The influence of selected redox dyes on the functionality of *Verticillium* and *Trichoderma* genera – a methodical aspect

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Abstract

The research goal was to **determine the redox dye type and its concentration** that reasonably and comparably reacts to the respiratory activity of *Verticillium* spp. and *Trichoderma* spp. isolates in the presence of a **model nitrogen source** ($(NH_4)NO_3$). Therefore, the commercial **D, E, and F Biolog™ redox mix dyes**, and 2,3,5-Triphenyltetrazolium chloride (**TTC**; Tetrazolium chloride), Iodonitrotetrazolium chloride (**INT**), 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (**MTT**) suspensions were evaluated in selected concentrations and their nutritive/toxic effects were described. The **1% F and 0.5% D Biolog™ dyes** were found to be the most appropriate for the **evaluation** of isolates belonging to the ***Verticillium* and *Trichoderma* genus**, using **Biolog™ PM-nitrogen plate** respiratory assays, for their extended functional evaluation.

Methods & Materials

Fungal isolates
The four 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 the four 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 characterized.

Redox dyes
The six redox dyes D, E, and F Biolog™ (marked further as D, E, and F dyes, respectively), and 2,3,5-Triphenyltetrazolium chloride (TTC, Tetrazolium chloride) (Chemat, Gdańsk, Poland), 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-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 the dye type were investigated following the nutrition and toxicity tests.

Nutrition test
The nutrition test was performed in 0.9% NaCl. Sterile transparent 96-well microplates were inoculated with a volume of 180 µl of fungal spores suspended in 0.9% NaCl (73% Transmittance). Next, 20 µl of 10x concentrated dyes were added and mixed precisely to obtain final concentrations: 0.1% 0.5%, 1% and 2% of D, E, F dyes, or 0.1% 0.5% and 1% of MTT, TTC and INT dyes. The 0% dye addition (20 µl 0.9% NaCl) was the control for each dye. The 10x concentrated stock of D, E, and F dyes was 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 through a syringe filter. Analyses were performed in three technical replications. The prepared microplate cultures with dyes addition were incubated in dark for ten days at 24°C. Every 24 hrs fungal functional response to dyes was measured. Absorbance readings were performed using Biolog™ MicroStation (Hayward, Canada) at 750 nm wavelength.

Model nitrogen substrate-based medium
The preliminary step was carried out, where a model nitrogen substrate concentration was established. The Ammonium nitrate ($(NH_4)NO_3$) (Sigma Aldrich™, Saint Louis, Missouri, USA) was proposed in the presented research. The following final concentrations: 0.05%, 0.1%, 0.5%, 1% and 0% (the control) were tested in the three media: PM3,5-8 inoculating fluid (PM) according to Biolog™ (Hayward, Canada) preparation protocol, IF-FF inoculating fluid (FF) (Biolog™, Hayward, Canada) and 0,9% NaCl (NaCl) each. The final concentrations of ingredients in PM were as follows: FF-IF 0.833x mM, D-glucose 100 mM, potassium phosphate (pH6.0) 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 a particular medium (73% T) and 10 µl of 10x concentrated $(NH_4)NO_3$ suspended in the tested media. The total volume of 100 µl was mixed thoroughly by pipetting. Analyses were performed in three technical replications. All ingredients were filtered through a syringe filter. Such prepared in microplates cultures were incubated in dark for ten days at 24°C. Every 24 hrs fungal absorbance readings were performed using Biolog™ MicroStation (Hayward, Canada) at 750 nm wavelength.

Toxicity test
The toxicity test was performed on a selected and described above PM medium with 0.1% $(NH_4)NO_3$ addition and the set of the redox dyes. Sterile transparent 96-well microplates were inoculated with a volume of 180 µl of fungal spores suspended in the 0.1% $(NH_4)NO_3$-PM medium (73% T). Then 20 µl of 10x concentrated dyes set with 0.1% $(NH_4)NO_3$ were added and mixed precisely to obtain final concentrations: 0.1% 0.5%, 1%, or 0.01% 0.05% and 0.1% of MTT and INT. The 10x concentrated stock of D, E, and F dyes were prepared using 0.9% NaCl, while MTT and INT by using 0.1 M HCl 10% Sodium Dodecyl Sulfate (SDS) solution. Analyses were performed in three technical replications. Such prepared microplate' cultures with different dyes supplementation were incubated in dark for ten days at 24°C. Absorbance readings were performed every 24 hrs using Biolog™ MicroStation (Hayward, Canada) at 490 nm and 750 nm wavelengths.

