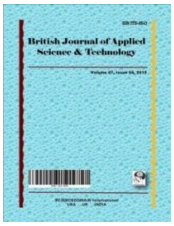

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# Growth and Phytohormones Production by Thermophilic *Aspergillus fumigatus* 2 and Thermotolerant *Aspergillus terreus* 8 Strains in Salt Stress

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***Authors' contributions***

*This work was carried out in collaboration between both authors. Both authors designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript and managed literature searches. Both authors read and approved the final manuscript.*

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## ABSTRACT

Few investigations have been made the biodiversity and biological properties of fungi in natural high salt and thermal environments. Halophilic and thermophilic fungi belong to extremophiles, which can survive in salt-affected habitats. Thermophilic and thermotolerant fungi are little known for indole-3-acetic (IAA) and gibberellins (GAs) production and their growth, development in salt-affected (salt-stress) environments. Aim of the current studies was to investigate the IAA and GAs synthesis by the thermophilic *A. fumigatus* 2 and thermotolerant *A. terreus* 8 strains, and their behavior in salt-stress. These fungal strains exhibited maximum IAA and GAs production and growth under different salt (NaCl and $Na_2SO_4$) concentrations. Furthermore, the fungal strains were able to synthesize the phytohormones in elevated temperatures – *Aspergillus fumigatus* 2 in 55ºC, *Aspergillus terreus* 8 in 45ºC. Salt tolerance estimations showed that *Aspergillus terreus* 8 was salt

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tolerant and exhibited ability to produce the phytohormones in the presence of salts. This is a first report on the isolation and description of thermophilic and thermotolerant *Aspergillus*, which synthesize IAA and GAs in the presence of NaCl and $Na_2SO_4$. In relatively low salt concentrations *A. terreus* 8 synthesized the phytohormones nearly as the same as in the control, but in presence of 0.5-1% salts, the synthesis of both metabolites sharply reduced, first of all, the production of GAs.

*Keywords: Thermophilic and termotolerant fungi; Aspergillus fumigates; Aspergillus terreus; indole-3-acetic acid; gibberellins; salinity.*

## 1. INTRODUCTION

Few investigations have been made regarding the biodiversity and occurrence of fungi in natural high salt and thermal environments in literature [1]. *Aspergillus fumigatus* and *Aspergillus terreus* have been isolated repeatedly and with high frequency from salty soil samples as well as from hypersaline water samples, with salinities ranging from 3-15% NaCl [1-4]. *A. fumigatus* is a widespread thermophilic and xerotolerant species, known to occur naturally on decomposing, self-heating plant material and organic debris from which it releases a high number of spores into the atmosphere [3]. It also produces several mycotoxins, the most known being fumitremorgins A and B and verruculogen [3], but little data exist on phytohormones – gibberellic acid (GAs) and indole-3-acetic acid (IAA) production of thermophilic *A. fumigatus*and thermotolerant (not mesophylic) *Aspergillus terreus*. Recently, Abdul Latif Khan et al. [5] reported synthesis of gibberellins by endophytic *A. fumigatus*.

Salinity is a major problem in many agricultural countries. It was estimated that about half of the arable land will be affected by salt stress by the year 2050 [6]. Saline soils possess high levels of sodium ($Na^+$) cations, and chloride ($Cl^-$) and sulphate ($SO_4^{-2}$) anions content [7]. Consecutively, presence of the salts in soil exerts abiotic stress not only to plants, but also on microorganisms, present therein. Salinity induce osmotic and ionic imbalance inside plant's cell, thus affecting plant growth and metabolism. Studies have been made on the biodiversity and occurrence of halophilic fungi [8,9,10] of different genus. Salt-tolerant fungi belong to extremophiles which can survive under the conditions of zero to high salinity. However, using salt-tolerant fungi to produce new secondary metabolites at high salinity was rarely reported [11-15]. In order to investigate the effect of high salt stress on fungal secondary metabolites, some authors tested even 10% salt with *Aspergillus variecolor PT06-1 and A. terreus* PT06-2 strains [11,16].

Aim of this study was to research the IAA and GAs synthesis by the thermophilic *A. fumigatus* 2 and thermotolerant *A. terreus* 8 strains, the salts (NaCl and $Na_2SO_4$) affect on growth and development of these fungi, and the phytohormones production under salt stress.

