishing the suitability of chromogenic dyes in the analysis of filamentous fungi with phenotype microarrays is described here. The research goal was to determine the most suitable redox dye indicator and its optimal concentration that reacts quantitatively to the respiratory activity of both *Verticillium* spp. and *Trichoderma* spp. isolates in the presence of a nitrogen source. The commercial Biolog™ Redox Dye Mixes D, E, and F and also TTC (2,3,5-Triphenyltetrazolium chloride), INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride), and MTT solutions were evaluated in selected concentrations. Both their nutritive and toxic effects were quantified. Results showed that 1% "F" and 0.5% "D" Biolog™ dyes were appropriate for the functional evaluation of isolates belonging to the *Verticillium* and *Trichoderma* genera. They can be used in Biolog™ PM-nitrogen plate respiratory assays for a comprehensive functional characterisation of these organisms.

Keywords: redox dyes, formazan, fungi, phenotype microarrays, nitrogen, metabolic profiling

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INTRODUCTION

Respiration is the process by which microorganisms consume organic matter and release carbon dioxide (CO_2) as a byproduct. This activity is a fundamental component of the microbial decomposition of organic materials and is crucial for nutrient cycling in soil and ecosystem functioning. Respiratory activity can be used as an indicator of microbial sensitivity to various environmental stimuli. Also, respiration combined with other activity indicators is used to understand how environmental conditions influence microbial metabolic activity (Zhang *et al*., 2023). Respiration-based microbial activity indicators have been applied to determine microbial efficiency in breaking down specific organic compounds or response to different environmental conditions, such as temperature and moisture levels (Li *et al*., 2023), to identify potential candidates for bioremediation or study the mechanisms of pollutant degradation (Kaushik *et al*., 2021), or individuate the most efficient strains promoting plant growth or suppressing plant pathogens, *e.g*. in the rhizosphere (Bhadrecha *et al*., 2023). In such a context, the degree of utilization of specific substrates, the effect of nutritional cofactors, the functional relationship between macronutrients such as carbon and nitrogen (Spohn, 2015), and also the synergy of specific carbon sources in determining the outcome of interspecies relationships (*e.g*., commensalism versus competition; Canfora *et al*., 2017), become information of primary importance.

In the rhizosphere, a delicate ecosystem that largely depends on plant exudates, thousands of species of fungi and bacteria compete and fight for space and resources (Broeckling *et al*., 2008). *Trichoderma* and *Verticillium* are fungi genera commonly found in soil and have important ecological roles (Akram *et al*., 2023). While there are some similarities in their ecological functionality, there are also some key differences. Both *Trichoderma* and *Verticillium* are important saprophytes that can decompose dead plant material in the soil. *Trichoderma* produces a range of extracellular enzymes, such as cellulases, hemicellulases, and ligninases, that can break down complex plant polymers, such as cellulose and lignin (Hu *et al*., 2023). *Verticillium* also produces similar enzymes for decomposition (Nuez *et al*., 2022). While some species of *Verticillium* can form mutualistic relationships with plants (Robb, 2007), this is not as well-documented as it is in *Trichoderma*. Some species of *Trichoderma* can colonise the roots of plants, where they can promote plant growth, enhance nutrient uptake or help the plants to tolerate environmental stressors such as drought (Adedayo and Babalola, 2023) or salt (Contreras-Cornejo *et al*., 2014). While *Trichoderma* and *Verticillium* can cause plant diseases (Brotman *et al*., 2010), *Verticillium* species are more well-known for their pathogenicity (Daayf, 2015). *Verticillium* species can invade the plant's vascular tissues, where they grow and produce toxins that interfere with the plant's water and nutrient transport systems, resulting in wilt symptoms and even plant death (Reusche *et al*., 2012). On the other hand, *Trichoderma* is not typically pathogenic to plants, and some species have beneficial effects on plant growth (Di Mola *et al*., 2023). There have been rare reports of *Trichoderma* causing disease symptoms in certain plant species under specific conditions (Poveda *et al*., 2020; Pfordt *et al*., 2020). Both *Trichoderma* and *Verticillium* have biocontrol activity against plant pathogens, but *Trichoderma* is more well-known for its biocontrol properties (Guzmán-Guzmán *et al*., 2023). *Trichoderma* produces a range of enzymes and secondary metabolites that can inhibit the growth of other fungi, including plant pathogens (Manzar *et al*., 2022). *Verticillium* can also produce secondary metabolites (verticillin) with biocontrol activity (Lu *et al*., 2019), but this is not as well-studied as it is in *Trichoderma*.

Fungal antagonisms provide natural and precise biotechnological weapons to protect crops from pathogens (Raaijmakers *et al*., 2009). Developing consistent and effective biological control strategies requires a comprehensive understanding of the ecological dynamics between microbial species in the rhizosphere. Berg *et al*. (2005) demonstrated, for example, the natural potential of certain *Trichoderma* strains for the biocontrol of some plant pathogenic species of *Verticillium*. The efficacy of many *Trichoderma* strains against some of the most widespread plant pathogens at the rhizospheric level is due to certain peculiarities of this genus, which can compete like a few others, both for space and nutrients (Pylak *et al*., 2019). This is due to their intense aggressiveness against other species (*Trichoderma* species are capable of mycoparasitism, antibiosis, and competition), forms of plant growth stimulation, successful defensive mechanisms, and, more generally, the ability to modify the rhizospheric microenvironment to their advantage (Tyśkiewicz *et al*., 2022).

The mechanism of action of *Trichoderma* spp. against phytopathogens that has been least studied and described in the literature is competition for nutrients (Oszust *et al*., 2020a). Effects of carbon concentration and carbon-to-nitrogen ratio on the growth and sporulation of several biocontrol fungi were previously described (Gao *et al*., 2007; Gao and Liu, 2009). Nitrogen strongly impacts fungal species' competitiveness in several ecological niches (Band *et al*., 2022). A tailored supplementation with carbon substrates was recently proposed to improve the effects of biocontrol preparations based on antagonistic bacteria (Pylak *et al*., 2021) or fungi (Oszust *et al*., 2021). These substrates are selected to positively affect antagonistic fungi included in biopreparations to implement their efficacy against plant pathogens' activity. In other words, the potential trophic niche overlap between pathogenic and antagonistic fungi increases competition in favour of the "good" fungi (Wallis, 2021).

The Phenotype MicroArray™ system (PM) can be efficiently applied to study the differential fungal use of substrates as carbon or nitrogen sources. It is a sensitive, reliable, and repeatable method based on functional fingerprinting (Cruz-Magalhaes *et al*., 2022) that also finds application in prebiotic supplement selection (Oszust *et al*., 2021; Pylak *et al*., 2021). However, carbon source-based panels have been more widely used, while nitrogen-based screening has been much less exploited, even though nitrogen is an essential element, especially in trophic competition among species (Maynard *et al*., 2017). Fungal phenotypic diversity is usually described based on differences in respiratory activity as an evidence of substrate catabolism or based on biomass production, but these parameters are rarely combined and considered together.

