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OPTIMIZATION OF *TRICHODERMA* STRAIN CULTIVATION FOR BIOCONTROL ACTIVITY

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Abstract
Pathogens cause world-wide economically significant diseases in numerous agricultural, horticultural and ornamental crops. Most of the pathogens are difficult to control by conventional fungicides. Biocontrol represents an economical, environmentally friendly alternative to chemical pesticides for diseases produced by phytopathogens. Trichoderma strains have received particular attention as biocontrol agent of fungal plant pathogens. The present work is focused on the optimization of growth and sporulation of antagonistic Trichoderma T36. Assessment of microbial cultures was done by measuring the fungal colony growth on solid medium. Likewise, visual and microscopically observations were performed. Trichoderma T36 was cultured on different nutrient media and M1 medium, a Czapek-Dox medium supplemented with sodium phosphate, ammonium chloride and malt extract, was selected for further experiments. A wide range of carbon sources has been tested replacing initial source in M1 medium. The best results were obtained in media with fructose as carbon source. As expected, ammonium dihydrogen phosphate was found to be the best nitrogen source for Trichoderma T36 cultivated on M1 medium and colony diameter decreases in the following order: $NH_4H_2PO_4$<NH_4Cl<NH_4NO_3=$NaNO_3$<KNO_2<KNO_3=$NaNO_2$<urea. Fungal growth was excellent at temperatures of 26 - 37°C and pH range of4.0 – 5.5.

Keywords: *biocontrol, microbial antagonism, phytopathogens, Trichoderma*

INTRODUCTION

Strains of *Trichoderma* can produce a great number of metabolites and because of these properties, the genus has high biotechnological potential [1; 2]. Several studies have been reported the capacity of *Trichoderma* to be used as biocontrol agent against phytopathogens, such as *Fusarium, Phytium, Rhizoctonia* and *Sclerotinia* species [3; 4;5; 6]. When planning the application of antagonistic *Trichoderma* strains for the purposes of biological control, it is very important to consider the parameters affecting the growth and sporulation [7; 8; 9]. The work is a continuance of a complex research to obtain an antagonistic *Trichoderma* and is focused on optimization of growth and sporulation of selected fungal strain.

MATERIAL AND METHOD

Microorganisms

A potential biocontrol agent *Trichoderma* strain T36 used in the study provides from Microbial Collection of ICECHIM. The strain isolated from forest soil was maintained on potato dextrose agar (PDA) slants medium at 4°C.

Cultivation conditions

The microbial growth was evaluated on solid agar media in Petri plates. The compositions of tested nutrient media are:
MEA medium [10] (g/l): malt extract malt, 30; peptone, 3, agar 20, 1ml $CuSO_4$ x 5 H_2O 0.5g/l; 1ml $ZnSO_4$ x 7 H_2O 1g/l; pH, 5.5.
PDA medium: potato, 250, dextrose, 20, agar, 18 [10].

