INFLUENCE OF PROCESS PARAMETERS ON THE PRODUCTION OF *TRICHODERMA* BIOCONTROL AGENT

ORIGINAL SCIENTIFIC PAPER

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DOI: 10.5281/zenodo.6371166

RECEIVED 2021-10-04 **ACCEPTED** 2021-10-25

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ABSTRACT:
Maize is an agricultural crop that is susceptible to infections by various phytopathogenic fungi, producers of mycotoxins harmful to humans and animals. Since this agricultural crop has an important place in the human diet, its health safety is very important. *Trichoderma* genus has great potential in the biocontrol of various phytopathogens however, the medium composition as well as the cultivation conditions, have a significant impact on the efficiency of the produced *Trichoderma* bioagents. In this work, influence of medium pH, temperature and mixing speed on the productivity of *Trichoderma* bioagent effective against two maize pathogens, *Fusarium graminearum* and *Aspergillus flavus*, was investigated. The results obtained by statistical processing show that the best productivity of *Trichoderma harzianum* bioagent was achieved when the pH of the medium was 6, the temperature was 28°C and the mixing speed of the rotary shaker was 180 rpm. By applying these cultivation conditions, the largest inhibition zone diameters of *F. graminearum* and *A. flavus* mycelial growth were formed. Also, the results show that the maize pathogen, *F. graminearum*, was more sensitive to the produced *Trichoderma* biocontrol agent compared to the other maize pathogen, *A. flavus*.

KEYWORDS: ***Trichoderma harzianum*; Bioprocess; Process parameters; pH; Temperature; Mixing.**

INTRODUCTION

The primary goal of maize production is to achieve stable and high grain yields of high nutritional value, which is at the same time healthy. However, maize is susceptible to attack by a number of pathogens. *Fusarium* and *Aspergillus* species are mentioned among the most important causes of maize disease. A big problem is the fact that these species are producers of mycotoxins harmful to human health and their presence on maize must be controlled [1]. Zearalenone is one of the most prevalent mycotoxins, mainly produced by *Fusarium* fungi (*F. graminearum*, *F. cerealis*, etc.) and has been proven to affect the reproductive capacity of animals [2]. Also, deoxynivalenol is one of several mycotoxins produced by certain *Fusarium* species that frequently infect maize, wheat, oats, barley, rice, and other grains in the field or during storage [3]. This mycotoxin leads to a number of side effects in animals and humans, but above all cytotoxicity [4]. On the other hand, according to the classification of the International Agency for Research on Cancer, aflatoxin is classified in group 1a of carcinogenic compounds for humans and animals [5]-[7].

The emergence of overpopulation and the growing number of people around the world has led to the intensification of agricultural activities aimed at direct food production and increased need for food has led to increased use of chemical pesticides and fertilizers [8]. One of the acceptable alternatives to the use of chemical plant protection products and the control of plant diseases caused by various microbiological phytopathogens is biological control [9]. Biological control, according to one of the most widely accepted definitions, represents the purposeful use of living organisms and their metabolites to suppress the activity and reduce the population of one or more phytopathogens [10]. Due to low toxicity to humans and the environment and a high degree of efficiency against phytopathogenic fungi, the biotechnological production of antifungal agents is progressively increasing with an emphasis on *Trichoderma* species as production microorganisms.

Fungi of the *Trichoderma* genus are used in various branches of biotechnology and in agriculture. *Trichoderma* genus inhabit different ecosystems, but are predominantly present in different types of soil, from agricultural to forests, pastures, orchards, but also deserts [11]. *Trichoderma harzianum* most often

inhabits the rhizosphere of different agricultural crops, where due to the exchange and recognition of signaling molecules, physiological and biochemical changes can occur in *Trichoderma*, but also in plants [12].

Among the first, their antagonistic ability should be emphasized, which is why they occupy a significant place in the biological control of plant pathogens. In addition to the interaction of *Trichoderma* - pathogen, certain isolates of this fungus have other positive effects on plant growth and development [12]. Products based on fungi of the genus *Trichoderma*, which are known as biocontrol agents, make up about 60% of the total number of registered biofungicides. *T. harzianum* stands out as the most used species in commercially available preparations [13].