The increase in fungal biomass measured in the PM method can occur with the consumption of a small amount of substrate, corresponding to a condition of high metabolic efficiency.

Conversely, an increased respiratory response of a fungus combined with low biomass production can potentially indicate a stressful condition (Pinzari *et al*., 2017). This phenomenon might be an additional value in selecting prebiotic supplements. The Phenotype MicroArray™ microplates dedicated to fungi are not provided with a redox dye responsive to the respiratory activity of fungi.

In fact, redox dyes can be toxic to some fungal species, or fungi can use them as carbon or nitrogen sources for biomass production, reducing their effectiveness or altering the method's performance. This study set up and applied

a methodology to measure respiration and biomass production of filamentous fungi at the same time through the Phenotype MicroArray™ technique. In particular, we were interested in defining an appropriate protocol for selecting a redox dye without toxic or nutritional effects on the strains studied and its optimal concentration to achieve reliable respirometric staining in functional screening. In particular, respirometric dyes were tested to select the one that would allow comparing the nitrogen metabolism of *Verticillium* and *Trichoderma* strains without being used as a nitrogen source or not consistently colouring the two fungal genera.

MATERIALS AND METHODS

Three experiments were conducted on four strains of both genera *Verticillium* and *Trichoderma* (pathogen and antagonist, respectively). A first test evaluated fungi's use of chromogenic compounds as carbon and nitrogen sources. Plates were prepared with the different chromogenic substances, at different concentrations, without other organic compounds, and the growth of the fungus was measured as an increase in biomass (turbidity measure), representing the use of the dye as a nutrient source. A second test aimed to define the proper nitrogen concentration to be used with the carbon source. Finally, an experiment was set up to evaluate the toxicity of different chromogenic compounds. In this case, the fungi were inoculated into the plates with different inoculation fluids supplemented with glucose and nitrogen for optimal growth conditions in each well. The different dyes were then added at different concentrations to assess, compared with a control, any detrimental effect of the dyes on fungal development.

Four phytopathogenic isolates of *Verticillium* sp. G293/18 (V1) (GenBank: MT133324.1), G296/18 (V2) (GenBank: MT133320.1), G299/18 (V3) (GenBank: MT133319.1), G319/18 (V4) (GenBank: MT133325.1), and four antagonistic isolates of *Trichoderma* sp. G63/18 (T1) (GenBank: MT558561), G64/18 (T2) (GenBank: MT558562), G70/18 (T3) (GenBank: MW233578.1), G78/18 (T4) (GenBank: MW205829.1) were tested as biological replications. These isolates were previously characterised (Malarczyk *et al*., 2020; Pytak *et al*., 2020; Oszust *et al*., 2021). Isolates were also identified at the species level using Whole Genome Sequencing (WGS) as following: G296/18 *Verticillium dahliae*, G63/18 *Trichoderma citrinoviride*, G64/18 *Trichoderma atroviride*, G70/18 *Trichoderma citrinoviride*, G78/18 *Trichoderma pseudokoningii* (data not published). Fungal isolates were cultured on a PDA medium (Potato Dextrose LAB-AGAR, Biomaxima, Lublin, Poland) until conidia spores were produced. Spores were separated from the mycelium using sterile filter bags (BagPage®100, Bionovo, Legnica, Poland) and then used to prepare inoculation suspensions.

The strains listed here and used in the experiment are part of the project EcoFruits BIOSTRATEG3/344433/16/NCBR/2018 and are currently stored in the microbial strains collection of the Institute of Agrophysics, Polish Academy of Sciences, Lublin, Poland. Some tested isolates are patent pending, but others are used in research.

Six redox dyes: Biolog™ Redox Dye Mixes D, E, and F (marked further as D, E, and F Biolog™ dyes, respectively), 2,3,5-Triphenyltetrazolium chloride (TTC, Tetrazolium chloride) (Chemat, Gdańsk, Poland), 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium (INT, Iodonitrotetrazolium chloride) (Chemat, Gdańsk, Poland), and 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT, Thiazolyl blue tetrazolium bromide) (Chemat, Gdańsk, Poland) were tested. Different concentrations of each dye were applied in both nutrition and toxicity tests.

The nutrition test was performed in 0.9% NaCl, with dyes used as the only carbon and nitrogen sources. Sterile transparent 96-well microplates (Nest Scientific™, Woodbridge, USA) were inoculated with 180 µl of fungal spores suspended in 0.9% NaCl (73% Transmittance). 20 µl of 10x concentrated dyes were added and mixed to obtain final concentrations of 0.1%, 0.5%, 1% and 2% of D, E, F Biolog™ dyes, or 0.1%, 0.5% and 1% of MTT, TTC and INT dyes. The controls for each dye were the trials with no dye addition (20 µl 0.9% NaCl). The 10x concentrated stocks of D, E, and F dyes were prepared using 0.9% NaCl, while MTT, TTC, and INT dyes were prepared using 0.1 M HCl 10% Sodium Dodecyl Sulfate (SDS) solution. All ingredients were filtered-sterilised through a syringe filter (the size of pores 0.22 µm). All analyses were performed in three technical replications. The microplate cultures with added dyes and controls were incubated in the dark for ten days at 24°C. Every 24 h, fungal functional responses to dyes were measured. Absorbance readings (optical density) were obtained using MicroStation™ (Biolog™, Hayward, USA) at 750 nm.

To evaluate the effect of nitrogen concentration on fungal development in the plate wells, we selected a mineral nitrogen source to be administered at different concentrations. Ammonium nitrate (NH_4NO_3) (Sigma Aldrich™, Saint Louis, Missouri, USA) was used in final concentrations of 0.05, 0.1, 0.5, 1 and 0% (the control) in three different liquid media: PM3,5-8 inoculating fluid (PM) according to Biolog™ (Hayward, USA) preparation protocol, IF-FF inoculating fluid (FF) (Biolog™, Hayward, USA) and 0.9% NaCl (NaCl). The final concentration of other ingredients in all the microplates was as follows: D-glucose 100 mM, potassium phosphate (pH 6.0) 5 mM, sodium sulfate 2 mM.

Sterile transparent 96-well microplates were inoculated with a volume of 90 µl of fungal spores already suspended on the chosen liquid medium (73% T) and 10 µl of 10x concentrated NH_4NO_3 also suspended in the same media. The total volume of 100 µl was mixed thoroughly by pipetting. Analyses were performed in three technical replications,

and all ingredients were filter-sterilised. The inoculated microplates were incubated in the dark for ten days at 24°C. Every 24 h, turbidity at 750 nm wavelength, corresponding to biomass quantification, was measured using MicroStation™ (Biolog™ Hayward, USA).