## 2. MATERIALS AND METHODS

### 2.1 Microorganism (Fungal Strains)

*Aspergillus fumigatus* 2 and *Aspergillus terreus* 8 were previously isolated from the rhizosphere soil samples of cotton and were cultured and maintained at the laboratory culture collection of the Institute of Microbiology, Uzbekistan Academy of Sciences, Department of Mycology. The fungal isolates were maintained on potato dextrose agar at +4ºC and subcultured at 30-day intervals.

### 2.2 Medium

Throughout the research, Czapek' medium with different modifications, in which sucrose (Suc), starch extract (SE), molasses (Mol) and glucose syrup (Glu Syr) were used as carbon source. Indole-3-acetic acid and gibberellic acid were produced on synthetic Czapek' broth media, and 0.1% tryptophan was added to the IAA production medium. The media was distributed to 250 ml Erlenmeyer flasks that contained 100 ml of medium and was sterilized in an autoclave at 121ºC and 1.5 atm pressure for 15 min [2].

### 2.3 Incubation

*Aspergillus fumigatus* 2 and *Aspergillus terreus* 8 ($2 \cdot 10^7$ spores/ml) were inoculated into 100 ml of production medium and incubated at 45ºC in dark conditions on a rotary shaker (150 rev min-1). After the incubation, indole-3-acetic acid and

gibberellic acid production and growth were measured [2].

## 2.4 Determination of Growth

The amount of growth in cultures was calculated as dry weight (re-cast). The growth media were filtered through pre-weighed filter paper, the biomass dried in an incubator at 30ºC for 24 h and weighed.

## 2.5 Physiological Conditions Affecting the Production

*Aspergillus fumigatus* 2 and *Aspergillus terreus* 8 were grown in a rotary shaker (150 rpm) under dark conditions at 45ºC [2]. The effect of temperature was studied by incubating the media at different temperatures. To determine the optimal pH values for indole-3-acetic acid and gibberellic acid productions, media with different pH values (3.0-7.0) were used. The incubations were carried out at $45^0$C in both sets; the first set was agitated at 150 rpm while the second was static.

## 2.6 Salt Tolerance of the Strains

Salt tolerance of the isolates was checked by inoculating the cultures in triplicate on Czapek' amended with salt up to concentrations of 10% w/v [3,4]. Growth was recorded after 7 days of incubation, in terms of colony diameter; plates that did not show growth in 7 days were further incubated for 15 days to check for any delayed growth and then sub-cultured onto corresponding media to confirm the salt tolerance level. Salt tolerance curves were obtained by plotting the arithmetic mean of colony diameter with the standard error.

## 2.7 IAA and GAs Determination

The culture liquids of fungal isolates, obtained via incubation in the L-tryptophan amended Czapek' broth were centrifuged at 3000 rpm for 30 min [7]. The supernatant (2 ml) was mixed with two drops of orthophosphoric acid and 4 ml of Salkowski reagent (50 ml, 35% of sulphuric acid, 1 ml 0.5 M $FeCl_3$). Intensity of the color was measured using a spectrophotometer at 530 nm. Concentration of IAA produced by cultures was measured using standard graph of IAA obtained in the range of 0.5 – 10 ppm.

Culture liquids were filtered for elimination of fungal biomass, then the pH value of supernatant was adjusted to 2.5 using stock (37%) HCl. The Supernatant was extracted using liquid-liquid ethylacetate/$NaHCO_3$. The amount of gibberellic acid in the ethylacetate phase was measured by the UV spectrophotometer (JENWAY 6105) at 254 nm [2].

For salt influence on the IAA and GAs synthesis the strains were incubated in the salt amended Czapek' broth in a shaker at 45ºC [2].

Statistically significant difference ($P<0.05$) on the effect of different salt concentrations on growth of all isolates, as well as in growth within the species, was analyzed by two-way ANOVA.

# 3. RESULTS AND DISCUSSION

IAA and GAs are secondary metabolites of many rhizospheric bacteria and fungi, and these compounds are usually produced near the end of the growth phase and during the stationary phase. In bacteria, IAA basically is synthesized in the relatively young cultures, whereas GAs are noticed usually in the old cultures [7]. Furthermore, as it is documented by some authors [7], the synthesis of these biologically active substances a takes long time. GAs production is maximized after the second week of incubation, whereas IAA production is usual at maximum during the first week of incubation. In similar studies on IAA and GAs production in various fungi, incubation times between 10 and 18 days were used [17]. In another study, the synthesis of IAA was maximized after 30 days of incubation [18]. Therefore, it was expected that the production times of these plant regulators were long.