Statistics
An analysis of variance (ANOVA) with the comparisons of the mean values (of ten-day readings) between dyes concentrations was used with Tukey's post hoc honestly significant differences (HSD) at $p < 0.05$. Statistica 13.1 software (StatSoft®, Tulsa, Oklahoma, USA) was used. The analysis was performed using every 10-days readings. The PRIMER-e v. 7 software (Albany, Auckland, New Zeland) was used for isolates clustering following Euclidean distance and Multidimensional Scaling analysis (MDS) for visualizing the level of similarity of individual isolates depending on their reaction to nitrogen concentration or the dye`s dataset.

Introduction

Some species of the ***Verticillium*** fungal genus are **key soil-borne pathogens** of soft fruits. Due to consumers' rising interest in organic farming products, there is a need for natural plant protection products based on antagonistic microorganisms, limiting the damage caused by *Verticillium* on some fresh products. Among the fungi already in use for **biological control** of fungal pathogens, the most significant interest is invariably in ***Trichoderma*** isolates. This is due to their strong aggressiveness against phytopathogens (mycoparasitism, antibiosis, competition), effective stimulation of plant growth and defense mechanisms, and the ability to modify the rhizosphere microbiome. The mechanism of action of *Trichoderma* spp. against phytopathogens that has been least studied and described in the literature is **competition for nutrients**.

Phenotype MicroArray™ system (PM) can be efficiently applied to study the fungal use of different substrates as carbon or nitrogen sources. It is a sensitive, reliable, and repeatable method based on **functional fingerprinting**. However**, nitrogen is considered only marginally** compared to carbon sources, even if its presence is necessary for fungal conidiation and chlamydospores production. Nitrogen has an impact on fungal species' competitiveness in any ecological niche. Fungal phenotypic diversity is usually described based on differences in substrate catabolism or biomass production, but these parameters **are rarely combined and considered together**. The increase in fungal biomass 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 in the corresponding well can potentially **indicate a stressful condition**.

Nevertheless, the Phenotype MicroArray™ system **is not commercially supplied with a redox dye responsive to the respiratory activity** of fungi. Therefore, its addition was determined experimentally. Since redox dyes can be **toxic** to some fungal species or fungi can easily **use them as carbon sources** for biomass production, the research goal was to indicate the particular redox dye and its concentration with **the least nutritive and toxic effects**, and simultaneously a reasonable color formation enabling to quantify the respiratory processes and thus extend the functional evaluation of *Verticillium* and *Trichoderma* genera.

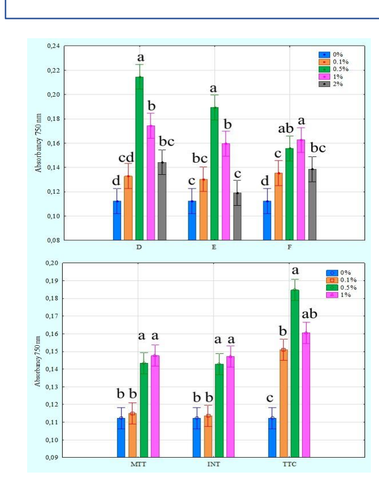

Figure 1. The nutritive effect of selected redox dyes on fungal growth in 0.9% NaCl medium, based on dyes` concentrations following 750 nm absorbance readings a) Biolog™ redox dyes, b) other dyes. *Explanations*: redox dyes D, E, and F Biolog™ (marked as D, E, and F, respectively), and 2,3,5-Triphenyltetrazolium 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

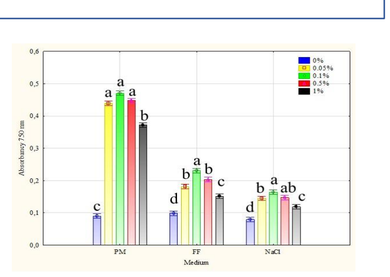

Figure 2. The biomass production in selected media of all tested *Verticillium* spp. and *Trichoderma* spp. isolates depending on the $(NH_4)NO_3$ concentrations based on absorbance readings at 750 nm.