The metabolic activity of the selected production microorganism in the medium with optimal composition significantly depends on the environmental conditions such as the pH value, oxygen content in the cultivation medium and the temperature of the bioprocess. *Trichoderma* species require special conditions in terms of the mentioned process parameters, so it is important to examine the impact of each factor on the production of the *Trichoderma* biological agents. In accordance with this fact, the influence of temperature, pH of the cultivacion medium and mixing on the production of *Trichoderma harzianum* biocontrol agent effective against phytopathogenic fungi *Fusarium graminearum* and *Aspergillus flavus*, was examined in this paper.

MATERIALS AND METHODS

Microorganisms.

The isolate *T. harzianum* obtained from soil sample is kept as a pure culture in the Microbiological Collection of cultures of the Institute of Field and Vegetable Crops, Novi Sad, Serbia.

Within the work, phytopathogenic isolates, *F. graminearum* and *A. flavus* were isolated from maize with typical symptoms of infection. Isolation of phytopathogenic fungi was performed according to the method described in the study by Tančić Živanov et al. [14]. Microorganisms are stored at the PDA medium in the Microbial Culture Collection of Faculty of Technology Novi Sad, Serbia.

Inoculum preparation.

Isolates were initially grown on PDA (potato dextrose agar) [15] for seven days at 25°C. A small amount of mycelium of each isolate was taken to inoculate 50 cm^3 of PDB. The Erlenmayer flakes were then incubated for 72 h on a rotary shaker (150 rpm) at 25°C.

Obtained *T. harzianum* inoculum was further used to inoculate experimental flasks for each individual experiment. On the other hand, after three days of cultivation, obtained cultivation broth of *F. graminearum* and *A. flavus* was filtered through double layer of sterile cheesecloth and used for *in vitro* testing.

Experiments.

Selection of the optimal pH of the medium, optimal cultivation temperature and optimal mixing speed on a rotary shaker were realized on a medium containing: dextrose (10 g/dm^3), soy flour (7 g/dm^3), K_2HPO_4 (3 g/dm^3), KCl (0.5 g/dm^3) and $MgSO_4$ x $7H_2O$ (0.5 g/dm^3).

The influence of medium pH on production was monitored through seven experiments with different pH (3-9 ± 0.1). Medium pH values were adjusted using HCl (1 M) and NaOH (1 M) before sterilization. The influence of cultivation temperature on the production of *T. harzianum* biocontrol agent was monitored through six separate cultivations (24, 26, 28, 30, 32 and 34°C). Examination of the effect of mixing on the production of *T. harzianum* bioagent was followed by change of rpm (rotation per minute) of the rotary shaker in five separate experiments (120, 150, 180, 210 and 240 rpm).

Experiments to select the optimal pH value of the medium were performed in Erlenmeyer flasks with a volume of 100 cm^3 with 40 cm^3 of culture medium. Cultivation was continued for 7 days at 26 ± 1°C on a rotary shaker with a stirring speed of 150 rpm.

Experiments to select the optimal value of the cultivation temperature were performed in 100 cm^3 Erlenmeyer flasks with 40 cm^3 of cultivation medium. Cultivation lasted 7 days on a rotary shaker with a mixing speed of 150 rpm.

Experiments aimed at selecting the optimal mixing speed on a rotary shaker were performed in 100 cm^3 Erlenmeyer flasks with 40 cm^3 of cultivation medium. Cultivation lasted 7 days at 26 ± 1°C.

Antifungal activity testing.

The antifungal activity of the produced *T. harzianum* bioagent was examined on the test phytopathogenic isolates, *F. graminearum* and *A. flavus*, in accordance with the method described in the paper Grahovac et al. [16].

Data analysis.

The results obtained in *in vitro* tests were performed in three replicates under identical

conditions, and the obtained results are presented as mean values with standard deviations. The results obtained in this experiment were processed by one-way ANOVA using Software Statistica, version 13.0 (StatSoft Inc., USA). Duncan multiple range test was used to test significance of differences ($p \leq 0.05$) between mean values of measured diameter of inhibition zones.

RESULTS AND DISCUSSION

The results shown in Figures 1-3 represent the mean values of the inhibition zone diameters of the tested isolates *F. graminearum* and *A. flavus*, obtained by the activity of *T. harzianum* biocontrol agent. For each test isolate, obtained diameters were statistically processed. Homogeneous groups and the significance of differences between the mean values of the groups were determined by post-hoc testing using the Duncan multiple range test. Results of the Duncan test are also shown in Figures 1-3 and the differences in significance are indicated with lowercase letter.

pH.