*A. fumigatus* 2 and *A. terreus* 8 synthesized basic amount of IAA in the first week of incubation. After 6-7 days the IAA synthesis slowed down and almost stopped (Fig. 1). As for *A. fumigatus* 2 the maximal synthesis was observed in the Czapek'-sucrose (Suc) and Czapek'-sucrose-molasses (Suc+Mol) media. In both media the strain synthesized 146 mg/l IAA in day 7.

Generally, between day 5-7, *A. fumigatus* 2 yielded almost all of the synthesized IAA. The same was observed with *A. terreus* 8. But, the strain synthesized higher amounts of IAA in the Czapek'-sucrose (Suc) and Chapeck-sucrose-glucose syrup (Suc+GluSyr) media. In these media *A. terreus* 8 synthesized 108 and 129,5 mg/l IAA in day 7. Like *A. fumigatus* 2, *A. terreus* 8 also yielded substantial amounts of IAA between day5-7 of incubation.

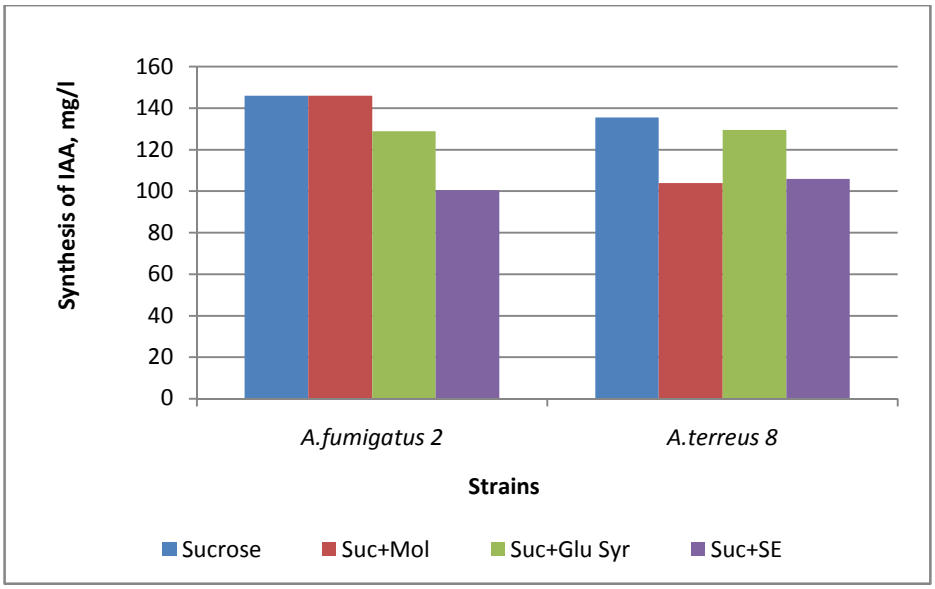


**Fig. 1. Synthesis of IAA by the fungal strains in different substrates**

Earlier, *A. fumigatus* was reported to promote plant growth and their pure culture had also secreted bioactive gibberellins [5]. The well-known functions of GAs are to promote vegetative growth, elongation of stems and roots, and the expansion of leaves [19]. In our research, both strains were active in GAs synthesis, and after the second week of incubation in the culture liquids were detected maximal production of GAs. It is necessary to point out the substrate dependent production of GAs. The maximal synthesis was observed when sucrose, sucrose-glucose syrup and sucrose-molasses were used as carbon source for the strains. In the Czapek'-sucrose (Suc) broth *A. terreus* 8 produced a total of 1.21 µg/ml GAs in the second week of cultivation, whereas *A. fumigatus* 2 synthesized 1.07 µg/ml GAs in total. In the Czapek'-sucrose-glucose syrup (Suc+GluSyr) *A. terreus* 8 synthesized total 1.13, in the Czapek'-sucrose-molasses (Suc+Mol) – 1.19 µg/ml. As for *A. fumigatus* 2 total GAs production was as following –1.07 µg/ml in the Czapek'-sucrose (Suc), and 1.19 µg/ml in the Czapek'-sucrose-molasses (Suc+Mol).

