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Effect of different carbon and nitrogen sources combination in medium for production of biocontrol agent *Trichoderma harzianum*

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Summary: The increasing usage of chemicals for plant protection in recent years has become a serious problem. One of the possible solutions is use of beneficial microorganisms instead of synthetic fungicides, which will contribute to the protection of the environment and human health. Since the fungi *Aspergillus flavus* and *Fusarium graminearum* are the most important pathogens that cause maize diseases and produce mycotoxins, the potential of *Trichoderma harzianum* for biocontrol of both phytopathogens was examined in this paper. The aim of this paper was to study the influence of different carbon and nitrogen combinations in the medium for *T. harzianum* production. *T. harzianum* was cultivated in Erlenmeyer flasks and the effect of cultivation broth against selected maize pathogens was tested using well diffusion method. The results of this study showed that the combination of different carbon and nitrogen sources in the *T. harzianum* cultivation medium statistically significantly affects the production of *Trichoderma* cultivation broth effective on two tested phytopathogens. Dextrose as a carbon source and soybean flour as a nitrogen source proved to be the best combination in the medium for production of *T. harzianum* cultivation broth effective on *A. flavus* and *F. graminearum*. Maximal inhibition zone diameters of 31 mm and 56.33 mm were registered in those medium formulations for *A. flavus* and *F. graminearum*, respectively. These researches represent an important step for further research in which a medium of low market value would be selected. This would reduce the price of the production process but also the final product.
Key words: *Aspergillus flavus*, biocontrol, carbon, dextrose, *Fusarium graminearum*, medium, nitrogen, soybean flour, *Trichoderma harzianum*, well diffusion method

Introduction

Maize is one of the most important grain crops (Mitrović et al., 2020). However, pre-harvest and post-harvest infections by different plant pathogens can contribute to yield reduction of this important crop. These losses in cereal crops can be over 30% (Silva et al., 2019). Also, among all plant pathogens, fungi occupy the largest place. Special and significant problems are the phytopathogenic fungi that produce toxins and threat to the health of both, humans and animals. Fungi from the genera *Aspergillus, Fusarium* and *Penicillium* are listed among the most important pathogens of maize, which are also mycotoxin producers (Garcia-Diaz et al., 2020). WHO (World Health Organization) classifies aflatoxins amongst the most poisonous mycotoxins which are produced by certain molds such as *A. flavus*. On the other hand, *F. graminearum* produce zearalenone that has been related to estrogenic activity, and deoxynivalenol that has been related to high cytotoxic and immunosuppressive properties, so these toxins pose a risk to human and animal health (Tatay et al., 2017; Beisl et al., 2020).

Due to all the above mentioned, the control of phytopathogenic fungi, which cause economically important maize diseases, is of great importance. Well-known negative impact of synthetic fungicides on environment and food chain has led scientists around the world to pay more attention to finding biological plant protection products. Compared to synthetic fungicides, where it is necessary to repeat treatments in order to be effective, in biological protection, the advantage is given to living organisms` ability to inhabit ecosystems, establish

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balance and thus provide continuous natural/eco-friendly protection (Silva et al., 2019). Also, under certain conditions, microorganism-based preparations can be used in organic agriculture. Organic agriculture increased production by 21% from 2010 to 2015 in Europe, increasing demand for biological preparations for crop protection (Babec et al., 2019). *Trichoderma* biocontrol activity is of great importance not only for agriculture, but also for the environment, because it does not accumulate in the food chain, and thus there is no harm to plants, animals and humans (Mohiddin et al., 2017; Chalieu et al., 2019). Data from the scientific literature show that *Trichoderma* species, especially *T. harzianum*, have great potential in the biocontrol of *Fusarium* species, isolated from different plants (Bunbury-Blanchette & Walker, 2019; Zhi-xiang et al., 2020; Larran et al., 2020). On the other hand, Kifle et al. (2016) showed that *T. harzianum* has a significant antagonistic effect on *Aspergilus flavus*. This important fungus is of great importance not only in means of biocontrol, but also as a growth promoter and in bioremediation (Zin & Badaluddin, 2020).

It is well known that the composition of the medium for cultivation of microorganisms plays a significant role in the bioprocess (Ahamed & Vermette, 2009; Mehta et al., 2012). Also, carbon and nitrogen are essential for microorganisms and it is especially important to examine the influence of different C:N combinations and ratio on productivity of *Trichoderma* isolates (Gao et al., 2007; Singh et al., 2014). Calistru et al. (1997) also found that the antifungal activity of certain *Trichoderma* isolates against phytopathogenic fungi of the species *Fusarium moniliforme* and *Aspergillus flavus* depends on the carbon and nitrogen source present in the medium. Accordingly, the aim of this paper was to examine the influence of different carbon and nitrogen combinations in the medium for the *Trichoderma harzianum* production intended for the maize protection from pathogens, *A. flavus* and *F. graminearum*. Therefore, *T. harzianum* was used as the antagonistic microorganism in this study.

