Effect of exudates excreted by *Trichoderma harzianum* on the nitrogen fixation (C2H2- reduction) rate of the cyanobacteria *Anabaena variabilis*

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Abstract

Exudate of the fungus *Trichoderma harzianum* stimulate the nitrogen fixation (C2H2-reduction) of the cyanbacteria *Anabaena variabilis*. Autoclaving the exudates caused a decrease in the stimulation of the nitrogenase activity.Chemical analysis of the exudates showed it contain 95µg ml^{-1} protein, no free amino acids were detected, no nucleotides or nucleosides were found. Acid hydrolysis of the exudates showed the presence of bound amino acids and ammonia.

Low concentrations (one n mole ml^{-1}) of glutamic acid, aspartic acid, valine, glycine , and serine enhanced the nitrogenase activity and increased the heterocyst frequency of the cyanobacteria while threonine and proline seemed to reduce (inhibit) the nitrogenase activity at the same concentration.

دراسة تأثير افرازات الفطر *Trichoderma harzianum* على كفاءة تثبيت النتروجين الجوي (C2H2-reduction) للبكتريا الخضراء المزرقة *Anabaena variabilis*

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الخلاصة

تمت دراسة تأثير افرازات الفطر *Trichoderma harzianum* على تنشيط عملية تثبيت النتروجين الجوي للبكتريا الخضراء المزرقة *Anabaena variabilis* حيث وجد ان التركيز المخفف 1/5000 اعطى اعلى زيادة في فعالية أنزيم النتروجينيز (200%) وزيادة في اعداد وتردد الخلايا المتخصصة بمعدل 37% (Heterocyst) لاتمام عملية تثبيت النتروجين الجوي, فيما لم يكن هناك تأثير معنوي على زيادة في نمو البكتريا الخضراء المزرقة.ان دراسة تأثير الحرارة العالية (التعقيم الحراري الرطب) على مكونات الافرازات الفطرية وتحليل مكوناتها الكيمياوية بينت طبيعتها البروتينية حيث انخفض تأثيرها المحفز بنسبة 60% , ولم يظهر التحليل الكيمياوي وجود احماض امينية حرة او نيوكليوتيدات او نيوكليوسيدات , وان التحليل الحامضي للافراز الفطري بين وجود احماض امينية مرتبطة اظافة الى الامونيا.

ان معاملةالمزارع البكتيرية بثمانية احماض امينية (تركيز 1 نانومول /سم^3) بصورة منفردة ادى الى زيادة معنوية في فعالية انزيم النتروجينيز وتردد الاجسام المتخصصة (Heterocyst) من قبل الاحماض الامينية الاتية: حامض الكلوتاميك, حامض الاسبارتيك, فالين, الكلايسين, السيرين و الالينين, في حين كان لحامضي الثريونين والبرولين تأثير مثبط ولنفس التركيز.

Introduction

In an earlier communication it was reported that the fungi *Trichoderma harzianum Rifai* and *Aspergillus flavus* link ex.Fries affect the nitrogen fixation and growth of the cyanobacteria *Anabaena variabilis* (kűtz). Stimulatory as well as inhibitory agent were released by the fungi , and the stimulating agents could be separated by dialysis from the fungal medium and were thus found to be of higher molecular weight than the inhibiting agents (1).

Exudates from the fungus *Sclerotinia sclerotiorum* (Lib) De Bary have been throughly investigated and were found to contain proteinaceous compounds , free amino acid ,ammonia and enzymes (2,3). Later (4) found soluable carbohydrates and different salts in the exudates excreted by the fungus *Fusarium culmorum* (W.G.Smith) Saccardo.

In this study exudates excreted by *Trichoderma harzianum* were collected and tested with regard to their influence on the nitrogen fixation rate of the cyanobacteria *Anabaena variabilis*.

Material and Methods

Microorganisms and culture condition:

The cyanobacteria *Anabaena variabilis* 1403/12 (Cambridge culture collection of algae & protozoa) was grown axenically on modified nitrogen –free ASM medium (5)supplemented with trace elements (6).The cyanobacteria was incubated at 26c˚ and 62 µEm $^{-2}$ S $^{-1}$.The fungus *Trichoderma harzianum rifai* ,was grown for 14 days on malt extract agar (difco),in the dark at 28c˚. All further experiments were conducted at 26c˚ and 62 µEm $^{-2}$ S $^{-1}$.