One of the key factors in metabolite synthesis is pH of broth. Considering this, the strains were cultivated at different pH levels in order to reveal optimal conditions for each strain. Throughout the research, the strains were active at pH 5.5-6.5 and in all cultivation variants, the acidity of culture liquids dropped down to 5.0, which indicated the enhanced synthesis of IAA and GAs. In conclusion, optimum production conditions of IAA and GAs in *A. terreus* 2 and *A. fumigatus* 8 were set as following: 45ºC and pH 6.0 was suitable for *A. terreus* 8 in Czapek'-sucrose (Suc) broth, whereas 55ºC and pH 5.5-6.0 was an ideal for *A. fumigatus* 2. Obtained these results were common findings of previous studies, but temperature-depended synthesis for thermotolerant and thermophilic strains reported for the first time.

The strains were isolated from the salt-affected soils, where soil salinity reaches a maximum of 2%. Besides, in these soils sulphate salinity exceeds chloride salinity [7]. Microbes that inhabit high-salt environments may be of salt tolerant or halophilic nature, being adapted to high levels of ions, as well as to low $a_w$ [20]. Salt-effected environments have been a focus of study of salt-tolerant microorganisms that are able to survive in these environments.

The evaluation of salt-effects on fungal growth and development showed that *A. fumigatus* 2 was very sensitive even to low concentrations of salt. Both, in NaCl and $Na_2SO_4$ supplemented media, the strain didn't grow. However, *A. terreus* 8 can grow and develop in high concentrations of the salts. In concentrations from 0.1 to 1% *A. terreus* 8 developed nearly as in the control. The growth assays of *Aspergillus terreus* 8 in terms of fresh and dry biomass production in the presence of NaCl and $Na_2SO_4$ were carried out after seven days of incubation.

The data on fresh biomass formation revealed a decrease in growth of *Aspergillus terreus* 8 from control (without salts) (Figs. 2 and 3). Maximum decrease was observed when 5 g/100 ml NaCl and 5 g/100 ml $Na_2SO_4$, and 5 g/100 ml NaCl+5 g/100 ml $Na_2SO_4$ applied. In these concentrations the cell biomass decreased down to 58,8% (when 5 g/100 ml NaCl and $Na_2SO_4$ applied), whereas in 5g/100 ml NaCl+5 g/100 ml $Na_2SO_4$ – 46,4%. In concentrations from 0.1 to 1% *A. terreus* 8 developed nearly as in the control.

Increasing the concentration of salt further, led to biomass weight decreasing and shortening the colony diameter (Fig. 4). When the salt concentration reached 2%, the strain considerably lost its biomass and yielded an average of 56.5% biomass. In 2% the strain formed its 51.5% biomass. It is necessary to point out that in high concentrations of the salts (<2%) the hyphae of the strain developed as in the in control.