The results presented in Figure 1 show that production of *T. harzianum* bioagent effective against *F. graminearum* was successful on medium with all applied pH (all results are greater than 22 mm in diameter of the mycelial growth inhibition zone) [17]. Certainly, at the highest level of statistical significance are three medium pH marks with lowercase letter *a* (pH 5-7). However, the largest formed diameter of the inhibition zone was formed by cultivating *T. harzianum* on medium with a pH value of 6 (50.67 mm).

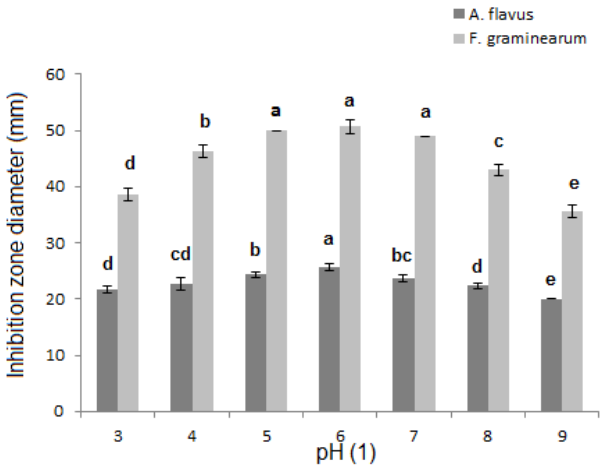

Figure 1. Variation of medium pH. Duncan multiple range test: mean values of inhibition zone diameter formed around wells for isolates *A. flavus and F. graminearum.*

On the other hand, observing the effect of *T. harzianum* bioagent against *A. flavus*, the conclusion is different. Namely, the pH values of the medium from 4 to 8 (diameters higher than 22 mm) are considered suitable for the production of *T. harzianum* bioagent. Medium pH values of 3 and 9 do not show adequate activity. In this case, the pH 6 of the medium was the most suitable for the production of *T. harzianum* bioagent effective against *A. flavus* isolate and this pH value is at the statistically highest level of significance for this tested isolate (25.67 mm).

In accordance with the obtained results, a slightly acidic medium (pH of 6± 0.1) was most suitable for the production of *T. harzianum* bioagent effective against *A. flavus* and *F. graminearum*. These results coincide with the research of Singh et al. In their research they have found that the production of *Trichoderma* spp. was the most suitable on medium with pH range of 5.5 to 7.5 [18].

Temperature.

Temperature is one of the most important parameters that affect the growth of microorganisms. The optimal temperature is the temperature at which cells multiply, grow and produce the appropriate metabolites. For the production of high value products using *Trichoderma* spp. the optimum temperature ranges from 25°C to 30°C. However, some members of this genus can inhabit areas with temperatures up to 40°C and even extreme areas with low temperatures (0°C) [18].

The results presented in Figure 2 show that at all applied bioprocess temperatures, the desired production of *T.harzianum* bioagent effective against isolate *F. graminearum*, occurs. However, at the highest level of statistical significance is only the inhibition zone obtained by activity of *T. harzianum* bioagent produced at a temperature of 28°C (marked with lowercase letter *a*) forming a zone diameter mean value of 61.33 mm. On the other hand, the weakest activity against *F. graminearum* isolate was formed by the production of *T. harzianum* bioagent at a temperature of 34°C (indicated by a lowercase letter *f*) and 24°C (indicated by a lowercase letter *e*).

Depending on the applied bioprocess temperature, the zones range from 19.33 mm (indicated by a small letter *d*) to 28 mm (indicated by a small letter *a*) was formed by activity of produced *T. harzianum* bioagent against *A. flavus*. The largest diameter of the inhibition zone was formed by applying a temperature of 28°C and this diameter is at the highest level of statistical significance together with the diameter formed at a temperature of 30°C. Thus, for isolate *A. flavus* two temperature values were at the highest level of

statistical significance. In these cases, a lower temperature is generally chosen to reduce the cost of producing *Trichoderma* bioagent.