Collection of fungal exudates:

When *T. harzianum* had grown for 14 days on malt extract slants, exudates had accumulated as yellowish watery droplets on the fungal surface . Such exudates were collected with Pasteur pipettes , and were then diluted with distilled water up to 5 or 10 ml using sterile conditions . The diluted exudates was then passed through Sartorius Millipore filters (0.2µm) in order to remove the fungal spores which might have adhered to the pipettes during the collection of the exudates ,1ml of 10 times diluted fungal exudate was analysed for free and combined amino acid contents .The samples were hydrolyzed for 24 hours by 6 N HCL, and the concentrations of the amino acids determined using Durrum D500 amino acid analyser.

Cyanobacteria nitrogenase activity (C2H2-reduction):

The nitrogenase activity was determined according to the method of Stewart *et al.* (7,8) . Acetylene gas equal to 10% of the total volume of the incubation flask was injected . After one hour , one

ml gas phase samples were withdrawn and analyzed for concentration of ethylene produced using Perkin – Elmer 880 gas chromatograph fitted with a Porapak T (50-80 mesh) , column run at 100c˚ . The flasks contained were aerated before and after each nitroginase activity measurement . Experiments were run accordingly , testing:

a) Effects of fungal exudates on cyanobacterial nitrogenase activity:

Effects of the exudates were studied by incubating 0.5 ml of an axenic cyanobacterial culture in 7ml , sterile glass serum bottles containing one ml ASM medium . 0.5 ml of sterile diluted fungal exudates being untreated or autoclaved , was added in a separate series . Nitrogenase activity (C2H2-reduction) was measured every 24 hours.

b) Effects of amino acids on nitrogenase activity , growth and heterocyst frequency:

Aliquots of an axenic cyonobacterial culture suspension were diluted to one n mole/ ml as a final concentration of the following amino acids:aspartic acid , threonine, serine , glutamic acid, proline , glycine, alanine and valine. The experiment was carried out in 70ml Erlemyer flasks containing 10 ml of cyanobacterial suspension . Nitrogenase activity was measured every 24hours ,chlorophyll a concentration ,heterocyst frequency was determined after 72hours.

Results & Discussion

Diluted fungal exudates had stimulatory effects on the nitrogen fixation (C2H2-reduction) rate of the cyanobacteria studied (Fig.1). Maximum effects was after 72 hours of treatment . The sterile , nonautoclaved exudates increase the nitrogenase activity by 200% comparing with the untreated control while the autoclaved exudates increase the activity rate by 40% ,this result indicate presence of more than one stimulatory factors in the fungal exudates , one is proteniaceous in nature, while the more effective once is the nonproteinaceous, Colotelo. *et al*. 1971(2) has characterized the chemical constiluents of exudates secreted by the fungus *S. sclerotiorum*.

The chemical analysis of the exudates shows that the total protein content was 95µg ml^{-1} , no free amino acids were detected . Acid hydrolysis of the exudates for 24hours showed presence of bound amino acids (table 1) and 1.25 µ moles ml^{-1} bound ammonia . These results partly agree with (2) and (8) who found free and bound amino acids and free ammonia in the exudates of *S. sclerotiorum* .Paper chromatography of the exudates

indicated no nucleotides and nucleosides when examined visually under an ultraviolet irradiant lamp according to the method of Bednar (9).

The fungal exudates had to be diluted before maximum effects on nitrogen fixation could be obtained. Out of the concentration tested , the dilution of 1:5000 gave the highest increase in nitrogen fixation , 50% higher than the control after 72 hours of treatment ,also the heterocyst frequency was increased by 44% (unpublished data).

Treatment of the cyanobacterial culture *A. variabilis* with one n mole ml^{-1} of eight amino acids seperatly ,which was found in the hydrolyzed exudates of the fungus *T. harzianum* showed that glutamic acid, aspartic acid ,valine, glycine, serine and alanine increase the heterocyst frequency and nitrogen fixation (Table 2), wherase proline and threonine acted as inhibitors.

No significant effect on growth of cyanobacteria at the end of the experiment was observed (Table 2) . A conclusion derived that low concentration of some kind of amino acids can act as stimulatory agents for nitrogen fixation of cyanobacteria .The above result confirmed the correlation between the nitrogen fixation and heterocyst frequency which has been shown in this association between *A. variabilis* and *T. harzianum* (1) . It is possible that the fungal exudates may serve as an inducer for heterocyst initation by breaking down the inhibitory zome described by (10) and (11), thus leading to an increase in heterocyst frequency and nitrogenase activity.

