

Gibberellin and auxin production by plant root-fungi and their biosynthesis under salinity-calcium interaction

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ABSTRACT

Rhizosphere and rhizoplane of fababean (*Vicia faba*), melochia (*Corchorus olitorius*), sesame (*Sesamum indicum*) and soyabean (*Glycine max*) plants are inhabited with fungi, mostly *Aspergillus flavus*, *A. niger*, *Fusarium oxysporum*, *Penicillium corylophilum*, *P. cyclopium*, *P. funiculosum* and *Rhizopus stolonifer*. All fungal species have the ability to produce gibberellin (GA) but *F. oxysporum* was found to produce both GA and indole-acetic acid (IAA). The optimum period for GA and IAA production by *F. oxysporum* was 10 days in the mycelium and 15 days in the filtrate at 28°C. The contents of GA and IAA were significantly increased at 0.5 and 1% NaCl after 5 days, but they were lowered at 4% (700 mM) NaCl. Cytochrome P-450 was significantly increased under salt stress at 0.5–7% NaCl. Calcium decreased NaCl stress on *F. oxysporum* by significant elevating GA biosynthesis at 40 mM Ca²⁺/700 mM Na⁺. GA at 10 µM and Ca²⁺ at 10 mM enhanced the germination of seeds under 175 mM Na⁺.

Keywords: GA; IAA; cytochrome; fungi; calcium; salinity

The microbial synthesis of plant growth regulators (gibberellin and auxin) is an important factor in soil fertility (Kampert and Strzelczyk 1975). Gibberellin (GA) and indole-acetic acid (IAA) are secondary metabolites, which are important biotechnological products, produced commercially from fungi for the agricultural and horticultural industry (Deacon 1984, Bruckner 1992). GA was isolated from *Gibberella fujikuroi* by T. Yabuta and IAA was isolated from *Rhizopus suinus* by K.V. Thimann in 1935. GA₃ is the dominant component of the gibberellin complexes isolated from fungi (Bozhkova et al. 1991). It is now generally agreed that GA and IAA are very widely distributed throughout the plant kingdom.

Salinity represents one of the most important factors exerting stress on fungal as well as plant cell metabolism. Kaur et al. (1998) found that GA₃ at 6µM concentration induced increased germination and seedling growth under salt stress. Calcium is particularly important nutrient in plants exposed to NaCl stress because of its role in maintaining the structural and functional integrity of membrane (Leopold and Willing 1984) and in cell wall extensibility (Taiz 1984).

No recent study explains the ability of filamentous fungi, associated with roots of plant crops, for the GA and IAA production and their role toward the host plant crops. Also, no studies explain the effect of salinity on the activity of rhizosphere and rhizoplane fungi and role of Ca²⁺ and GA in alleviation the salt stress.

The present work was commenced by collecting four plant crops for isolation of both rhizosphere and rhizoplane fungi, and screening their efficiencies for GA and IAA production. Also, effect of salinity on GA, IAA and cytochrome P-450 biosynthesis by *F. oxysporum* was studied. The roles of GA and Ca²⁺ in reversing the toxicity of salt stress effect in seedlings were also studied.

MATERIAL AND METHODS

Collection of samples. Ten samples of fababean (*Vicia faba*), melochia (*Corchorus olitorius*), sesame (*Sesamum indicum*) and soyabean (*Glycine max*) plants were collected from the Agriculture Garden of Assiut University during 1998. Samples of root-tips were collected from these plants, placed in plastic bags, and transferred immediately to the laboratory.

Determination of rhizosphere fungi. The samples were placed in sterile flasks containing a suitable amount of water to maintain the desired final dilution. The flasks were shaken by hand in a rotating motion for ten minutes. One ml of the rhizosphere soil suspension was transferred aseptically into 5 sterile Petri dishes with glucose-Czapek's agar medium. The dishes were rotated by hand in a broad swirling motion so that the suspension was dispersed in the agar medium, and then incubated at 28°C for 7 days.

Determination of rhizoplane fungi. The procedure described by Guzeva and Zvyagiutsev (1977) was adopted. The roots were subjected to repeated washings with sterile water. Thereafter they were thoroughly dried between sterile filter papers. The roots were blended with a suitable amount of water agar (2 g agar.L⁻¹ distilled water) to maintain the desired final dilution. One ml of the dilution was transferred aseptically into each of the 5 Petri dishes, with the glucose-Czapek's agar medium. The dishes were rotated by hand in a broad swirling motion so that the suspension was dispersed in the agar medium, and then incubated at 28°C for 7 days.