Czapek medium [10] (g/l): $NaNO_3$, 3, K_2HPO_4, 1, $MgSO_4x7H_2O$, 0.5, $FeSO_4$, 0.01; KCl, 0.5; saccharose, 30g; agar, 18.
Czapek-Dox medium [10] (g/l): $NaNO_3$, 3; K_2HPO_4, 1; $MgSO_4x7H_2O$, 0.5; $FeSO_4$, 0.01; KCl, 0.5; glucose, 30; agar, 18.
OM medium [11] (g/l): starch, 5; glucose, 5; peptone, 5; yeast extract, 7.5; after autoclaving, 10 ml of solution A and B and 1 ml of solution C and D. Composition of solution A g/l): KH_2PO_4, 5; K_2HPO_4, 5. Composition of solution B (g/l): $MgSO_4$, 17, NaCl, 1; $MnSO_4$, 0.7; $CuSO_4$, 0.06. Composition of solution C (g/100 ml): $FeSO_4$ x 7 H_2O, 0.1:, sodium citrate, 2,2; ammonium acetate, 2; sodium succinate, 3.3. Composition of solution D (mg/100 ml): biotin, 10 mg; p-aminobenzoic acid, 20; vitamin B_{12}, 5; calcium pantothenate, 10; pyridoxal hydrochloride, 10; nicotinamide acid, 35.
Mediul Mandels [12] (g/l): glucose, 10; urea, 0.3; $(NH_4)_2SO_4$, 1.4; KH_2PO_4, 2; $CaCl_2$ x $2H_2O$, 0.4; $MgSO_4$ x $7H_2O$, 0.3; peptone, 0.75; yeast extract, 0.25; $FeSO_4$ x $7H_2O$, 0.005; $MnSO_4$ x $4H_2O$, 0.0016; $ZnSO_4$ x $7H_2O$, 0.0014; $CoCl_2$ x $6H_2O$, 0.02..
Miller medium [13] (g/l): Na_2HPO_4, 6; KH_2PO_4, 3; NaCl, 0.5; agar, 15; pH, 6.5. Salts and agar are autoclaved separately and then supplemented with 2 ml of 1M $MgSO_4$ x 7 H_2O, 10 ml 20% glucose, 0,1 ml 1M $CaCl_2$ and 0.5 ml vitamin B_1.
M1 medium (g/l): Czapek-Dox medium supplemented with $NaHPO_4$, 3; NH_4Cl 1; malt extract, 3.
The media were sterilized at 1 atm (121^0C), 20 minutes. Petri plates were inoculated with 20 µl of sporal suspension ($1,2x10^7$ conidii/ml) and incubated for 120 hours at 26±2°C. All the culturing experiments were carried out in triplicate.
In selected medium, carbon and nitrogen sources were replaced and the effects were determined. The concentration of carbon and nitrogen source was 0.2% (w/v) and 0.1% (w/v), respectively. The effect of several aminoacids and vitamins (B1 and B12) was analyzed by medium supplementation with 0.1 % (w/v) of each compound tested.

Influence of pH values

Fungal cultures were incubated at different pH values, from 4.0 to 9.0. OAKTON pH- meter was used.

Influence of temperature

Fungal cultures were incubated in static incubator Heidolph Unimax 1010 at 2°, 4°, 6°C, 10°, 16°, 22°, 26°, 30° and 37°C.

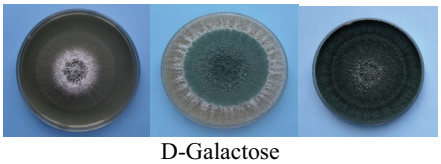
RESULTS AND DISCUSSIONS

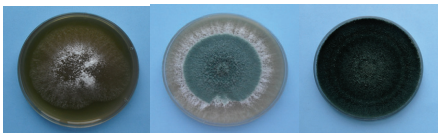
Nutritional requirements and psychological parameters of antagonistic *Trichoderma T36* were studied.
Initially, *Trichoderma* T36 was cultivated in Petri plates on different solid nutrient media. The fungal growth was measured as colony diameter. The results presented in Table 1 reveal that M1 medium offers conditions for faster and better growth of *Trichoderma* T36. The difference between media is obviously at 48 hours of cultivation, although at the end of incubation period most of tested media present similar colony diameters. According to these results, M1 medium was selected for further experiments.

Table 1. Influence of nutrient medium on the radial growth and sporulation of *Trichoderma* T36

Culture solid medium	Colony diameter (cm)		Sporulation
	48 h	120 h	
MEA	5.4	8.5	+++
PDA	5.0	8.5	+++
Czapek	2.4	6.1	+
Czapek-Dox	2.2	7.2	+
OM	5.4	8.5	+++
Mandels	5.4	8.5	+++
Miller	4.5	8.5	+++
M1 medium	5.5	8.5	+++

Based on composition of M1 medium, several carbon sources was tested for *Trichoderma* T36 growth.


D-Galactose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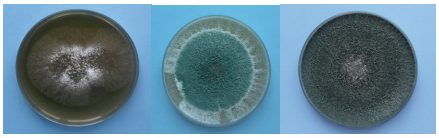
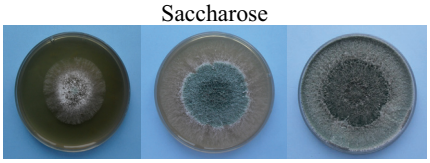