Material and Methods

Microorganisms

T. harzianum originating from the soil was used as the antagonistic microorganism. *T. harzianum* was isolated from soil sample according to method described by Tančić Živanov et al. (2017). The isolate is kept as a pure culture in the Microbial Culture Collection of the Institute of Field and Vegetable Crops, Novi Sad, Serbia. *T. harzianum* was identified according the isolates morphological characters and identification key of Samuels & Hebbar (2015).

In this study, the analysed fungal species *F. graminearum* and *A. flavus* were isolated from maize plants with typical symptoms of infection. Isolation of fungi was performed according to the method described in Tančić Živanov et al. (2017). The obtained monosporic isolates were incubated for three days on PDA (potato dextrose agar) slants at 20°C and afterwards kept in refrigerator at 4° C until use with regular revitalization every six months. Microorganisms are stored in the PDA medium in the Microbial Culture Collection of Faculty of Technology Novi Sad. *F. graminearum* isolate was morphologically identified according to Leslie & Summerell (2006), while isolate of *A. flavus* was identified according to Watanabe (2010).

Inoculum preparation

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

The obtained *T. harzianum* inoculum was further used to inoculate 20 experimental flasks with a different combination of carbon and nitrogen sources in the medium. On the other hand, after three days of cultivation, the obtained cultivation broth of *F. graminearum* and *A. flavus* was filtered through double layer of sterile cheesecloth and used for *in vitro* testing.

Experiment

In order to select the best carbon and nitrogen source in the medium for *Trichoderma* cultivation broth production, five different carbon sources and four nitrogen sources were selected. The selected carbon sources were: dextrose, starch, fructose, lactose and glycerol, while selected nitrogen sources were: soybean flour, yeast extract, NH_4NO_3 and $(NH_4)_2SO_4$. The combination of each carbon source with each nitrogen source was investigated, which totally included 20 experimental flasks.

Each carbon source was added to the medium in the amount of 25 g/L. On the other hand, nitrogen source was added in the amount of 2.5 g/L in medium used for growth of *T. harzianum*. In addition to combinations of carbon and nitrogen sources, the following components have been added in the medium for the *T. harzianum* production: KH_2PO_4 (2 g/L), KCl (0.5 g/L) and $MgSO_4$ x $7H_2O$ (0.5 g/L). The pH value of the media was adjusted to 6 before sterilization.

The experiment was performed in 100 ml Erlenmeyer flasks with 30 ml of medium and inoculated with a previously prepared *Trichoderma* inoculum in an amount of 10% relative to the amount of medium. The inoculum was cultured for 72 h on PDB medium. The production of *Trichoderma* cultivation broth was carried out at temperature of 25°C for 7 days of cultivation on a rotary shaker at 175 rpm (Al-Taweil et al., 2009; Singh et al., 2014).

Well diffusion method

Well diffusion method was applied in order to examine the activity of the *T. harzianum* cultivation broth on test isolates, *F. graminearum* and *A. flavus* (Grahovac et al., 2020). Three wells with a diameter of 15 mm represented one treatment. To each well 100 µl of cultivation broth pre-filtered on two layers of sterile cheesecloth was added. In control plates, 100 µl of sterile distilled water was added to wells. The formed inhibition zone diameters were measured after 7 and 14 days of incubation at 25°C.

Data analysis

The results obtained in this experiment were processed by factorial 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 the measured diameter of inhibition zones.

Results and discussion

Since the obtained results measured after 7 days of incubation compared to those measured after 14 days of incubation did not show a statistically significant difference, only the results measured after 14 days of incubation were selected for further analysis in this study.

The results presented in Table 1 show that the combination of different carbon and nitrogen sources in the medium for *T. harzianum* production had a statistically very significant effect on the formed inhibition zone diameters for both tested fungi ($p<0.01$). This is expected given that microorganisms have different affinities for different carbon and nitrogen sources and the C:N combination and ratio has an important influence on the efficiency of bioprocesses (Kobori et al., 2015). On the other hand, there is a statistically very significant difference between the sensitivity of both tested fungi on produced *Trichoderma* cultivation broth effect as well as interaction of these two factors. Also, the results show that the biggest source of variation of inhibition zone diameter occurs as a consequence of *A. flavus* and *F. graminearum* sensitivity to the produced *T. harzianum* cultivation broth. Namely, *F. graminearum* isolate showed significantly higher sensitivity to produced *T. harzianum* cultivation broth in all combinations of carbon and nitrogen in the medium than *A. flavus* isolate.