Identification of fungi. The developed colonies were examined microscopically and identified using the following references: Raper and Fennel (1965) for *Aspergillus*, Booth (1977) for *Fusarium*, Christensen and Raper (1978)

for *Emericella*, and Pitt (1985) for *Penicillium* species. The average number of colonies per dish was multiplied by the inverted dilution factor to obtain the number of colonies per g of fresh root.

GA and IAA production by fungi. Twenty isolates belonging to ten fungal species isolated from rhizosphere and rhizoplane were subjected to GA and IAA screening during this investigation. Fifty ml of 1% peptone and 1% glucose-Czapek's liquid medium in 250 ml Erlenmeyer flask was sterilized, inoculated with a disc of 1 week-old culture of each tested isolates and incubated at 28°C for 7 days as stationary cultivation. Extraction and analysis of GA and IAA in culture filtrates were carried out as detailed below.

Procedure for GA and IAA bioassay. The crude GA and IAA were tested on viability of fababean, sorghum and wheat seeds. The seeds were surface sterilized by shaking in a 3% NaOCl solution for 3 min and then rinsed in three changes of sterile distilled water. Thereafter, seeds were allowed to germinate in Petri dishes on 5 mL of 10 µM concentration of GA crude, GA₃ standard, IAA crude and IAA standard. Control seeds were germinated in sterilised water. The dishes were incubated in the dark at 25°C. Three dishes were used for each treatment and each contained 10–15 seeds. The germination percentage and length of shoot and root were determined after 3 days of growth. The viability of seedlings was determined by calculating the vigour index [VI = length of shoot + root (cm) × germination %] of the seeds.

GA, IAA and cytochrome P-450 production by *F. oxysporum* in NaCl-treated broth. Fifty ml of glucose mineral medium (g.L⁻¹: glucose 30, ammonium sulphate 2, KH₂PO₄ 3, MgSO₄ 0.5) were dispensed in 250 ml Erlenmeyer conical flask. NaCl was added to give final concentrations of 0.5, 1, 4, 7 and 10%. After autoclaving, flasks

were inoculated by 1ml of spore suspension of 7-day old culture of *F. oxysporum* and incubated at 28°C for 5, 10 and 15 days, statically. Three flasks were used for each concentration and control without NaCl. The culture filtrate was used for assaying GA and IAA. The mycelia were used for estimation of GA, IAA, and cytochrome P-450 as detailed below.

Effect of Ca²⁺ on GA and IAA production by *F. oxysporum* in NaCl-treated broth. The previous medium was supplied with 700 mM NaCl (4%) and different concentration of CaCl₂ at 10, 40 and 70 mM. The medium was sterilized and inoculated with *F. oxysporum*, and incubated at 28°C for 10 days statically. Three flasks were used for each treatment. The mycelia and filtrate, separately, were used for estimation of GA and IAA contents.

Effect of GA, IAA and Ca²⁺ on germination of NaCl-treated seed. The seeds were soaked in 10 µM GA, 10 µM IAA and 10 mM Ca²⁺ for 10 hours. Control seeds were soaked in 175 mM NaCl as control I and in sterile distilled water as control II for 10 hours. The soaked seeds were then placed in 5 ml of 175 mM NaCl and germinated in Petri dishes at 25°C. The seeds were evaluated after 3 days of imbibition for the emergence of radicle and plumule.

GA and IAA determination. The culture filtrate and mycelium homogenate, separately, were adjusted to pH 2.5 with 1 M HCl and then extracted with equal volume of ethyl acetate. The ethyl acetate layer was dried over Na₂SO₄, and evaporated in a rotary evaporator. The residue was taken up in acetone and developed on thin layer chromatography using isopropanol-ammonia-water (10:1:1, v/v/v). The plates were sprayed with the reagent (3% H₂SO₄ in methanol + 50 mg FeCl₃), heated in oven at 80°C for 10 min. GA showed greenish fluorescence under UV light. IAA showed violet-red colour in VL and orange in UV light after spraying. GA and IAA were spec-

Table 1. Fungal species in rhizosphere (Rs) and rhizoplane (Rp) of some plants (colony forming units/g fresh root wt)

