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GIBBERELLIN AND AUXIN-INDOLE PRODUCTION BY PLANT ROOT-FUNGI AND THEIR BIOSYNTHESIS UNDER SALINITY-CALCIUM INTERACTION

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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, IAA and cytochrome P-450 were increased at 0.5 and 1% NaCl after 5 days, but GA and IAA were lowered at 4% (700 mM) NaCl. Calcium decreased NaCl stress on *F. oxysporum* by significant elevating GA biosynthesis at 40mM Ca^{2+}/700 mM Na^{+}. GA at 10 μM and Ca^{2+} at 10 mM enhanced the germination of seeds under 175 mM Na^{+}.

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

Introduction

The microbial synthesis of plant growth regulators (gibberellin and auxin) is an important factor in soil fertility [1]. 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 [2, 3]. GA was isolated by T. Yabuta from *Gibberella fujikuroi* and IAA was isolated by K. V. Thimann from *Rhizopus suinus* in 1935. GA_3 is the dominant component of the gibberellin complexes isolated from fungi [4]. 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. [5] found that GA_3 at 6μM concentration induced increase in germination and seedling growth under salt stress. Calcium is a particularly important nutrient in plants exposed to NaCl stress because of its role in maintaining the structural and functional integrity of membrane [6], and in cell wall extensibility [7].

No recent studies explain the ability of filimentous fungi, associated with roots of plant crops, for the GA and IAA production. Also, no studies explain the effect of salinity on the activity of rhizosphere and rhizoplane fungi and the role of GA and Ca^{2+} in alleviation the salt stress.

The present work was performed by collecting four plant crops for isolation of both rhizosphere and rhizoplane fungi and screening their efficiencies for GA and IAA production. Also the effect of salinity on GA, IAA and cytochrome P-450 biosynthesis by *F. oxysporum* and recovering the salinity stress by Ca^{2+} were studies. The roles of GA and Ca^{2+} in reversing the toxicity of salt stress effect in seedlings were also investigated.

Materials 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 sufficient 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 each of the 5 sterile Petri dishes poured 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. The dishes were then incubated at 28°C for 7 days.

Determination of rhizoplane fungi

The procedure described by Guzeva and Zvyagiutsev [8] 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 (2g 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, which were poured 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. The dishes were 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 Fennelel [9] for *Aspergillus,* Booth [10] for *Fusarium,* Christensen and Raper [11] for *Emericella,* and Pitt [12] 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 species belonging to ten fungal species isolated from rhizosphere and rhizoplane were subjected to GA and IAA screening. Fifty ml of 1% peptone and 1% glucose-Czapek's liquid medium in 250 ml Erlenmeyer flask were 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, GA_3 standard, IAA crude and IAA standard. Control seeds were germinated in sterilized water. The dishes were incubated in the dark at 25 °C. Triplicate 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, KH_2PO_4 3, $MgSO_4$ 0.5) were dispensed in 250 ml Erlenmeyer conical flasks. 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 Ca^{2+} 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 $CaCl_2$ 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 Ca^{2+} on germination of NaCl-treated seed

The seeds were soaked in 10 μM GA, 10 μM IAA and 10 mM Ca^{2+} 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 kept for germination in Petri dishes at 25 °C. The seeds were demonstrated 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 Na_2SO_4, and evaporated to dryness 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% H_2SO_4 in methanol + 50 mg $FeCl_3$), heated in oven at 80 °C for 10 min. GA showed greenish fluorescence under UV light after spraying. IAA showed violet-red colour in VL and orange in UV light after spraying. GA and IAA were spectrophotometrically determined according to Bruckner et al. [13] and Wohler [14], respectively. The values obtained were computed from the standard curves of GA_3 and IAA.

Table I

Fungal species in rhizosphere (Rs) and rhizoplane (Rp) of some plants (count/g fresh root wt)[a]

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

[a]OR=average occurrence remarks of rhizosphere and rhizoplane; H= high occurrence remark (> 50% samples); M= moderate occurrence remark (50–30% samples); L= low occurrence remark (<30% samples).