In order to obtain more information about differences in the significance of carbon and nitrogen sources combinations on the antifungal activity of the produced *T. harzianum* cultivation broth, a more detailed post-hoc analysis was performed using

Table 1. Results of factorial analysis of variance

Source of variation	Univariate Test of Significance for the 14th day Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degrees of Freedom	MS	F	P
Intercept	156746.4	1	156746.4	170996.1	0.0000
CN combination	2144.1	19	112.8	123.1	0.0000
Test fungi	21574.0	1	21574.0	23535.3	0.0000
CN * Fungi	1103.2	19	58.1	63.3	0.0000
Error	73.3	80	0.9		

Table 2. Duncan's test: mean values of inhibition zone diameter formed around wells for isolate *A. flavus*

C:N combination	Inhibition zone diameter (mm)	C:N combination	Inhibition zone diameter (mm)
G+SO_4	20.00±0.00 ^{a}	F+SO4	23.00±0.00 ^{cde}
G+NO_3	20.00±0.00 ^{a}	D+Y	23.00±0.00 ^{cde}
S+NO_3	20.33±0.58 ^{a}	L+NO_3	23.00±1.00 ^{cde}
S+SO_4	20.67±1.15 ^{ab}	G+Y	23.00±1.00 ^{cde}
L+SO_4	21.00±1.00 ^{ab}	F+Y	23.33±0.58 ^{de}
D+NO_3	21.33±0.58 ^{abc}	F+SF	24.00±1.00 ^{de}
D+SO_4	21.33±0.58 ^{abc}	G+SF	24.33±0.58 ^{e}
F+NO_3	21.33±0.58 ^{abc}	S+Y	24.67±1.15 ^{e}
L+Y	22.33±0.58 ^{bcd}	S+SF	24.67±2.08 ^{e}
L+SF	22.33±0.58 ^{bcd}	**D+SF**	31.00±1.00 ^{f}

*Values in same column followed with the same letter are not significantly different at 0.05 level
(D-dextrose, G-glycerol, F-fructose, L-lactose, S-starch,
Y-yeast extract, SF-soybean flour, NO_3-NH_4NO_3 and SO_4-$(NH_4)_2SO_4$)

Table 3. Duncan's test: mean values of inhibition zone diameterformed around wells for isolate *F. graminearum*

C:N combination	Inhibition zone diameter (mm)	C:N combination	Inhibition zone diameter (mm)
G+SO_4	29.00±0.00 [a]	S+NO3	51.67±2.08 [f]
D+NO_3	41.33±0.58 [b]	G+Y	53.00±0.00 [fg]
F+NO_3	41.67±1.15 [b]	D+Y	54.33±0.58 [gh]
G+NO_3	42.33±0.58 [bc]	D+SO_4	54.67±1,15 [hi]
L+SF	43.67±1.15 [c]	F+SO_4	55.00±0.00 [hi]
L+Y	46.00±1.00 [d]	L+NO_3	55.00±0.00 [hi]
F+Y	47.33±0.58 [de]	L+SO_4	56.00±0.00 [hi]
S+SO_4	48.00±0.00 [e]	S+Y	56.00±1.00 [hi]
G+SF	51.67±1.15 [f]	S+SF	56.33±0.58 [i]
F+SF	51.67±1.15 [f]	**D+SF**	56.33±0.58 [i]

*Values in same column followed with the same letter are not significantly different at 0.05 level
(D-dextrose, G-glycerol, F-fructose, L-lactose, S-starch,
Y-yeast extract, SF-soybean flour, NO_3-NH_4NO_3 and SO_4-$(NH_4)_2SO_4$)

Duncan's multiple range test. Tables 2 and 3 show the results of Duncan's multiple range test obtained for *A. flavus* and *F. graminearum*, respectively.

Due to the fact that all inhibition zone diameters larger than 22 mm show that the applied agent is highly effective (Tadijan et al., 2016), from the results obtained for isolate *A. flavus* (Table 2) it can be concluded that more than half of the tested C:N combinations proved to be good choice in the medium for the production of *T. harzianum* cultivation broth. Certainly, it can be noticed that the application of inorganic nitrogen sources in the medium for the production of *T. harzianum* cultivation broth effective against maize pathogen *A. flavus* does not show a positive effect. On the other hand, the reason for the formation of larger inhibition zone diameters by using organic nitrogen sources may be in their composition. Namely, soybean flour and yeast extract contain a number of amino acids, which may be responsible for better production of *T. harzianum* (Kobori et al., 2015). The most important amino acids that can be found are leucine, arginine, lysine, isoleucine, asparagine and glutamine (Tadijan et al., 2016).