The toxicity test was performed with the PM3,5-8 inoculating fluid (PM) medium with added 0.1% NH_4NO_3 the different redox dyes separately. Sterile transparent 96-well microplates were inoculated with 180 µl of fungal spores' suspension in 0.1% NH_4NO_3-PM medium (73% Transmission). 20 µl of 10x concentrated dyes were added to each well and mixed to obtain final concentrations of 0.1 0.5, 1, or 0.01 0.05 and 0.1% for MTT and INT. The 10x concentrated stock solutions of D, E, and F Biolog™ dyes were prepared using 0.9% NaCl, while MTT and the INT with a 0.1 M HCl 10% Sodium Dodecyl Sulfate (SDS) solution. Analyses were performed in three technical replications. The plates inoculated with the fungi and the different dyes were incubated in the dark for ten days at 24°C. Absorbance readings were performed every 24 h using MicroStation™ (Biolog™, Hayward, USA) at 490 nm and 750 nm wavelengths, thereby obtaining both biomass (turbidity) and chromogenic compound colouration measurements based on respiratory activity.

Analysis of variance (ANOVA) followed by Tukey's *post hoc* honestly significant differences (HSD) test at $p<0.05$ were used to compare the treatments. Statistica 13.1 software (StatSoft®, Tulsa, Oklahoma, USA) was used. The analysis was performed by reading the plates every hour during a 216 h incubation.

Principal component analysis (PCA) and discriminant analysis (DA) techniques were applied to the biomass dataset obtained by measuring the growth of all the *Verticillium,* and *Trichoderma* isolates with increasing concentrations of nitrogen (NH_4NO_3). PCA was used to summarise the variability associated with incubation time (24 to 216 h) and to reduce the number of variables for further statistical analyses (Massart *et al*., 1998). The factor scores obtained for the first principal components (PCs) resulting from PCA were then used to run DA (XLSTAT 2019.3.2, Addinsoft, New York, USA), using first the strains and then the nitrogen concentrations as *apriori* grouping factors. The DA was applied to verify: 1) whether the response to nitrogen concentration may outweigh differences in nitrogen use by individual strains; 2) whether, in the face of different nitrogen concentrations, individual strains were still distinguishable based on their growth.

RESULTS

The nutritional effects of selected redox Biolog™ dyes (Fig. 1a) and other dyes (MTT, INT, TTC) (Fig. 1b) on fungal growth in 0.9% NaCl medium was documented for 0.5 and 1% of all dye types, with 0.1 % of F and TTC, and 2% of F dye. After adding 0.1% D, E, MTT, and INT dyes, no fungal growth observed. Since, instead, the TTC dye addition caused a clear nutritional effect at all tested concentrations, TTC was excluded from the following experiments.

Three media were compared with four nitrogen concentrations each. Figure 2 shows biomass production in the different media for *Verticillium* spp. and *Trichoderma* spp. isolates, with different NH_4NO_3 concentrations. When comparing media, the largest growth of fungi was observed

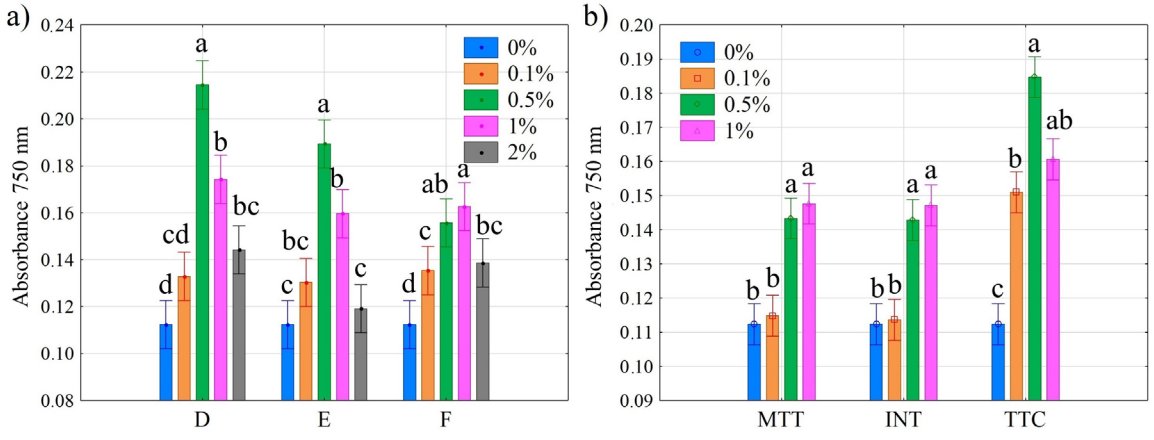

Fig. 1. Nutritive effect of selected redox dyes on fungal growth in 0.9% NaCl medium. Different dye concentrations were tested at 750 nm absorbance readings: a) Biolog™ redox dyes; b) other dyes. Redox dyes D, E, and F Biolog™ (marked as D, E, and F, respectively), and 2,3,5-Triphenyltetrazolium chloride (TTC, Tetrazolium chloride), 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium (INT, Iodonitrotetrazolium chloride), and 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT, Thiazolyl blue tetrazolium bromide). Statistics: ANOVA with Tukey's *post hoc* honestly significant differences (HSD) at $p<0.05$; mean values and standard deviation bars are presented; $n=3$; different small letters above bars indicate significant differences among different nitrogen concentrations for each redox dye type.

on the PM medium. The highest optical density values (at 750 nm) were found for 0.05, 0.1 and 0.5% nitrogen concentrations, and these were significantly ($p<0.05$) higher than 1 and 0% (the control). As expected, NaCl medium alone supported the most negligible biomass production. The 0.1% nitrogen concentration produced the greatest fungal biomass values in all tested media (Fig. 2).

Figure 3 shows the fungal growth rate during two weeks of turbidity measurements recorded every 24 h. The 0.1% NH_4NO_3-PM medium produced the highest biomass. These results allowed us to preliminarily point to the 0.1% NH_4NO_3-PM medium as the best model for the toxicity tests.

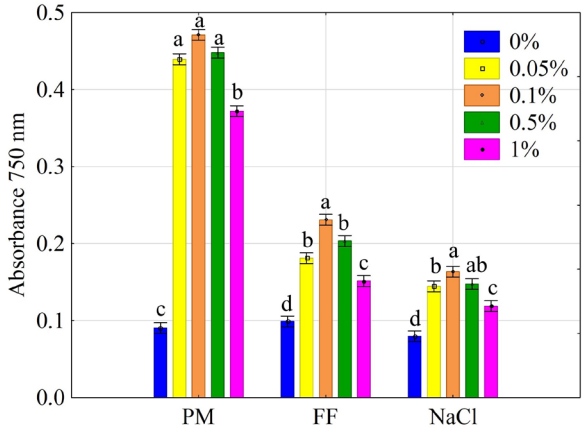

Fig. 2. Biomass production by *Verticillium* spp. and *Trichoderma* spp. isolates in the different media and NH_4NO_3 concentrations. Values were obtained as absorbance readings at 750 nm optical density. Statistics: ANOVA with Tukey's post hoc honestly significant differences (HSD) at $p<0.05$; mean values and standard deviation bars are presented; $n=3$; different small letters above bars indicate significant differences among different nitrogen concentrations for each medium type. Explanations: PM3,5-8 inoculating fluid (PM), IF-FF inoculating fluid (FF) and 0.9% NaCl (NaCl).