Cytochrome P-450 determination

The mycelia were removed, chilled on ice, washed with phosphate buffer (pH 7) and homogenized separately in 3 volumes in the same buffer. The crude homogenate was centrifuged at 10,000×g for 10 minutes at 4 °C [15]. The supernatant fraction was used as the enzyme source. Cytochrome P-450 was measured by the method of Omura and Sato [16] 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 performed 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 I). *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 species of filamentous fungi isolated from rhizosphere and rhizoplane of plants were tested for their GA and IAA production (Table II). 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 [17, 18] found that several filamentous fungi were well known for their GA production.

The bioassay effects of GA and IAA on the seedling viability of fababean, sorghum and wheat seeds are shown in Figure 1. GA at 10 μM increased the seedling viability, however IAA decreased it as compared to that 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. [19] had explained the beneficial effects of endophytic bacteria to the host plant. Karabaghli et al. [20] 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 the 10th day. The maximum quantity of GA and IAA was registered after 10 days and decline after 15 days in mycelium. However in filtrate, GA and IAA reached its maximum production after 15 days. The declined 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 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 at 1–10% concentrations after 15 days. GA and IAA production were significantly increased at 0.5% after 5 days in filtrate (Table III). This increase may act as adaptive response in order to maintain stability and proper function of the membrane and they may partially elevate the internal osmolality of these stressed cells that consequently enables them to copy 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 [21] has found that the mycelial growth of *A. wentii* was also increased at 1–6% NaCl, and fatty acids and sterol increased in mycelium at 1% as an adaptive response maintaining membrane stability and function.

Cytochrome P-450 production was significantly increased under salt stress at 0.5–7% NaCl after 5 days (Table III). The pathway of GA is cytochrome P-450-catalyzed [22]. 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.

Table II

Production of gibberellin (GA) and indole-acetic acid (IAA) by different species of fungi, grown in peptone Czapek's medium, after 7 days at 28°C

Fungal species	Source of isolation	GA (mg/50 ml)	IAA (mg/50 ml)
Aspergillus carneus	Melochia	10	0
A. flavipes	Sesame	10	0
A. flavus	Fababean	5	0
A. flavus	Melochia	6	0
A. flavus	Sesame	3	0
A. flavus	Soyabean	3	0
A. niger	Fababean	11	0
A. niger	Melochia	12	0
A. niger	Sesame	6	0
A. niger	Soyabean	9	0
A. tamarii	Melochia	5	0
Emericella nidulans	Soyabean	1	0
Fusarium oxysporum	Melochia	10	5
F. oxysporum	Sesame	12	6
F. oxysporum	Soyabean	13	7
Penicillium corylophilum	Melochia	10	0
P. corylophilum	Soyabean	9	0
P. cyclopium	Melochia	1	0
P. cyclopium	Soyabean	7	0
Rhizopus stolonifer	Sesame	10	0

Effect of Na^+/Ca^{2+} interaction on GA and IAA production

The role of supplemental Ca^{2+} in altering the stressing effects of salinity on *F. oxysporum* was studied (Table IV). At 4% (700 mM) NaCl, GA production was retarded. Ca^{2+} when added to the nutritive medium decreased NaCl stress by elevating the GA content at the specific optimum Na^+/Ca^{2+} ratios (700/40 mM). Lowering the toxicity of NaCl by Ca^{2+} may be due to its lower Na^+ absorption by fungal cell. Brodin et al. [23] have found that Ca^{2+} cause a decrease in Na^+ absorption across principal cells by activation Cl^- permeability and inhibition of Na^+ permeability.

Table III

Effect of NaCl on GA and IAA production by F. oxysporum *in glucose mineral medium after 5, 10 and 15 days at 28 ° C*

NaCl %	Mycelial dry wt (mg/50 ml)			GA and IAA content												Cytochrome P-450 activity after 5 days (nM/mg protein)
				Filtrate (mg/50 ml)						Mycelium (mg/g dry wt)						
				5 days		10 days		15 days		5 days		10 days		15 days		
	5 days	10 days	15 days	GA	IAA	GA	IAA	GA	IAA	GA	IAA	GA	IAA	GA	IAA	
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	328	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 0.1% NaCl at 5% level.