Fungal species	Fababean			Melochia			Sesame			Soyabean		
	Rs	Rp	OR ^a	Rs	Rp	OR	Rs	Rp	OR	Rs	Rp	OR
<i>Aspergillus carneus</i> (V. Tiegh.) Blochwitz	0	0	–	390	0	M	0	0	–	0	0	–
<i>A. flavipes</i> (Bain & Sart.) Thom & Church	0	0	–	0	0	–	0	85	L	0	0	–
<i>A. flavus</i> Link	400	250	H	240	0	M	204	238	H	150	25	H
<i>A. fumigatus</i> Fresenius	0	0	–	120	1880	H	0	0	–	0	0	–
<i>A. niger</i> van Tieghem	400	120	H	1190	705	H	1360	1224	H	500	100	H
<i>A. tamarii</i> Kita	0	0	–	390	0	M	0	0	–	0	0	–
<i>Curvularia lunata</i> (Wakker) Boedijn	0	0	–	0	0	–	0	0	–	0	25	L
<i>Emericella nidulans</i> (Eidam) Vuillemin	0	0	–	0	120	L	0	0	–	0	0	–
<i>Fusarium oxysporum</i> Schecht	0	0	–	476	0	M	680	578	H	3000	225	H
<i>Penicillium corylophilum</i> Dierckx	30	50	M	715	590	H	0	0	–	1900	25	H
<i>P. cyclopium</i> Westling	0	0	–	240	0	L	0	0	–	250	50	M
<i>P. funiculosum</i> Thom	100	130	M	0	0	–	0	0	–	300	25	M
<i>Rhizopus stolonifer</i> (Ehrenb. ex Fr.) Lindt	5	5	H	240	0	M	68	68	H	100	25	H
<i>Trichoderma</i> sp.	0	0	–	0	0	–	0	0	–	100	0	L

^a average occurrence in rhizosphere and rhizoplane, H = high occurrence (> 50% samples), M = moderate occurrence (50–30% samples), L = low occurrence (< 30% samples)

Table 2. Production of gibberellin (GA) and indole-acetic acid (IAA) by different species of fungi

Fungal species	Source of isolation	GA (mg/50 ml)	IAA
<i>Aspergillus carneus</i>	melochia	10	0
<i>A. flavipes</i>	sesame	10	0
<i>A. flavus</i>	fababean	5	0
<i>A. flavus</i>	melochia	6	0
<i>A. flavus</i>	sesame	3	0
<i>A. flavus</i>	soyabean	3	0
<i>A. niger</i>	fababean	11	0
<i>A. niger</i>	melochia	12	0
<i>A. niger</i>	sesame	6	0
<i>A. niger</i>	soyabean	9	0
<i>A. tamaritii</i>	melochia	5	0
<i>Emericella nidulans</i>	soyabean	1	0
<i>Fusarium oxysporum</i>	melochia	10	5
<i>F. oxysporum</i>	sesame	12	6
<i>F. oxysporum</i>	soyabean	13	7
<i>Penicillium corylophilum</i>	melochia	10	0
<i>P. corylophilum</i>	soyabean	9	0
<i>P. cyclopium</i>	melochia	1	0
<i>P. cyclopium</i>	soyabean	7	0
<i>Rhizopus stolonifer</i>	sesame	10	0

trophotometrically determined according to Bruckner et al. (1991) and Wohler (1997), respectively. The values obtained were computed from the standard curves of GA₃ and IAA.

Cytochrome P-450 determination. The mycelia were removed, chilled on ice, washed with phosphate buffer (pH 7) and homogenized separately in 3 volume in the same buffer. The crude homogenate was centrifuged at 10 000 × g for 10 minutes at 4°C (Sheweita and Mostafa 1995). The supernatant fraction was used as the enzyme source. Cytochrome P-450 was measured by the method of Omura and Sato (1964) with modification. CO was carefully bubbled through the sample for about 20 seconds (20 bubbles) at 20°C; this was sufficient to saturate the sample with the gas. CO was prepared by dropping concentrated sulfuric acid on sodium formate and purified by bubbling through a NaOH solution. Reduction of samples was effected with a few milligrams of solid sodium metabisulfate.

Statistical analysis of the results. Data of each experiment were analyzed statistically using a program of one-way analysis of variance (PC-state computer program).

RESULTS AND DISCUSSION

Rhizosphere and rhizoplane mycoflora of crop plants.