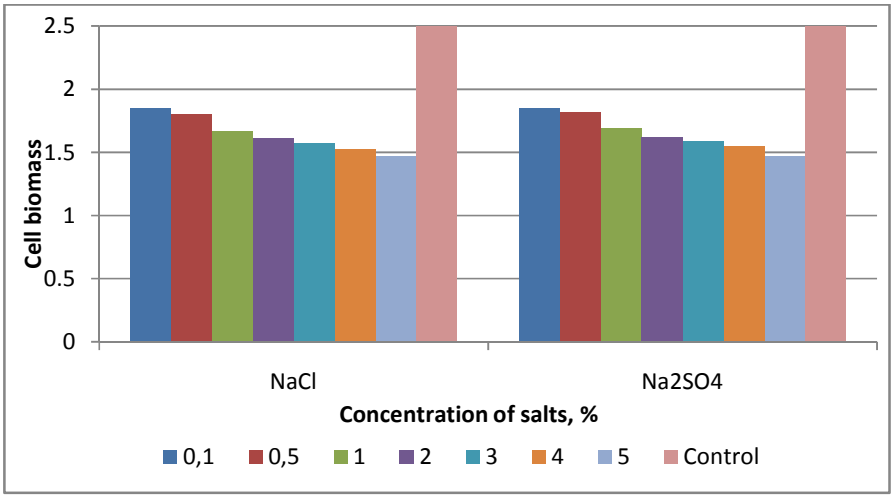


**Fig. 2. Cell biomass of *Aspergillus terreus* 8 in different salt concentrations**

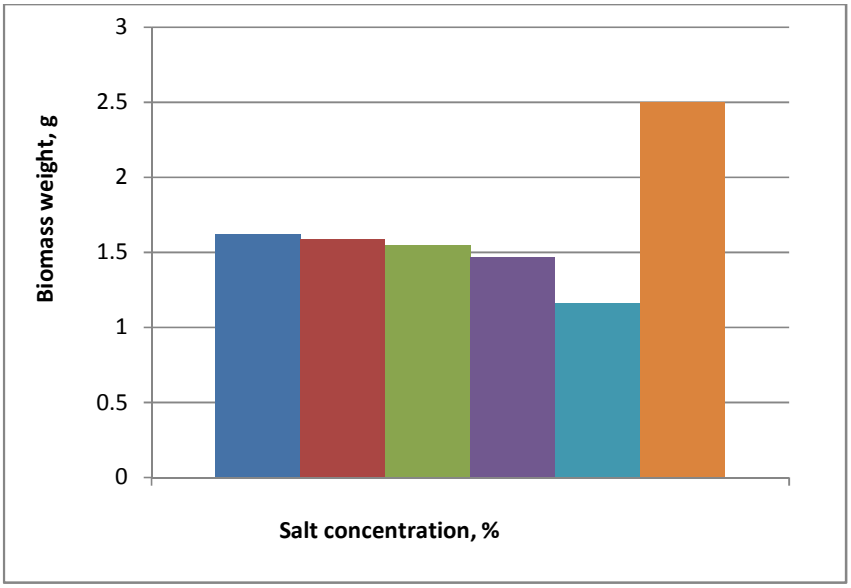


**Fig. 3. Cell biomass of *Aspergillus terreus* 8 in different concentrations of NaCl+$Na_2SO_4$**

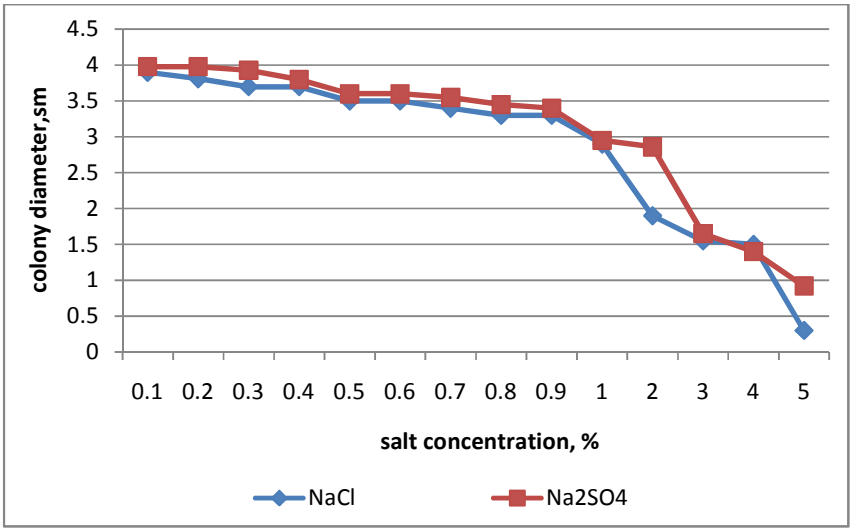


**Fig. 4. Single colony diameter of *A. terreus* 8 on solid Czapek' medium in different salt concentrations**

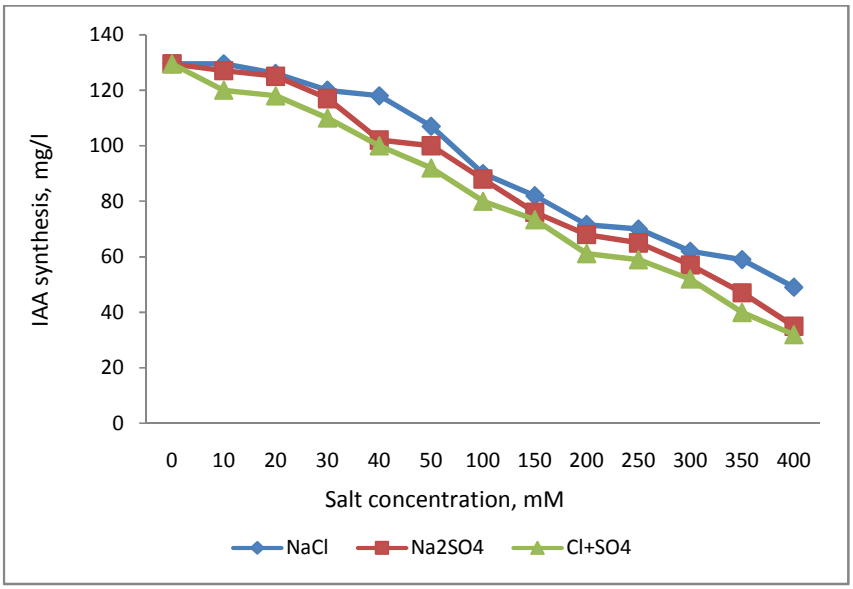


**Fig. 5. IAA synthesis by *A. terreus* 8 in different salt concentrations**

These findings almost correlate with earlier reports Gonsalves et al. [1] investigated halophilic fungal strains of *Aspergillus* and *Penicillium*. An analogical data was obtained by S. Nasmetova et al. [2] with halophilic *A. terreus*, isolated from the hyper saline environments, but the strain's salt-tolerance exceeded than that of thermotolerant *A. terreus* 8.

As it is known that salinity affects not only to growth and development of microorganisms, but also inhibits the secondary metabolites synthesis [7]. Other studies the salt-affected inhibition was reported both in bacteria and fungi. Earlier, Nasmetova S. M. et al. [2] reported isolation of halophilic *Aspergillus terreus*-9 and *Penicillium restrictum*-1 strains, which could not only survive and grew in high concentrations of NaCl, but also synthesized plant hormones such as indole-3-acetic acid and gibberellins. Similar case was observed with *A. terreus* 8 in different salts and concentrations. In relatively low salt concentrations *A. terreus* 8 produced nearly as the same as in the control, but in presence of