Figure 4 presents the diversified performance of *Verticillium* spp. (V1-V4) (Fig. 4a) and *Trichoderma* spp. (T1-T4) (Fig. 4b) individual isolates in terms of biomass production (based on absorbance readings at 750 nm) at different NH_4NO_3 concentrations in the PM medium. The two plots were obtained by reducing the time variable (daily absorbance readings) with a PCA and using the new principal coordinates in a discriminant analysis (DA).

Principal component analysis reduced the variance of the dataset due to readings over time (24-216 h of incubation, with readings every 24 h). The first four new orthogonal components explained more than 97% of the total variance and were used in discriminant analysis to compare the data clustering based on the nitrogen concentration and the fungal isolate.

The discriminant analysis produces a predictive model for membership in the apriori-defined grouping. It allows an assessment of whether the data succeeded in separating the observations into the initially assigned groups. The model consists of a set of discriminant functions (one for each group to be identified) based on linear combinations of the predictor variables that provide the highest or best discrimination between groups. In both *Verticillium* and *Trichoderma* datasets, each DA successfully separated the different fungal isolates. Conversely, the different concentrations were not grouped, indicating that the amount of nitrogen is not a determining variable in the dataset. In the DAs shown in Fig. 4c and 4d, the only group classified as 100% (all the observations initially assigned to that group resulted in a correct classification) in both datasets is the 0% nitrogen group. The 0.1% concentration was the second better classified (75% in the *Verticillium* dataset, 50% in the

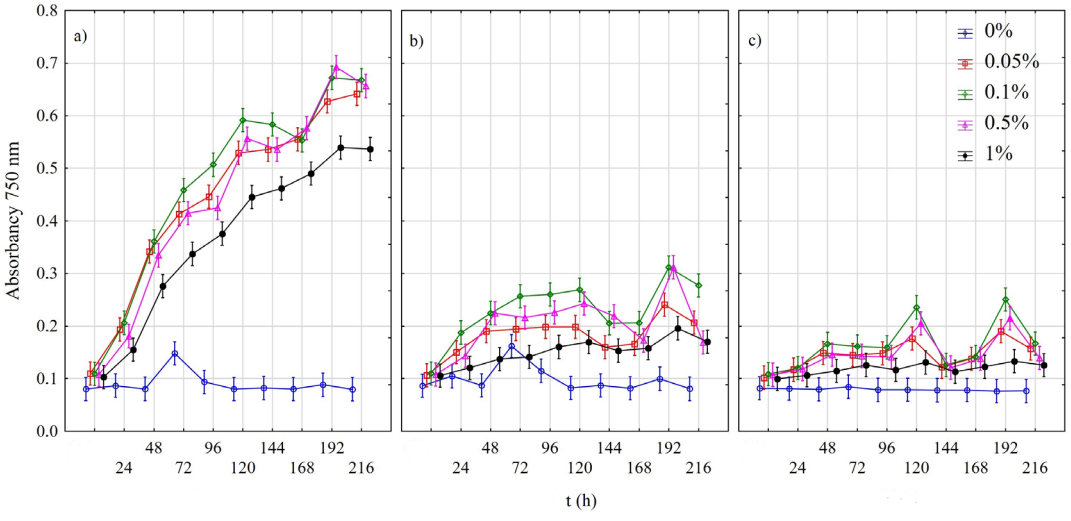

Fig. 3. Fungal growth rate within the 24 h in selected media of all tested *Verticillium* spp. and *Trichoderma* spp. isolates depending on the NH_4NO_3 concentrations based on absorbance readings at 750 nm: a) PM – PM3,5-8 inoculating fluid, b) FF – IF-FF inoculating fluid and c) NaCl – 0.9% NaCl.

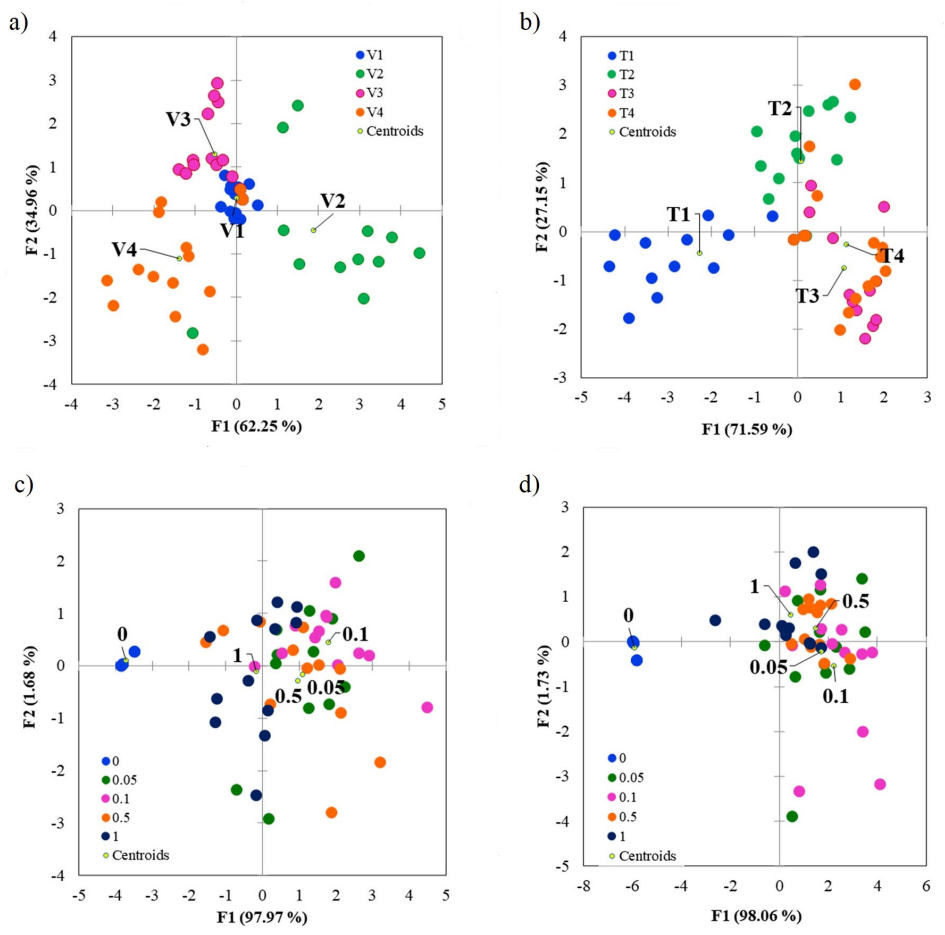

Fig. 4. Discriminant analysis plots were obtained using the first four components of a PCA that reduced the variable "incubation time". The diversity of: a) *Verticillium* spp. (V1-V4) and b) *Trichoderma* spp. (T1-T4) individual isolates in terms of biomass production (based on absorbance readings at 750 nm) depending on their reaction to different NH_4NO_3 concentrations in PM medium. The different concentrations of nitrogen supplemented to the isolates did not outweigh nitrogen use by individual strains. For both: c) *Verticillium* and d) *Trichoderma* strains, the different nitrogen concentrations did not affect fungal growth, as can be seen by the absence of clustering in the discriminant analysis according to the concentration used.