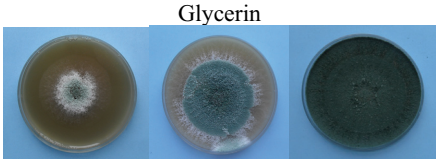
D-mannose

Lactose

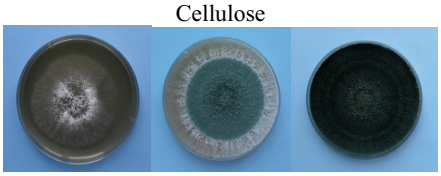
Saccharose



Glycerin



Cellulose



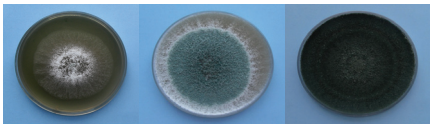
Dextrose

Glucose

Citric acid

Mannitol

Ribose



Arabinose

Figure 1. Effects of carbon source on the radial growth and sporulation of *Trichoderma* T 36; photos taken at 48, 72 and 120 hours of cultivation in Petri plates on solid M1 medium

The values of colony diameters corresponding to the photos depicted in Figure 1. are presented in Table 2.

Table 2. Influence of carbon source on the radial growth and sporulation of *Trichoderma* T36

Carbon source	Colony diameter (cm)		Sporulation
	48 h	120 h	
D-glucose	5.90	8.5	
D galactose	5.20	8.5	+++
Fructose	6.10	8.5	+++
Ribose	5.95	8.5	+++
D mannose	5.20	8.5	+++
Arabinose	4.10	8.5	+++
Mannitol	5.82	8.5	+++
Saccharose	3.61	8.5	++
Lactose	4.16	8.5	+++
D maltose	5.26	8.5	+++
Starch	4.82	8.5	+++
Cellulose	5.42	8.5	+++
Glycerin	3.10	8.5	+

It can be seen that the highest value of colony diameter is obtained on the medium with fructose as carbon source.

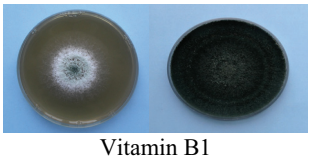
The effects of different nitrogen sources on fungal growth were studied by replacing initial nitrogen source of M1 medium with one of eight alternative nitrogen sources.

Ammonium dihydrogen phosphate was found to be the best nitrogen source for *Trichoderma* T36 growth. Colony diameter is decreasing in the following order: $NH_4H_2PO_4$< NH_4Cl< NH_4NO_3=$NaNO_3$<KNO_2<KNO_3=$NaNO_2$<urea. It is generally agreed that ammonium-nitrogen is the preferred form for microbial metabolism as it requires less energy to be assimilated. After 120 hours of cultivation the colony diameters are similar for all nitrogen sources tested

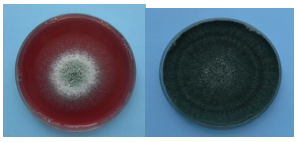
The results of the investigations dealing with the influence of the aminoacids and vitamins on fungal growth are presented in Figure 2, concluding that at 48 hours after inoculation, the highest value was obtained with valine.

Table 3. Influence of nitrogen source on the radial growth and sporulation of *Trichoderma* T36

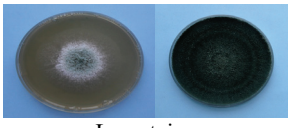
Nitrogen source in	Colony diameter (cm)		Sporulation
	48 h	120 h	
$NH_4H_2PO_4$	5.6	8.5	+++
NH_4NO_3	5.3	8.5	++
$NaNO_2$	3.2	8.5	++
$NaNO_3$	4.6	8.5	++
NH_4Cl	5.4	8.5	+++
KNO_2	3.5	8.5	++
KNO_3	3.2	8.5	++
Urea	2.8	8.5	++