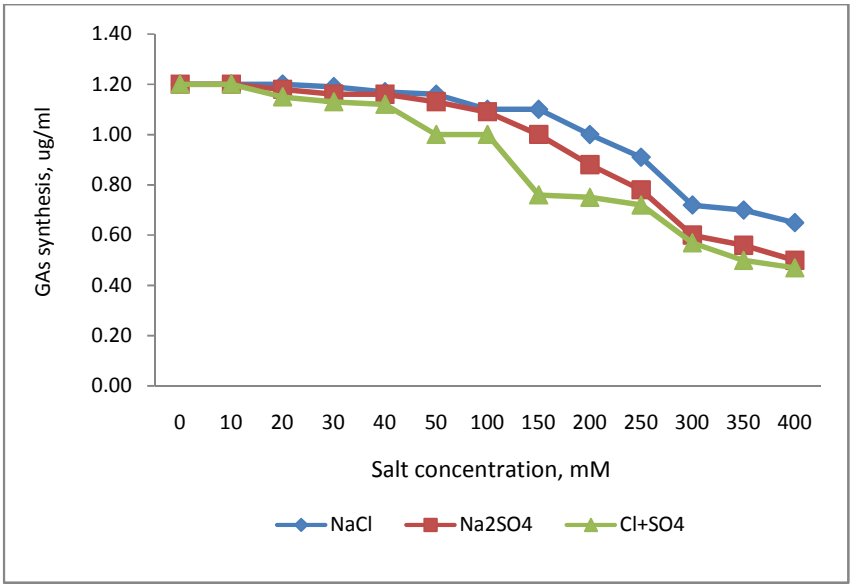


**Fig. 6. GAs synthesis by *A. terreus* 8 in different salt concentrations**

0.5-1% salts, the synthesis of both metabolites sharply reduced, first of all, the production of GAs (Figs. 5 and 6 above).

In higher salt concentrations (2-5%) no GAs were detected in the culture liquid, whereas the IAA synthesis continued. $Cl^-$ and $SO^{-2}_4$ ions effect on the synthesis was almost similar. This might be due to inhibition by the salts in the GAs synthesizing metabolic pathways.

## 4. CONCLUSION

This work demonstrates the occurrence of facultative salt-tolerant aspergilli in the salt-affected agricultural soils. This is a first report on the isolation and description of thermophilic and thermotolerant *Aspergillus*, which synthesize IAA and GAs in salt stress. It can be concluded that IAA production in *A. terreus* 8 is advantageous when compared to GAs. In relatively low salt concentrations *A. terreus* 8 produced nearly as the same as in the control, but in presence of 0.5-1% salts, the synthesis of both metabolites sharply reduced, first of all, the production of GAs. In concentrations from 0.1 to 1% *A. terreus* 8 developed nearly as in the control. But, further increasing the salt concentration led to biomass weight decreasing and shortening the colony diameter. It can be concluded that *A. terreus* 8 is able to growth and synthesize IAA and GAs in salt-affected soils when it was used as a biopreparation.

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## COMPETING INTERESTS

Authors declare that there are no competing interests.

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