Role of GA and Ca2+ in improvement seed germination under salt stress

The germination of sababean, sorghum and wheat seeds was completely inhibited with 175mM Na+. Imbibition of seeds for 10h in 10 μM GA and 10mM Ca^{2+}, separately, were significantly effective in enhancing the germination under 175 mM NaCl, but IAA decreased it as compared to those in water (Fig. 3). GA and Ca^{2+} could be regarded as a defense adaptation mechanism of plant to salinization. The results also explained that the reduction in enzyme activity as amylase in stressed seeds might be reversed by GA and Ca^{2+}. Martinez-Pastur et al. [24] have found that treatment containing 60 mg L^{-1} Ca^{2+} and 1mg L^{-1} boron produced root formation in fewer days and improve micro-cutting rooting.

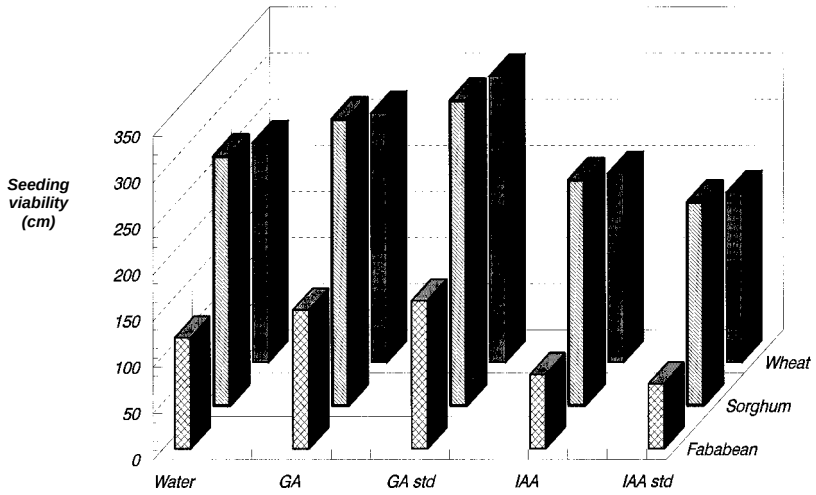

Fig. 1. Ga and IAA (10 μM) effect on seedling viability after 3 days of seed imbibition

Conclusion

Different fungal species of *Aspergillus*, *Fusarium*, *Penicillium* and *Rhizopus* are associated with rhizosphere and rhizoplane in 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 the presence of high salinity concentration may reduce the hazardous effect of salinity to plant crops, whereas gibberellin could be regarded as

a defense adaptation mechanism of 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.