Fourteen species belonging to seven genera were isolated as the fungal flora from fababean, melochia, sesame and soyabean rhizosphere and rhizoplane. *Aspergillus*

flavus, *A. niger* and *Rhizopus stolonifer* were common species isolated from all plants (Table 1). *Fusarium oxysporum* was common in melochia, sesame and soyabean. *Penicillium corylophilum* was common in fababean, melochia and soyabean. To our knowledge, no recent report exists on the incidence of mycoflora in rhizosphere and rhizoplane of these plants.

GA and IAA potentialities. Twenty isolates of filamentous fungi, isolated from rhizosphere and rhizoplane of plants, were tested for their GA and IAA production (Table 2). While all cultures produced GA in variable amounts, *F. oxysporum* represents the best producers of both GA and IAA. Isolates of *A. carneus*, *A. flavipes*, *A. niger*, *P. corylophilum* and *R. stolonifer* have the potential for production of GA in reasonable amounts. Previously, Hasan (1994a, b) found that several filamentous fungi were well known for their GA production.

The bioassay effect of GA and IAA on the seedling viability of fababean, sorghum, and wheat seeds is shown in Figure 1. GA at 10 µM increased the seedling viability, however IAA decreased it in comparison to those in water. GA may stimulate the hydrolytic enzymes promoting hydrolysis of storage reserves. The results show the importance of studying phytohormonal production when the interrelationships between plants and microorganisms are analyzed and may help explain the beneficial effects of fungi to the host plant. Bastian et al. (1998) had explained the beneficial effects of endophytic bacteria to the host plant. Karabaghli et al. (1998) found that the ectomycorrhizal and bacterial species enhanced the number of roots formed per rooted hypocotyl of spruce.

Optimization of GA and IAA production. The result in Figure 2 shows that, in glucose mineral medium at 28°C, the optimum growth of *F. oxysporum* was recorded on 10th day. The maximum quantity of GA and IAA was observed after 10 days and declined after 15 days in mycelium. However in filtrate, GA and IAA reached their maximum production after 15 days. The decline of GA and IAA in mycelium and rise of them in filtrate after 15 days may be due to the autolysis of mycelium in the late phase

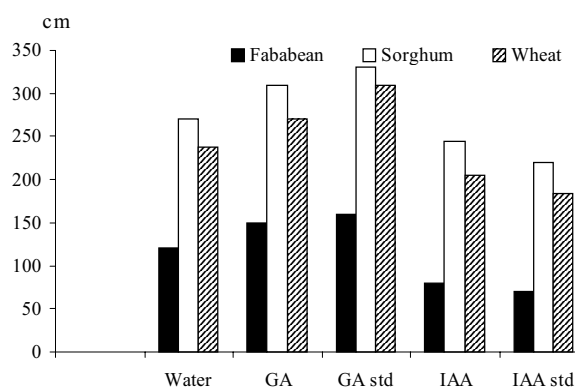


Figure 1. GA and IAA(10 µM) effect on seedling viability (cm) after 3 days of seed imbibition

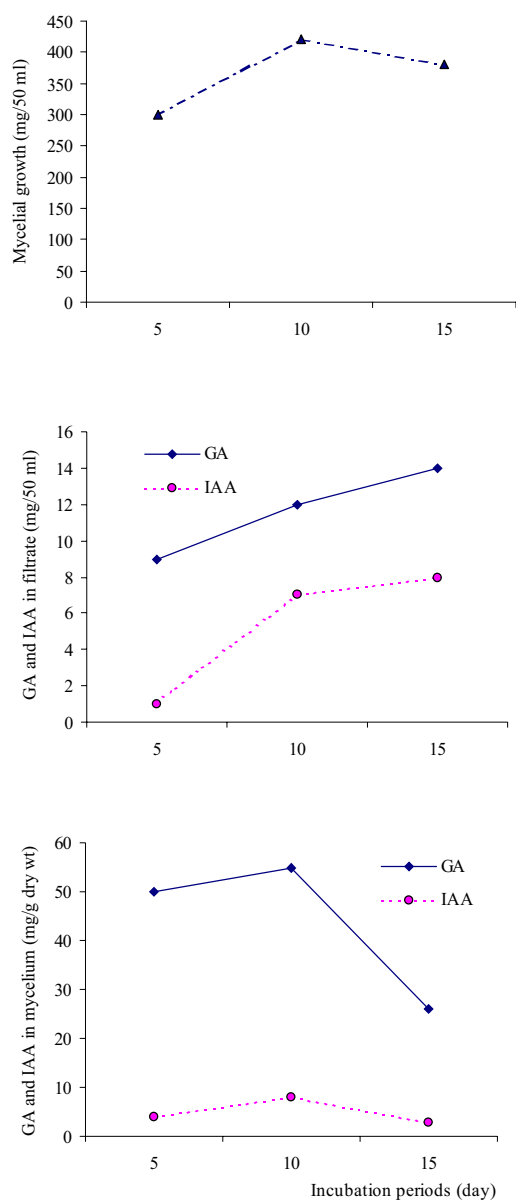


Figure 2. GA and IAA production by *F. oxysporum* in glucose mineral medium at 28°C