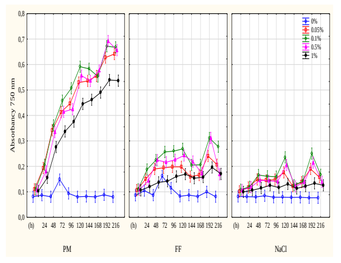

Figure 3. The rate of fungal growth within the 24-hrs in selected media of all tested *Verticillium* spp. and *Trichoderma* spp. isolates depending on the $(NH_4)NO_3$ concentrations based on absorbance readings at 750 nm.

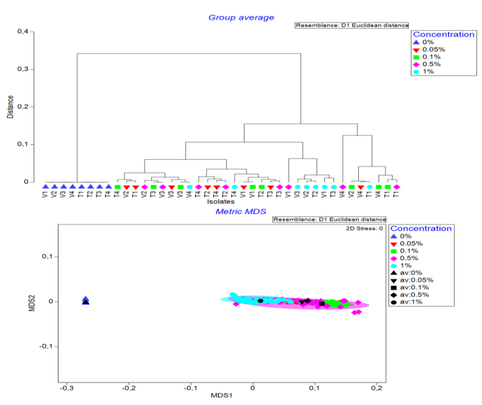

Figure 4. The diversity of *Verticillium* spp. (V1-V4) and *Trichoderma* spp. (T1-T4) individual isolates in terms of the biomass production (based on absorbance readings at 750 nm) depending on their reaction to different $(NH_4)NO_3$ concentrations in PM medium a) the clustering following the Euclidean distance; b) the metric Multidimensional Scaling analysis (MDS) based on the Euclidean distance.

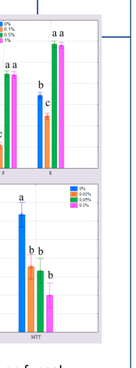

Figure 5. The toxicity effect of selected redox dyes on fungal growth in 0.1% $(NH_4)NO_3$-PM medium, based on dyes` concentrations following 750 nm absorbance readings, a) and b) and respiration based on absorbance 490 nm readings, c) and d). *Explanations*: redox dyes D, E, and F Biolog™ (marked as D, E, and F, respectively), and 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

Table 1. The toxicity dyes test schema**.** *Explanations*: 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-2H-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.

V/T (x)	dye	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%

Results & Discussion

Achieving the overall aim of the work required ensuring that the proper dye is not toxic to fungi and simultaneously dye is not used as a carbon source to produce their biomass. An especially negative situation is where it is little or no additional carbon source available. Therefore, **the nutritive and toxicity effects** were tested. Appropriately, in the nutrition test, the expected convenient output was when fungi are relatively not capable to increase their biomass production. Therefore, the experiment was performed using saline as a low substrate medium.

Conversely, in the **toxicity test**, the expected positive result was the dye does not inhibit fungal growth significantly in the medium, where normally (without the dye added) the biomass production is observed. Since the medium would be eventually suitable for biomass density measurements, it was assumed the adequate one should meet the translucence conditions. Namely, it must transmit light on a level that allows measuring the rate of fungal growth. Therefore, the appropriate one was at the beginning selected in **the model medium and nitrogen selection step.** Importantly, simultaneous observation of color formation correlating with the respiratory processes of the examined should be possible. Additionally, obtaining sensible and reliable results in the toxicity test required attention paid to designating such a medium and nitrogen concentration that the tested fungi differentiate bare minimum. Thus, isolates' diversity was described.

The nutritive effect of selected redox Biolog™ dyes (Figure 1a) and other dyes (MTT, INT, TTC) (Figure 1b) on fungal growth in the low substrate (0.9% NaCl) medium, based on dyes` concentrations are presented. An evident nutritive effect was noted for 0.5% and 1% of all dyes types and 0.1 % of F and TTC, and 2% of F dye. No nutrition phenomena (a significant biomass production increase) was met for 0.1% D, E, F, MTT, and INT dyes addition. Since the TTC dye addition caused the nutritive effect in any tested concentration the TTC was excluded from the next main step of the experiments (the toxicity test).