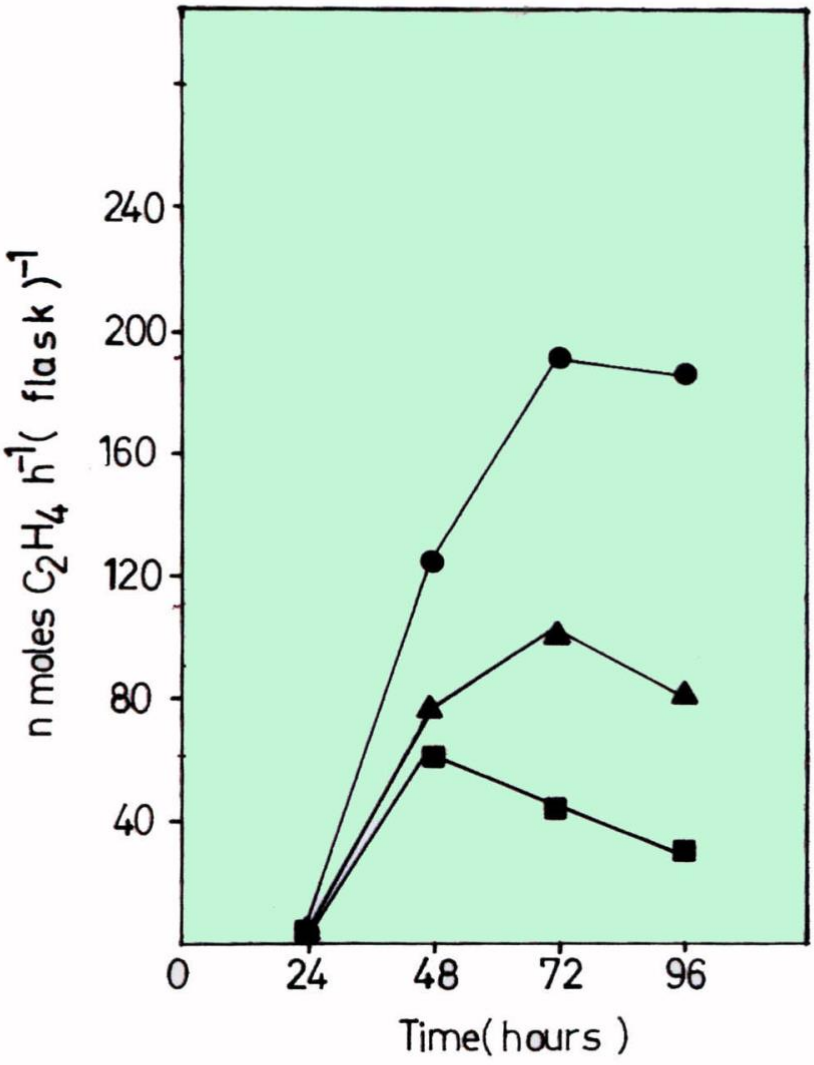

Fig. 1. Effect of the exudate excreted by the fungus *T. harzianum* on the nitrogen fixation (C_2H_2-reduction) of the cyanobacteria *A.variabilis*. ■—■ control, ●—● sterile filtered exudate, ▲—▲ autoclaved exudate.

Table 1 : Amino acids found in the hydrolyzed exudates excreted by the fungus *T. harzianum*

Amino acids	n moles ml^{-1}
Aspartic acid	96
Threonine	75
Serine	101
Glutamic acid	109
Proline	65
Glycine	112
Alanine	103
Valine	53
Methionine	7
Isolucine	30
Lucine	39
tyrosine	13
Phenylalanine	19
Histidine	12
Lysine	21
Arginine	12
Total	867 n moles

Total ammonia concentration =1250 n moles ml^{-1}

Table 2 : Nitrogen fixation (C2H2- reduction) , heterocyst frequency and growth of the cyanobacteria *A. variabilis* treated with one n mole of 8 amino acids separately

Amino acids	* µg Chlor. a ml^{-1}	** % of heterocyst	*** N-ase activity n moles h^{-1}/ µg Chlor. a
Control	5.78	4.6	8.02
Glutamic acid	6.14	8.0	17.40
Aspartic acid	5.79	7.2	10.28
Serine	5.69	5.8	14.24
Threonine	5.81	5.6	6.18
Valine	6.06	6.6	15.54
Glycine	5.78	6.8	10.48
Alanine	5.87	5.5	9.02
Proline	5.85	5.0	5.72

* the result are of three replicates for each treatment and after 160 hours of starting the experiment.

** Heterocyst are percentage of 1000 counted cells.

*** Maximum nitrogenase activity after 41-65 hours of treatment.

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