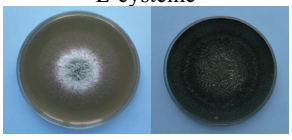
Vitamin B1



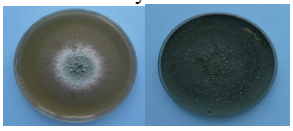
Vitamin B12



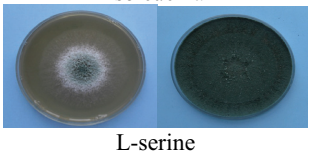
L-cysteine



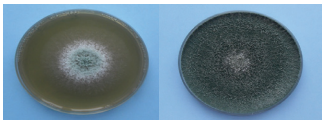
L lysine



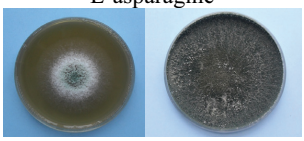
Isoleucina



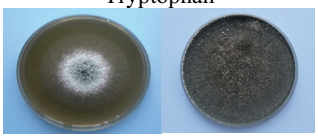
L-serine



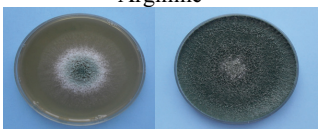
L-asparagine



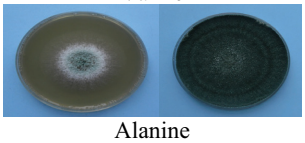
Tryptophan



Arginine



Valine



Alanine

Figure 2. Influence of aminoacids and vitamins on the radial growth and sporulation of *Trichoderma* T36

The values are decresing in following order: valine>alanine>L-lisine=isoleucine=L-serine, with colony diameter variying form 6.4 to 6.0 cm.

The next step was the investigation of physical factors affecting ***fungal growth***.

The pH values of nutrient medium belong to the most important parameters affecting microbial cultivation. As it can be shown in Figure 3, the pH values between 4.5–5.5 produce colonies larger than 6.5 cm in diameter, whereas for higher pH values the diameter did not exceed 4.2 cm. The strain grew best at pH 5.5, results in agreement with other scientific reports [14, 15].

For optimum growth, temperatures must be in a range that allows the most efficient progression of the chemical reactions necessary for growth. In this respect, the ability of *Trichoderma* T36 to grow at 2, 4, 6, 10, 16, 22, 26, 30 and 37˚C was tested (Figure 4.).

It was observed that *Trichoderma* T36 did not grow at lower temperatures, such as 2, 4 and

6˚C. As expected, the increasing temperature facilitates the growth, reaching the maximum growth and sporulation between 26 and 37˚C. This behavior is normal for mesophilic fungal strain at it has been reported [15].

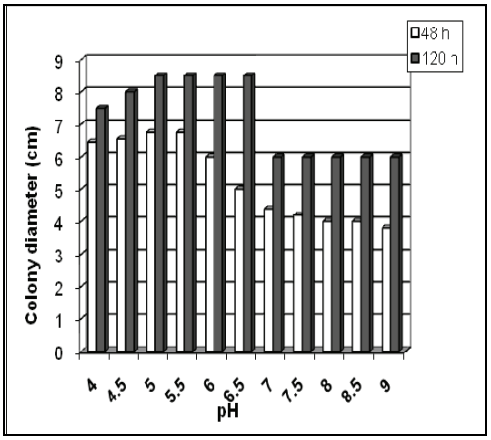


Figure 3. Influence of pH values on the radial growth and sporulation of *Trichoderma* T36

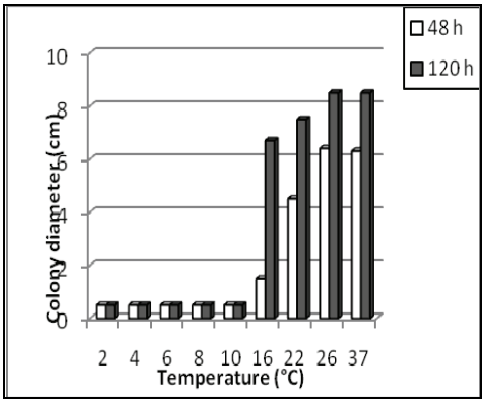


Figure 4. Influence of temperature on the radial growth and sporulation of *Trichoderma* T36

CONCLUSIONS

The optimization study covered physical parameters (pH and temperature) and nutrients composition (carbon and nitrogen source) on a selected solid medium. The nutrient solid medium that promoted the highest growth (estimated as colony diameter) was used for the subsequent steps of the investigation. The composition of selected M1 medium was modified as regarding the nature of carbon and nitrogen source. The highest colony diameter is obtained on M1 medium with fructose. The ammonium-nitrogen salts $NH_4H_2PO_4$, NH_4Cl and NH_4NO_3 are preferred for microbial metabolism, $NH_4H_2PO_4$ being the best nitrogen source. Among the aminoacids and vitamins tested, the best result was obtained in medium supplemented with valine. *Trichoderma* T36 has a maximum growth and sporulation between 26 and 37˚C at pH 5.5. The studied strain is a potential candidate for the biological control of plant diseases. In this respect, further researches will be dedicated to production of volatile and non-volatile metabolites [16], capable of inhibiting mycelial growth at several pathogens.

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