Trichoderma one). In the case of the DAs in Figs 4a and 4b, the different isolates showed varying classifications, with V1 classified at 93.33%, V4 and T2 at 80%, and the least well-classified being the T4 (26.67% classification, meaning that many biomass observations belonging to this fungal isolate were assigned to other isolates).

Once the most suitable inoculum fluid and the nitrogen concentration capable of providing greater reproducibility were identified, the effect of adding the different dyes on fungal functionality was evaluated. Figure 5 illustrates the toxic effect of different redox dyes on fungal growth in a medium with 0.1% nitrogen substrate (NH_4NO_3). In particular, the effect of different dye concentrations on absorbance values at 490 nm (Fig. 5a and b) and 750 nm (Fig. 5c and d) is shown.

Similar behaviour was observed for all redox dyes provided by Biolog$^{TM.}$ with no apparent toxic effects. Biomass production and respiratory activity were higher with dye E compared with dyes D and F.

Figure 6 shows that colour development was obtained for all redox dyes marketed by Biolog^{TM}, while no staining was observed for MTT and INT dyes (Table 1 shows the plate inoculation scheme). Significant differences in colour development were found between the different isolates of both *Trichoderma* and *Verticillium*, especially for the former. In addition, *Trichoderma* T2 and T3 isolates showed different sporulation capacities depending on the redox dye used and its concentration (Fig. 5).

DISCUSSION

Tetrazolium salts are compounds used to measure the redox activity of metabolically active cells (Braissant *et al.*, 2020). They are chemically different molecules that are initially colourless but, in the presence of metabolically active cells, are reduced to red-violet formazan derivatives by reduced nicotinamide adenine dinucleotide (NADH)-dependent oxidoreductases and dehydrogenases or phosphorylated derivatives (NADPH). There are various chemical

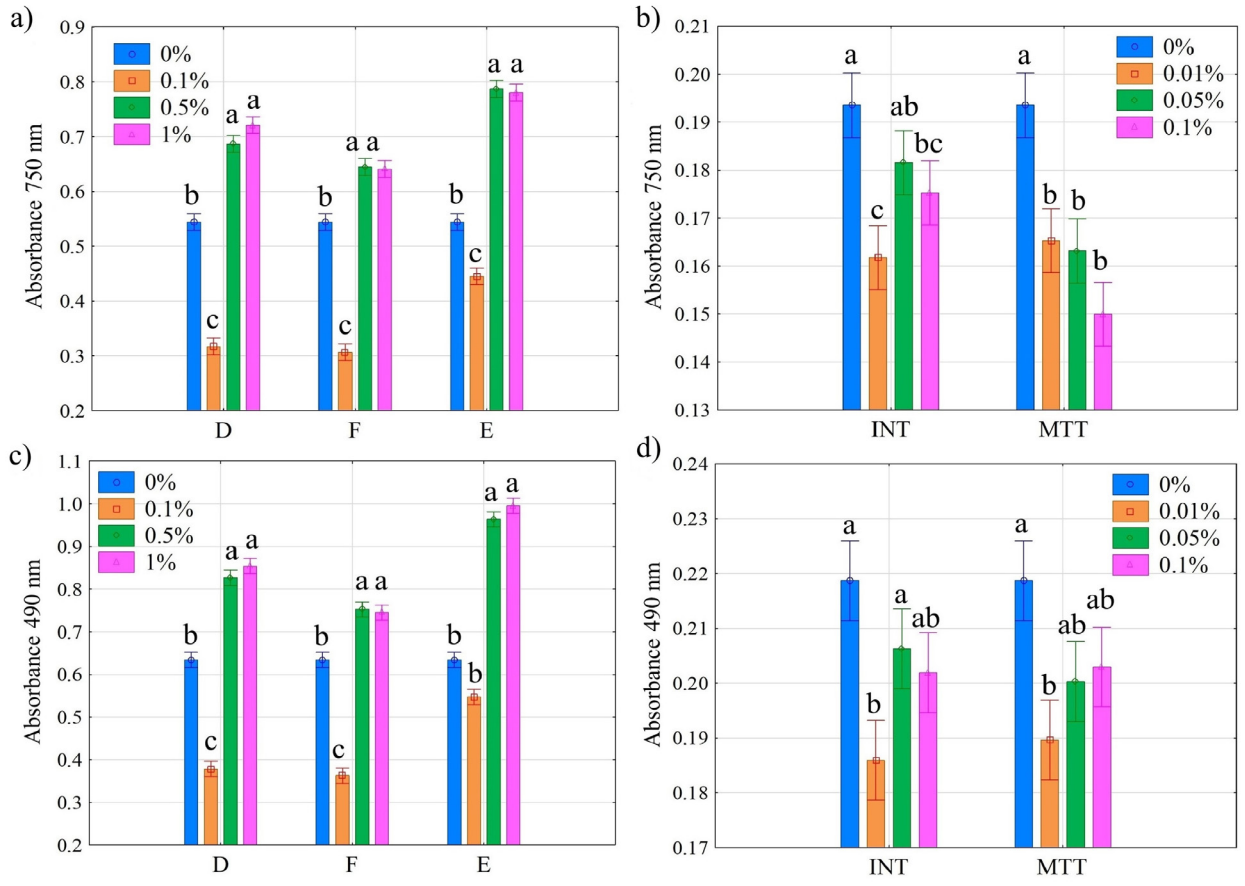

Fig. 5. Toxicity effect of selected redox dyes on fungi in 0.1% NH_4NO_3-PM medium, based on (a-b) biomass production on different dyes concentrations (750 nm absorbance readings) and (c-d) respiration (490 nm absorbance readings). Redox dyes D, E, and F Biolog™ (marked as D, E, and F, respectively), and 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium (INT, Iodonitrotetrazolium chloride), and 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT, Thiazolyl blue tetrazolium bromide). Statistics: ANOVA with Tukey's *post hoc* honestly significant differences (HSD) test at $p<0.05$; mean values and standard deviation bars are shown; $n=3$; different small letters above bars indicate significant differences between different nitrogen concentrations for each redox dye type.