In Figure 2. there are biomass production results in selected media of all tested *Verticillium* spp. and *Trichoderma* spp. isolates depending on the $(NH_4)NO_3$ concentrations presented. When comparing media, the greatest growth of fungi was noted for PM medium. Thus, the highest values of optical density (at 750 nm) were found for 0.05%, 0.1% and 0.5% nitrogen concentration. These were statistically higher than 1% and 0% (control). As it was predicted NaCl medium provoked the smallest biomass production. It was a trend noticed that for all tested media 0.1% nitrogen addition caused the greatest fungal biomass production.

Figure 3. shows the rate of fungal growth within the 24-hrs readings. The 0.1% $(NH_4)NO_3$-PM medium is once again the most noticeable with distinguished dynamics of fungal growth. These results allowed us to preliminarily point out the 0.1% $(NH_4)NO_3$-PM medium as the model one for the toxicity test.

Figure 4. presents the diversity of *Verticillium* spp. (V1-V4) and *Trichoderma* spp. (T1-T4) individual isolates in terms of biomass production (based on absorbance readings at 750 nm) depending on their reaction to different $(NH_4)NO_3$ concentrations in PM medium. Both presented graphs indicate the diversity among tested isolates occurred. All tested isolates cultured in control conditions (0%) were separated as well as most isolates cultured with 1% nitrogen addition. 0.05%, 0.1% and 0.5% nitrogen addition do not implicate clustering (Fig. 4a). However, the data transformation using the MDS analysis emphasized and confirmed that 0.1% nitrogen addition provokes quite similar fungal biomass production. It is because 0.1% nitrogen cultured isolates create the most compacted group compering to the other nitrogen concentrations, and what is more, the average value of biomass production for 0.1% has the most positive influence on MDS1.

Toxicity test results are presented in Figure 5. Firstly, the trend is the same for respiratory and biomass production functionality for all redox dyes tested. The predicted toxic influence, when Biolog™ dyes were considered, was met only for the lowest dyes concentration (0.1%). For higher concentrations (0.5% and 0.1%) the opposite effect was noted. Inevitably, increased production of biomass and respiratory activity were observed, especially much higher for E dye than D or F dyes.

As for MTT and INT, the general trend was that all proposed dye doses were toxic (0.01%, 0.05%, and 0.1%) since the inhibition of the fungal growth was noted, even 0.01% and 0.05% these were doses 10x lowered compering to the nutritional test. For 0.1% MTT and INT dose, the same significant inhibitory effect was also previously met. It indicates that MTT and INT are generally toxic for tested *Verticillium* spp. and *Trichoderma* spp. isolates, irrespectively to the used medium. This toxicity reached the point that fungi did not reach the positive readings with the threshold. In the PM methods (e.g. FF plates), it is A≥0.25. MTT and INT have failed to meet the requirements for redox dyes for the PM technic. For Biolog™ dyes potentially F and D. Substantially, 0.5% D and 1% F Biolog™ dyes could be acceptable for extended functional PM-nitrogen plate applications. This was confirmed by the satisfactory color formation presented in pictures collected in Figure 6.

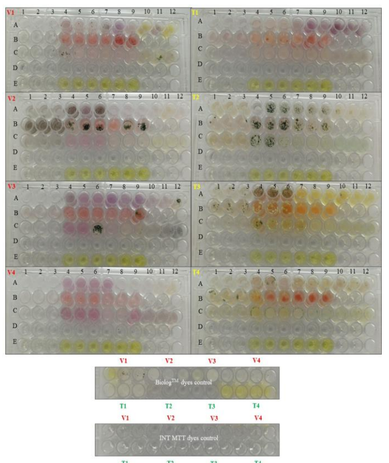

Figure 6. The color formation in the toxicity dyes test carried out in a 0.1% $(NH_4)NO_3$-PM medium**.** *Explanations*: *Verticillium* spp. (V1-V4) and *Trichoderma* spp. (T1-T4) isolates, Biolog™ redox dyes D, E, and F a) fungi + dyes, b) controls - fungi without dye; n=3. For dyes arrangement in a particular plate please see Table 1.

Conclusions

The 1% F and 0.5% D Biolog™ dyes were found to be the most appropriate for the functional evaluation of isolates belonging to the *Verticillium* and *Trichoderma* genus, using Biolog™ PM-nitrogen plate respiratory assays, for their extended functional evaluation

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