Furthermore, given that PDA medium, the standard medium for fungal growth, contains dextrose and starch, it was expected that these carbon sources would show the best production in this experiment. However, it is important to analyse their mutual influence on the production of *Trichoderma* cultivation broth, and based on Duncan's test, it can be observed that the combination of dextrose and soybean flour (D+SF) in the medium has the best effect on the production of *Trichoderma* cultivation broth effective against pathogen *A. flavus*, isolated from maize (marked with a lowercase letter *f*). Rajput et al. (2014) have also proven that dextrose is one of the best carbon sources in the medium for growth of *T. harzianum*. By applying this medium composition, the largest inhibition zone on *A. flavus* isolate reached the value of 31 mm.

Analysing Table 3, which shows the results of the Duncan's test for isolate *F. graminearum*, it can be observed that different combinations of carbon and nitrogen sources in the medium for the *Trichoderma* cultivation broth production showed a significant effect on this tested isolate (all inhibition zone diameters were larger than 22 mm). Therefore, the potential application of any C:N combination would give the desired result. Certainly, it can be noticed that in the experiment with *F. graminearum*, as well as with *A. flavus*, the weakest result was shown by a combination of glycerol and $(NH_4)_2SO_4$ (G+SO_4) in the medium for *T. harzianum* production.

In the experiment with *F. graminearum* the largest zone of inhibition was formed by the application of dextrose and soybean flour (D+SF) in the medium, forming the largest zone of 56.33 mm. However, in addition to the combination of D+SF, medium with the combination of D+SO_4, F+SO_4, L+NO_3, L+SO_4, S+Y and S+SF (marked with a lowercase letter *i*) are at the same level of significance. Mehta et al. (2012) have also proved that dextrose is the best carbon source in the medium for *Trichoderma* production.

The same result obtained for the best medium formulation (D+FS) for the production of *Trichoderma* cultivation broth effective on both tested maize pathogens significantly facilitates the selection of the medium for cultivation.

Conclusions

The results of this study showed that combination of different carbon and nitrogen sources in the *T.*

harzianum cultivation medium statistically significantly affects the production of *Trichoderma* cultivation broth effective on the tested isolates *A. flavus* and *F. graminearum*. Combination containing dextrose as a carbon source and soybean flour as a nitrogen source proved to be the best medium formulation for production of *T. harzianum* cultivation broth effective on two maize pathogens, *A. flavus* and *F. graminearum*, forming maximal inhibition zone diameters of 31 mm and 56.33 mm, respectively. Selecting the best combination of carbon and nitrogen sources in the medium for the production of biocontrol agent is an important step for further research in which a medium of low market value (e.g. wastewater) would be selected based on the obtained results. Using a low-cost medium instead of a well-defined, synthetic medium would significantly reduce the production cost, making the potential product more competitive in the market.

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Uticaj različitih kombinacija izvora ugljenika i azota u podlozi za proizvodnju biokontrolnog agensa *Trichoderma harzianum*

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Sažetak: Sve veća upotreba hemijskih sredstava za zaštitu bilja poslednjih godina postala je ozbiljan problem. Upotreba korisnih mikroorganizama umesto sintetičkih fungicida u biološkoj zaštiti sigurno je jedno od rešenja koje doprinose zaštiti životne sredine i ljudskog zdravlja. S obzirom na to da se gljive *Aspergillus flavus* i *Fusarium graminearum* pominju kao najvažniji prouzrokovači bolesti kukuruza, proizvođači mikotoksina, u ovom radu ispitan je antifungalni efekat *Trichoderma harzianum* na obe fitopatogene gljive. Cilj ovog rada je ispitati uticaj različitih kombinacija ugljenika i azota u podlozi za proizvodnju *T. harzianum*. Kultivacija *T. harzianum* izvedena je u erlenmajerima na rotacionoj tresilici, a aktivnost dobijene kultivacione tečnosti na odabrane fitopatogene kukuruza ispitana je difuzionom metodom sa bunarima. Rezultati ovog rada pokazali su da kombinacija različitih izvora ugljenika i azota u podlozi statistički značajno utiče na proizvodnju *Trichoderma* kultivacione tečnosti efikasne na dve testirane fitopatogene gljive. Kombinacija koja sadrži dekstrozu kao izvor ugljenika i sojino brašno kao izvor azota pokazala se najboljom za formulaciju podloge za proizvodnju *T. harzianum* kultivacione tečnosti efikasne na patogene kukuruza, *A. flavus* i *F. graminearum*, formirajući maksimalne prečnike zona inhibicije od 31 mm, odnosno 56,33 mm.
Ključne reči: *Aspergillus flavus*, biokontrola, ugljenik, dekstroza, *Fusarium graminearum*, podloga, azot, sojino brašno, *Trichoderma harzianum*, difuziona metoda sa bunarima