Table 3. Effect of NaCl on GA and IAA production by *F. oxysporum* at 28°C

NaCl (%)	Mycelial dry wt (mg/50 ml)			GA and IAA content												CA			
				filtrate (mg/50 ml)						mycelium (mg/g dry wt)									
	5 day		10 day		15 day		5 day		10 day		15 day		5 day		10 day		15 day		
																			GA
Control	331	435	390	10.8	0.7	11.5	7.0	13.5	8.0	49.0	4.0	53.5	6.0	27.7	1.7	0.4			
0.5	338	460	438	13.5*	1.3*	12.6	4.0	12.1	4.0	49.4	4.1	39.2	6.1	30.8	1.5	1.5*			
1.0	368	427	455*	11.8	2.0*	11.1	4.0	10.8	2.7	51.4	4.1	37.9	6.2	29.7	1.4	2.4*			
4.0	264	562*	535*	8.1	0	7.4	0	6.8	0	41.0	0	19.2	0	20.2	0	1.6*			
7.0	142	548*	544*	8.0	0	5.4	0	5.4	0	29.0	0	14.8	0	9.9	0	0.9*			
10.0	0	295	704*	0	0	5.0	0	4.1	0	0	0	13.7	0	5.8	0	-			

* mean significant increase compared to control at 5% level
CA = cytochrome P-450 activity after 5 day (nM/mg protein)

of growth. This leads to leaching of GA and IAA from the vacuole and so their increase in filtrate.

Effect of NaCl on GA, IAA and cytochrome P-450 production. The mycelial growth of NaCl salinized *F. oxysporum* significantly increased with 1–10% concentrations after 15 days. GA and IAA production were significantly increased at 0.5% after 5 days in filtrate (Table 3). Increasing of GA and IAA may act as adaptive response in order to maintain stability and proper function of membrane. Also, they may partially elevate the internal osmolality of the stressed cells that consequently enables them to cope with this new environment. Increasing the salinity and time decreased the content of GA and IAA. The content of IAA was completely inhibited at 4% NaCl. Hasan (1998) has found that the mycelial growth of *A. wentii* was also increased at 1–6% NaCl, and fatty acids and sterol were increased in mycelium at 1% as an adaptive response maintaining membrane stability and function.

Cytochrome P-450 production was significantly increased under salinity stress at 0.5–7% NaCl after 5 days (Table 3). The pathway of GA is cytochrome P-450-catalyzed (Coolbaugh et al. 1978). The participation of cytochrome P-450 is indicated by the characteristics of carbon monoxide inhibition and also interaction with other inhibitors. Cytochrome P-450 was also involved in activation and detoxification of organic molecules.

Effect of Na⁺/Ca²⁺ interaction on GA and IAA production. The role of supplemental Ca²⁺ in altering the stressing effects of salinity on *F. oxysporum* was studied (Table 4). Ca²⁺ when added to the nutritive medium decreased NaCl stress by elevating GA content at the specific optimum Na⁺/Ca²⁺ ratios (700/40 mM). Lowering the toxicity of NaCl by Ca²⁺ may be due to its lower Na⁺ absorption by fungal cell. Brodin et al. (1996) have found that Ca²⁺ cause a decrease in microscopic Na⁺ absorption across principal cells by activation Cl⁻ permeability and inhibition Na⁺ permeability.