forms of tetrazolium salts, and many of them cannot penetrate through the walls of fungi or react in the presence of their dehydrogenases, while most tetrazolium salts penetrate bacterial cells (Braissant *et al*., 2020). The analysis of microbial cell metabolism and phenotype profiling employing sensitivity tests to various compounds, especially nutrients, is a powerful and rapid approach that allows for the functional differentiation of morphologically similar microbial strains without relying on more time- and resource-consuming genetic methods (Acin-Albiac *et al*., 2020). Phenotypic profiling is based on the use of tetrazolium salts and, to a lesser extent, on turbidity measurements, *i.e*. the development of biomass by the microorganism, which can be measured by spectrophotometry. However, using metabolic profiling with fungi presents more difficulties than for bacteria. Fungi can degrade complex organic compounds with extracellular enzymes such as laccase, manganese peroxidase and lignin peroxidase (Przystas *et al*., 2015; Singh, 2017). These fungal abilities are often applied to decolourise and detoxify effluents treated with azo dyes (*e.g*., from textile industries) (Sen *et al*., 2016). Moreover, formazans have a structure with 4 nitrogen (N) atoms: [-N=N-C(R)=N-NH-] and are closely related to azo dyes with amine nitrogen (−N=N−) (Sen *et al*., 2016). Thus, fungi can decompose formazan derivatives and use nitrogen for sustenance, making fungi's metabolic profiling unreliable. Furthermore, redox dyes could be toxic to some fungal isolates (Bayineni, 2022).

This study aimed to define the best experimental approach to analyse the metabolic profile of filamentous fungi employing the Phenotype MicroArray™ technique. In particular, we were interested in selecting redox dye without toxic or nutritional effects on the strains studied and testing its optimal concentration to achieve reliable respirometric staining in functional screening. Specifically, we were also interested in selecting a respirometric dye that would allow us to compare the nitrogen metabolism of *Verticillium* and *Trichoderma* strains without itself being used as a nitrogen

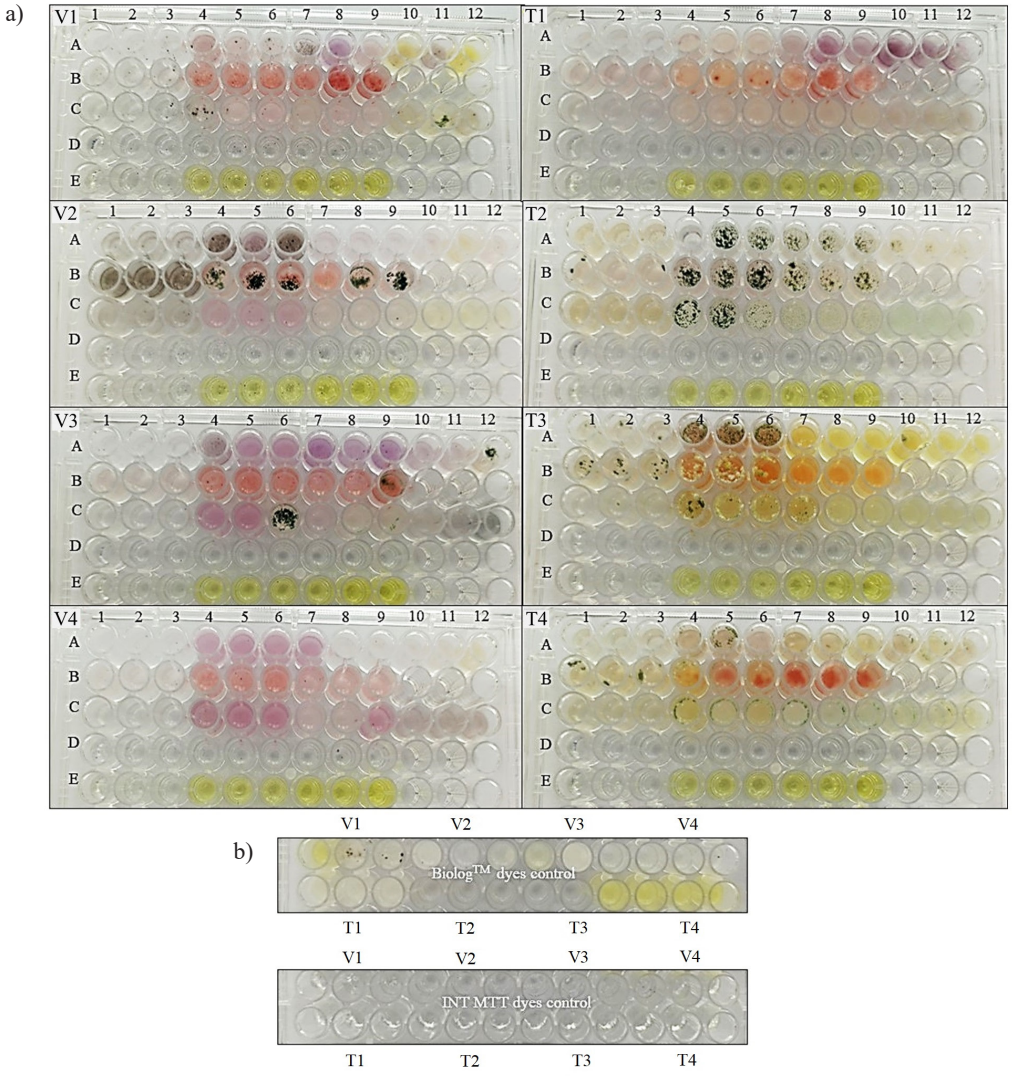

Fig. 6. Colour formation in the toxicity dyes test was carried out in a 0.1% NH_4NO_3-PM medium. *Verticillium* spp. (V1-V4) and *Trichoderma* spp. (T1-T4) isolates, Biolog™ redox dyes D, E, and F (marked as D, E, and F, respectively), and 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium (INT, Iodonitrotetrazolium chloride), and 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT, Thiazolyl blue tetrazolium bromide) with their different concentrations; a) fungi + dyes, b) controls - fungi without dyes; n=3. For dyes arrangement in a particular plate, see Table 1. Photos were taken at the end of the incubation (216 h).

source or differently colouring the two fungal genera. The experimental work consisted of testing and selecting chromogenic molecules that were not a source of nutrition and, at the same time, were not toxic for the fungi. If there is little or no carbon source available, the fungus tends to use the dye more as a nutritional source. In the nutritional test, it was necessary to evaluate the chromogenic compounds' performance without other carbon or nitrogen sources. Therefore, we tested fungal biomass development in the presence of the chromogenic compound alone as evidence of the fungus's ability to metabolise it. The experiment used a simple saline solution as the inoculation fluid, with no other nutrients but the dye. The absence of biomass production by the fungus was considered a positive result, that is, the inability of the fungus to use the dye to grow.

In contrast, in the toxicity test, the expected positive result was that the dye would not significantly inhibit fungal growth in the culture medium, where biomass production should be observed to the same extent as without the dye. Therefore, this test used a culture medium to ensure optimal fungal growth. In addition, since the culture medium must also be suitable for absorbance and turbidity measurements in microplates, it must also meet translucency conditions.