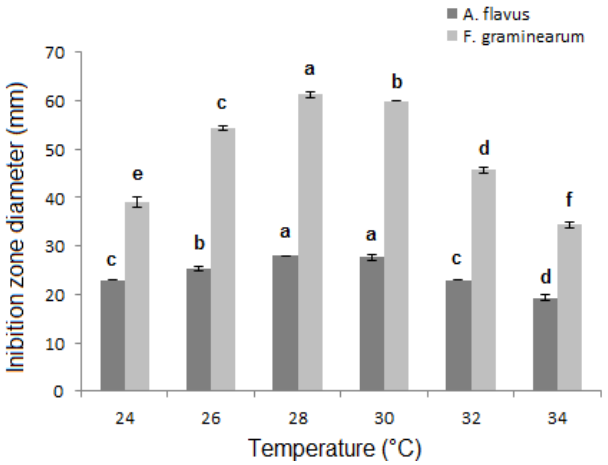

Figure 2. Temperature variation. Duncan multiple range test: mean values of inhibition zone diameter formed around wells for isolates *A. flavus and F. graminearum.*

Considering that in both cases the temperature of 28°C proved to be the most suitable for the production of *T. harzianum* bioagent effective against isolates *F. graminearum* and *A. flavus*, this temperature was selected as optimal for production.

Mixing.

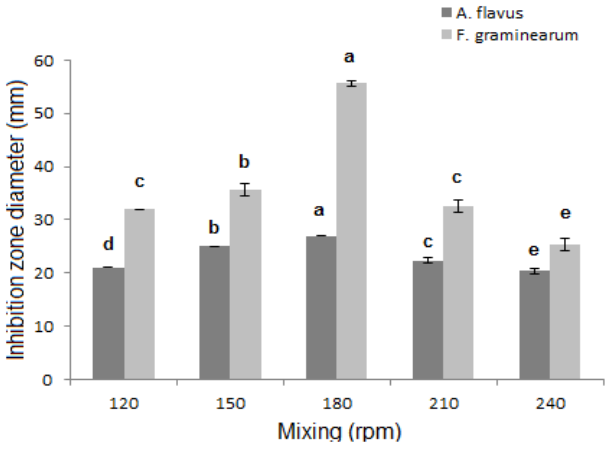

Figure 3. Mixing variation. Duncan multiple range test: mean values of inhibition zone diameter formed around wells for isolates *A. flavus and F. graminearum.*

Mixing of the cultivation medium enables dispersion of the gas phase, homogenization of the composition (nutrient and metabolite content), pH and temperature, suspension of solid ingredients (cells of the production microorganism and insoluble ingredients) and dispersion of liquid ingredients that do not mix with water.

The desired degree of homogenization is achieved by mixing that provides turbulent movement throughout the volume of the cultivation medium.

For the production of *T. harzianum* on an enlarged scale, mixing over 200 rpm [19] and even up to 500 rpm [20], have been obtained as optimal in the scientific literature. Certainly, the mixing of the cultivation fluid on a rotary shaker cannot be fully compared with the conditions in enlarged scale (laboratory bioreactors). However, obtaining the optimal mixing speed on a small scale can be an indicator of the microorganism's need for oxygen.
Figure 3 shows the influence of the rotational shaker speed on the production of *T. harzianum* bioagent effective on phytopathogenic isolates *F. graminearum* and *A. flavus*. The figure shows that isolate *F. graminearum* was more sensitive to produced *T. harzianum* bioagent in relation to isolate *A. flavus*.

At the highest level of statistical significance for both tested isolates, marked is a mixing speed of 180 rpm. This means that at this speed of shaker rotation the best production of *T. harzianum* bioagent occurs. By applying these conditions, the maximum formed diameter of the mycelial growth inhibition for isolate *F. graminearum* was 55.67 mm and 27.00 mm for isolate *A. flavus*. Higher and lower mixing speeds than this, show a decrease in the efficiency of the produced *T. harzianum* bioagent on the tested pathogenic isolates.

CONCLUSION

The results obtained in this study indicate that for the production of *Trichoderma harzianum* agent for biological control of maize pathogens, *F. graminearum* and *A. flavus*, the optimal process conditions were: medium pH 6, bioprocess temperature 28°C and rotary shaker mixing speed of 180 rpm. Application of these cultivation conditions leads to the highest production of T. *harzianum* bioagent, which also affects the formation of the maximum inhibition zone diameters of *F. graminearum* and *A. flavus* mycelial growth.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Education, Science and Technological development of the Republic of Serbia (451-03-9/2021-14/200134), and the Provincial Secretariat for Higher Education and Scientific Research, Autonomous Province of Vojvodina, Republic of Serbia (142-451-3213/2020-03).

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