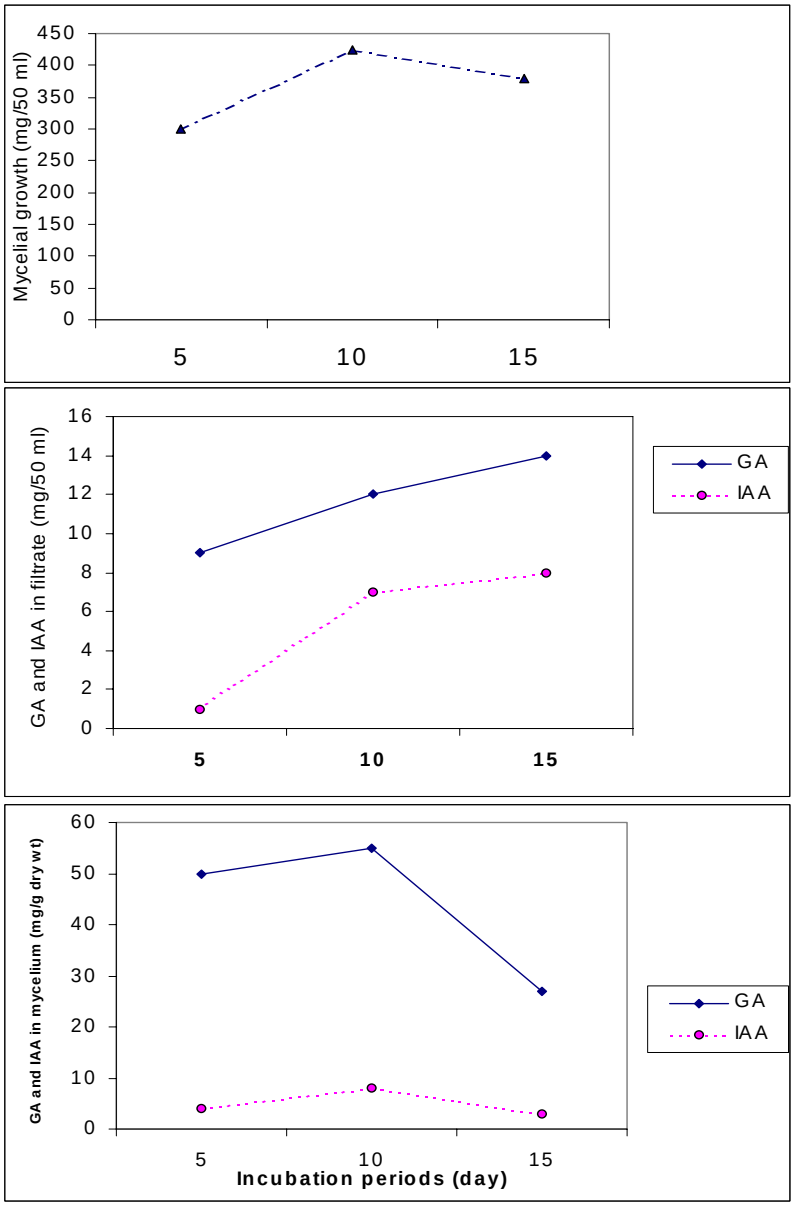

Fig. 2. GA and IAA production by *F. oxysporum* in glucose mineral medium at 28 ºC

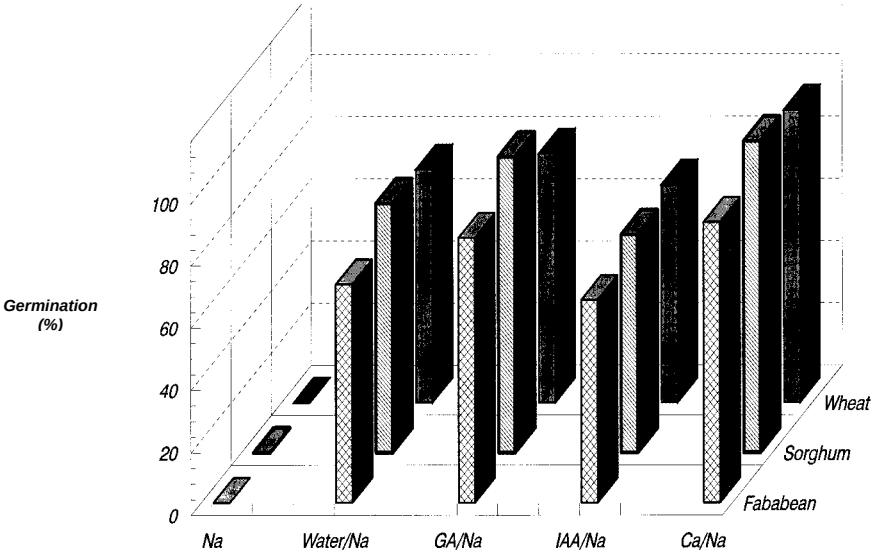

Fig. 3. Seed germination under GA/Na, IAA/Na (10 μM/175 mM) and Ca/Na (10 mM/175 mM) after 3 days of seed imbibition

Table IV

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

$Na^+ : Ca^{2+}$ (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.

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