For inoculum preparation, the FF inoculum fluid proposed by Biolog™ for filamentous fungi (Oszust *et al*., 2020; Pertile *et al*., 2020) and the PM3.5-8 inoculum fluid (Joseph *et al*., 2021) that, as previously demonstrated, are adequate for the evaluation of fungal response in microplate functional assays, were compared.

Table 1. Toxicity dyes test schema

V/T(x)	Dye	Concentration (%)								
		1	2	3	4	5	6	7	8	9
A	D	0.1	0.1	0.1	0.5	0.5	0.5	1	1	1
B	E	0.1	0.1	0.1	0.5	0.5	0.5	1	1	1
C	F	0.1	0.1	0.1	0.5	0.5	0.5	1	1	1
D	INT	0.01	0.01	0.01	0.05	0.05	0.05	0.1	0.1	0.1
E	MTT	0.01	0.01	0.01	0.05	0.05	0.05	0.1	0.1	0.1

Dye doses are given in %, V/T (x) - *Verticillium* spp./*Trichoderma* spp. (isolate acronym); Biolog™ redox dyes D, E, and F (marked as D, E, and F, respectively), and 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium (INT, Iodonitrotetrazolium chloride), and 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT, Thiazolyl blue tetrazolium bromide) with their different concentrations; n=3.

In addition to the inoculum fluid and the type of indicator compound, it was necessary to test different concentrations of a nitrogen source. Ammonium nitrate (NH_4NO_3) was chosen as the model compound for fungal nitrogen nutrition (Oszust *et al*., 2021).

The experiments were conducted in sterile transparent 96-well flat-bottom plates. Compared with commercial plates containing only the chromogenic compound (MT2, Biolog™), the setup chosen in this experiment allowed simultaneous manipulation of nutrient concentrations and redox dye type (Oszust *et al*., 2018).

Another critical element that had to be considered in the experimental setup was the type of solvent needed to dissolve the different redox dyes. For MTT and INT, SDS-0.01M HCl was used as a solvent, according to Septisetyani *et al*. (2014). Biolog™ dyes are sold in formulations readily soluble in water, as Touchette *et al*. (2022) described.

Finally, the wavelengths used to measure dye formation and biomass production were 490 nm and 750 nm, respectively. They were chosen because they have previously been shown to provide repeatable results for functional comparison between *Trichoderma* spp. and *Verticillium* spp. isolates (Oszust *et al*., 2020b). In this regard, absorbance values at 490 nm reflect the respiration rate, corresponding to substrate utilisation, while the absorbance value at 750 nm measures biomass production (growth pattern) (Oleszek *et al*., 2019; Pinzari *et al*., 2017).

As for the toxic effect of dyes MTT and INT, the general trend was that all proposed doses (0.01, 0.05 and 0.1%) significantly limited fungal growth compared to the control (without dye). This indicated that MTT and INT dyes are toxical to the *Verticillium* spp. and *Trichoderma* spp. isolates tested so far, regardless of the culture medium used. The manufacturer's protocol for commercial phenotype microarray (PM) plates (*e.g*., FF plates) considers A≥0.25 as positive values absorbances (Oszust and Frąc, 2021; Chou *et al*., 2022; Frąc *et al*., 2022). MTT and INT did not meet the requirements of redox dyes for the PM technique. Conversely, the Biolog™ 0.5% D and 1% F dyes were reliable indicators for PM-nitrogen plate measurements, even after long incubations (*e.g*., 216 h). The duration of incubation and, thus, the consistency of redox compounds' staining in repeated respiration measurements is an indispensable factor in the case of filamentous fungi compared to bacteria and yeasts. This is because the growth of fungi in phenotype microarrays can be slow, and measurements typically have to be protracted over a week, with daily readings, unlike bacteria that can reach full-colour development in 24-48 h (Pinzari *et al*., 2016).

Differences in colour development in the wells result from a sum of factors, among all the ability of the fungus to biodegrade or biotransform the redox dye, the type of enzymes and species-specific processes in the respiratory chain, or the influence of extracellular enzymes on the mechanism of oxidoreduction of the dye compound (Oszust and Frąc, 2021).

Furthermore, in the face of an initial formation of colour in the wells indicating respiratory activity, appreciable later discolouration of the wells by degradation of the formazan precipitated in the mycelium has sometimes been observed, especially during prolonged incubations. The discolouration by the fungus of the redox dye may influence and mislead the interpretation of results in such studies (Lasinio *et al*., 2021). Phenotype Microarrays experiments must rely on a robust protocol, not affected by the toxicity or nutritional effect of the dye or the biodegradability of the coloured compound. Reliable indicators are a necessary condition in the study of metabolic processes in fungi using redox respirometric compounds.

The described experiment selected the most suitable tetrazolium salt and its concentration for a robust and reliable respirometric analysis of fungal strains in *Verticillium* and *Trichoderma* genera. Among the formulations and chemical compounds tested, the 1% F Biolog™ and 0.5% D dyes were shown to form stable staining in both *Verticillium* and *Trichoderma* trials without giving rise to toxicity phenomena and without being used as a nitrogen source by the fungi. This makes them suitable for studying

the nitrogen metabolism of these species. Furthermore, in the presence of a nitrogen source at different concentrations, these indicator compounds provided proportional and consistent colourimetric responses, allowing quantitative and repeatable measurements of fungal respiration and biomass development.

The use of Phenotype Microarray™ plates produced by Biolog™ with filamentous fungi is problematic compared to bacteria due to some factors related to the fungi's mode of development and the complexity of their metabolism (Pinzari *et al*., 2016). The measurement of the metabolic profile of bacteria is based on the development of colour in the wells, which is directly considered a response to substrates use in the wells. In the case of fungi, the only reliable measure is usually the development of biomass through turbidity readings. It is necessary to avoid using respirometric dyes with fungi because of their variability of response in different fungal species and their frequent toxicity. However, an essential piece of information is lost in this way, which does not necessarily correlate directly with biomass development. Instead, the relationship between respiration and biomass often provides essential details about the energy coefficient of the organism growing on a specific substrate (Pinzari *et al*., 2016; 2017).

Through a prior assessment of redox dyes' reliability, toxicity and nutritional effect, it is possible and scientifically sound to use respiration in the phenotypic profiling of filamentous fungi. In the case exemplified, the results obtained will allow assessing the functionality of the genera *Verticillium* and *Trichoderma* with the Biolog™ Phenotype Microarray. The protocol here developed allows the measurement of the respiratory properties of fungi as an added value to the measurement of biomass production.

What has been shown outlines the substantive experimental steps that should be performed whenever a redox dye is used to measure respiration in filamentous fungi. Particular attention should be paid to testing redox dye's toxic and nutritional effects on each strain under study. The results showed a very high species- or genus-specific variability in the colouring of the dyes. Nonetheless, it was possible to identify respirometric indicators suitable for comparing the fungal isolates on a metabolic basis, both as a function of respiratory activity and biomass production. The results obtained so far will help to select suitable nitrogen sources as prebiotic supplements in *Trichoderma* biopreparations with antagonistic activity towards phytopathogenic strains of *Verticillium* spp.