Role of GA and Ca²⁺ in improvement of seed germination under salt stress. The germination of fababean, sorghum and wheat seeds was completely inhibited with 175 mM Na⁺. Imbibition of seeds for 10 h in water was

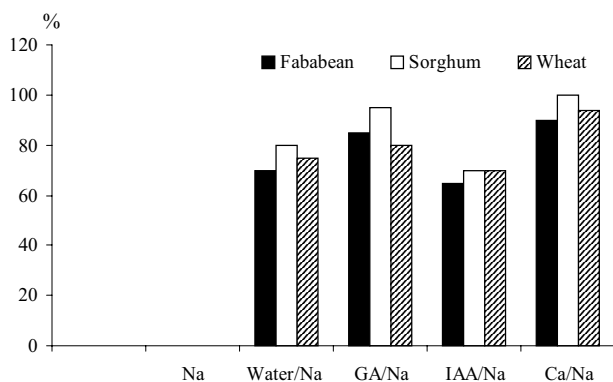


Figure 3. Seed germination (%) under GA/Na, IAA/Na (10 μ M/175 mM) and Ca/Na (10 mM/175 mM) after 3 days of seed imbibition

effective in recovering the germination when re-germinated under 175 mM NaCl (Figure 3). Imbibition of seeds for 10 h in 10 μ M GA and 10 mM Ca^{2+} , separately, were significantly effective in enhancing the germination under 175 mM NaCl, but IAA decreased as compared to those in water (Figure 3). GA and Ca^{2+} could be regarded as a defense mechanism of plant to salinization. The results also explained that the reduction in amylase activity in stressed seeds might be reversed with GA and Ca^{2+} . Martinez-Pastur et al. (2000) found that treatment containing 60 mg.L⁻¹ Ca^{2+} and 1 mg.L⁻¹ boron produced root formation in fewer days and improved micro-cutting rooting.

Different fungal species of *Aspergillus*, *Fusarium*, *Penicillium* and *Rhizopus* are associated with rhizosphere and rhizoplane of fababean, melochia, sesame and soyabean plants. These fungi may be responsible for the maintenance of soil fertility by production gibberellin that stimulates the growth and development of plants. The results show the beneficial effects of fungi to the host plant. The results indicate that the production of gibberellin in presence of high salinity may reduce the hazardous effect of salinity to plant crops, whereas gibberellin could be regarded as a defense mechanism of

Table 4. Na^+/Ca^{2+} interaction on GA and IAA production by *F. oxysporum* after 10 days at 28°C

$Na^+:Ca^{2+}$ (mM)	Mycelial dry wt (mg/50 ml)	GA and IAA production			
		filtrate (mg/50 ml)		mycelium (mg/g dry wt)	
		GA	IAA	GA	IAA
700:0	560	5.3	0	9.5	0
700:10	550	6.3	0	13.3*	0
700:40	530	7.3*	0	16.4*	0
700:70	564	6.7*	0	11.9*	0
000:70	506	13.3*	6.7*	15.8*	0

* mean significant increase compared to 700 mM NaCl at 5% level

plant to salinization. The results indicate also the important role of Ca^{2+} in lowering the toxicity of salinity towards fungi as well as plants.

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Received on April 23, 2001

ABSTRAKT

Produkce giberelinu a auxinu houbami na kořenech rostlin a jejich biosyntéza v podmínkách interakce salinity a vápníku

Rhizosféru a rhizoplán rostlin bobu obecného (*Vicia faba*), jutovníku zeleninového (*Corchorus olitorius*), sezamu indického (*Sesamum indicum*) a sóje (*Glycine max*) kolonizují houby, především *Aspergillus flavus*, *A. niger*, *Fusarium oxysporum*, *Penicillium corylophilum*, *P. cyclopium*, *P. funiculosum* a *Rhizopus stolonifer*. Všechny druhy hub měly schopnost produkovat giberelin (GA), ale zjistili jsme, že *F. oxysporum* produkuje jak GA, tak indolyloctovou kyselinu (IAA). Optimální doba pro produkci GA i IAA houbou *F. oxysporum* byla 10 dní v myceliu a 15 dní ve filtrátu při 28°C. Po přidavku 0,5% a 1% NaCl se obsahy GA a IAA po pěti dnech významně zvýšily, ale použití 4% NaCl (700 mM) vedlo k jejich snížení. Vlivem stresu, způsobeného přidáním 0,5–7% NaCl, se významně zvýšil obsah cytochromu P-450. Vápník snižoval vliv NaCl na *F. oxysporum* tím, že při dávce 40 mM Ca²⁺/700 mM Na⁺ významně zvyšoval biosyntézu GA. GA v dávce 10 μM a Ca²⁺ v dávce 10 mM zvyšovaly klíčivost semen při 175 mM Na⁺.

Klíčová slova: GA; IAA; cytochrom; houby; vápník; salinita

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