While *Verticillium* and *Trichoderma* have different modes of action, they may compete for similar nitrogen sources in their environment. Both *Verticillium* and *Trichoderma* are capable of decomposing organic matter and utilising various nitrogen sources for their growth and survival. This includes proteins, amino acids, peptides, and other nitrogen-containing compounds found in the soil or plant debris. They produce a range of enzymes, among other proteases, that enable them to break down complex nitrogen compounds into simpler forms that can be assimilated (Morán-Diez *et al*., 2019).

In environments where both *Verticillium* and *Trichoderma* coexist, there can be competition for limited nitrogen resources. This competition may arise when nitrogen sources are scarce or when the two fungi occupy similar ecological niches. Factors that influence the outcome of this competition can include their growth rates, nutrient uptake efficiencies, and ability to produce extracellular enzymes (Dutta *et al*., 2023).

It's important to note that the specific outcome of competition between *Verticillium* and *Trichoderma* can vary depending on the environmental conditions, the availability of nitrogen sources, and the specific strains or species involved. In some cases, one fungus may outcompete the other and dominate the niche, while in other situations, they may coexist without significant competitive interactions.

Verticillium dahliae is a soil-borne fungus that thrives and endures in soil environments. It is renowned as a soil-borne plant pathogen responsible for inducing vascular wilt disease in a broad range of plant species, including economically significant crops. During its saprophytic phase, *Verticillium dahliae* can persist in the soil even without a susceptible host. It exploits organic matter present in the soil, such as plant debris, root exudates, and other organic materials, as a source of nourishment. To enhance its survival in adverse conditions, the fungus forms compact survival structures called microsclerotia, enabling it to endure for extended periods in the soil. Upon encountering a suitable host, *Verticillium dahliae* invades the plant's roots and colonises its vascular system, giving rise to the telltale wilting symptoms associated with Verticillium wilt. The fungus reproduces and produces spores within the host tissue, facilitating its continued survival and propagation within the soil (Zhang *et al*., 2022).

However, in addition to its pathogenic tendencies, *Verticillium dahliae* engages in various interactions with other soil microorganism's species such as *Trichoderma citrinoviride*, *Trichoderma atroviride*, and *Trichoderma pseudokoningii* that are often used as beneficial microorganisms for biocontrol purposes (Guzmán-Guzmán *et al*., 2023). These interactions encompass competition for resources, associations with beneficial microorganisms, and responses to environmental factors. Thus, the soil is the ecological niche where *Verticillium dahliae* and *Trichoderma* spp. compete, as species typically do not colonise the xylem, as they are primarily rhizosphere-associated fungi and do not have the ability to invade the vascular system of plants. Their interactions and activities are primarily focused on the soil and root surfaces (Sohrabi *et al*., 2023).

Overall, the competition for nitrogen sources between *Verticillium* and *Trichoderma* is a complex ecological process that can be influenced by multiple factors. Further

research is needed to better understand the dynamics of this competition and its implications for plant health and disease suppression. In this direction, reliable methods and protocols are needed to better understand the interaction mechanisms between microorganisms.

CONCLUSIONS

1. Fungal competition for nutrients is measurable and exploitable in biocontrol. Competition among fungi for nutrients is well-documented in various ecosystems, including soil. In nature, complex ecological mechanisms resolve and regulate contrasts, such as compartmentalization into different ecological niches. However, when fungal strains are to be used in the biocontrol of crop pathogenic species, it becomes functional to exploit the abilities of competition and contrast between species to select strains that are particularly efficient in dominating others. In addition, understanding the mechanisms of fungal competition may hint at developing targeted strategies to improve biocontrol efficiency. For example, manipulating nutrient availability, introducing specific nutritional stimuli, or supporting microbial strains alongside biocontrol fungi could promote their dominance and strengthen their ability to challenge pathogens.

2. Toxic and nutritional effects of dyes affect the results of metabolic profiling. The use of phenotype microarrays to study the metabolism of fungi allows many applications in a variety of research areas, as the comparison of different strains for their functional selection to specific biotechnological fields. However, this work showed that dyes typically used as markers of respirometric activity may be toxic to some species of fungi or have stimulatory or nutritional effects on others, thereby affecting their enzymatic activity and altering their metabolic pathways. As a result, these dyes may lead to inaccurate or biased results, as they may interfere with the functional processes they are intended to study. The selection of dyes to be used to highlight dehydrogenase activity within multiwell plates must be made with great care, especially when comparing different species that may have different sensitivity to the dyes and thus provide for the same panel of nutritional compounds tested for metabolic profiling different results because of different reaction to the dye and not because of actual functional differences.

3. 1% F and 0.5% D Biolog^{TM} dyes are reliable with *Verticillium* and *Trichoderma* spp. This study selected dyes without toxic effects and their correct concentration to obtain repeatable and reliable results with *Trichoderma* and *Verticillium* species strains. Such an approach is necessary whenever the metabolic profile of fungal strains in microplates has to be compared using colourimetric markers of dehydrogenase activity based on formazan derivatives. Indeed, it was shown in this study how there is high variability in the interspecific and intraspecific response of filamentous fungi to different types of dyes. Therefore, in phenotypic comparisons between strains, there is a risk of confounding a different toxicity response to the dye by differences in metabolic response to administered nutrients. For *Verticillium* and *Trichoderma* species, it was seen that commercial Biolog^{TM} type "F" dyes at 1% concentration and "D" dyes at 0.5% concentration are to be considered reliable and capable of comparable responses in the two genera of fungi compared.

4. Implications for selecting nitrogen sources as prebiotic supplements in *Trichoderma*-based biopreparations. Functional profiling of filamentous fungi by multiwell plates used as phenotype microarrays is helpful in many biotechnological applications. To exploit the full potential of profiling, it is necessary to measure both their biomass production and the respirometric activity of the strains for certain nutrient compounds. It is, therefore, necessary to measure both well turbidity and colour formation. In this study, we demonstrated how formazan compounds, typically used as colourimetric markers of fungal dehydrogenase activity and respiration indicators, can also be used as nitrogen sources by fungi. Fomazan and its derivatives have a chemical structure that includes four nitrogen atoms, similar to amine dyes (azo dyes) that some fungal strains can decolorize. Some species of fungi possess enzymes capable of using colourimetric indicators as nitrogen sources. Therefore, if the purpose of profiling is to analyze and compare the performance of different nitrogen sources for multiple strains, the choice of the correct indicator molecule and its concentration becomes essential for the reliability of the results. In this work, indicator substances were selected that allow, without substantial nutritional interference, testing of different nitrogen sources for use as prebiotic supplements in *Trichoderma*-based biopreparations intended to contrast phytopathogenic strains of *Verticillium* spp.

Conflict of interest: The Authors declare they have no conflict of interest.

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