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Production of Cytochalasin B by *Helminthosporium solani* under Different Culture Conditions and its Effect on the Mitosis of Onion Root Cells

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THE OPTIMUM temperature for maximum production of cytochalasin B (CB) by *Helminthosporium solani* was 30°C. The optimum pH value was 5.5 - 6.0, and the maximum CB production was attained after nine days of incubation. Glucose and sodium nitrate were the best carbon and nitrogen sources for CB production. CB at the concentration of 30 µg/ml decreased the mitotic index (MI %) of *Allium cepa* L. (onion, Giza, 20) roots from 8.42 to 4.83 %. The spindle constituents (microtubules) were affected in a way that gave rise to a number of chromosomal abnormalities, namely; stickiness, un-oriented, bridges, free and distributed spindles without multinucleated cells production. CB at this concentration decreased onion seed production by 5.7 % compared with the control. CB also induced morphological changes to roots and decreased the root length.

Keywords: *Helminthosporium solani*, Mycotoxins, Cytochalasin B (CB), *Allium cepa* L., Mitosis.

Cytochalasins and related chaetoglobosins constitute a class of more than 24 structurally and functionally related mould metabolites. The cytochalasin, phomin was the first discovered biologically active compound produced by *Phoma* species (Rothweiler & Tamm, 1966). El-Kady & Mostafa (1995) found that cytochalasins C, D, and E are produced from dematiaceous hyphomycetes. Evidente *et al.* (2002) found that cytochalasin Z_1, Z_2 and Z_3 are new three cytochalasins produced by *Pyrenophora semeniperda*, a fungus proposed to biologically control grass weeds. Liu *et al.* (2002) isolated cytochalasin D and 19, 20 epoxy- cytochalasin D from the fruiting bodies of the *Ascomycetes engleromyces*. Decognet & Citharel (1991) found that CB could be isolated from culture filtrates of *Phoma exigua* var. *linicola* and from flax plants. Zohri & Saber (1994) found that five species of fungi produced cytochalasins A and / or B. The species occurred in five genera; *Alternaria chlamydospora, Cochliobolus spicifer, Diploococcum spicatum, Phoma herbarum, Phoma multipora* and *Setosphaeria rostrata*. Lopez & Flannigan (1997) found that cytochalasin E was produced by 4 strains of *Aspergillus clavatus* and detected only on malted barley medium at 25°C. Fujii *et al.* (2000) found that zygosporin D and two new cytocalasins were isolated from *Metarrhizium anisopliae*. Of these cytochalasins,

only zyogsporin D was an effective inhibitor of shoot elongation of rice seedlings. Kanematsu *et al.* (1997) found that cytochalasin E is a secondary metabolite secreted *in vitro* by *Rosellinia necatrix* (the causal organism of white root rot disease), which is toxic to plants.

Cytochalasins are characterized as macrolide antibiotics with cytostotic activity that inhibits actin polymerization (Srinivasan *et al.*, 1996) and induce

cytochalasin is a metabolite of *Phoma exiguea* and showed toxic activity in the brine shrimp assay. Cytochalasin D delayed the accumulation of PAL mRNA and inhibited the production of a potato phytoalexin (rishitin) in potato tuber discs induced by an elicitor (Furuse *et al.*, 1999 and Takemoto *et al.*, 1999). Suryanarayanan *et al.* (1986) found that cytochalasin D inhibited the growth of *Bipolaris sorokiniana* and *Rhizoctonia solani* at concentrations of 25 and 50 µg/ml, respectively; and induced profuse branching of the hyphae. Bottalico *et al.* (1990) found that two derivatives of CB purified from *Ascochyta heteromorpha* had the ability to inhibit the growth of tomato seedlings and toxic to brine shrimp. CB has been found to have a morphogenetic effect on *Allium cepa* L. roots by producing a reversible inhibition of mitosis and axis elongation (Thomas *et al.*, 1973; Eleftheriou & Palevitz, 1992 and Behboodi & Samadi, 2002). Cytochalasins were found to induce morphological and cytogenetic effects on other plants such as maize (Zholkevich & Chugunova, 1995), flax (Decogent & Citheral , 1991) and cotton (Seagull, 1998). Moreover, Choi *et al.* (2005) reported that the action of cytochalasins, actin-disrupting agents on human voltage-gated K^+ channel (Kv1.5 channel)(hKv1.5) stably expressed in Ltk^- cells was investigated using the whole cell patch-clamp technique. CB inhibited hKv1.5 currents rapidly and reversibly at +60 mV in a concentration-dependent manner with an IC_{50} of 4.2 µM.

The objective of this study was to study the role of cultural conditions on growth and CB production by *Helminthosporium solani* and its effect on the mitosis of onion root cells.

Material and Methods

Microorganism

Helminthosporium solani was isolated from a soil sample collected from Zagazig, Egypt and identified according to Barron (1968).

Culture media

Using Potato dextrose agar (PDA) with the following constituents (g / l): Potato slices, 200; glucose, 20.0; agar, 20.0 and distilled water up to 1000 ml. PDA was used for isolation and purification of the experimental fungus (Johnson *et al.*, 1959). Czapex`s - Dox agar: It has the following constituents (g / l): Sucrose, 30.0; sodium nitrate, 3.0; potassium dihydrogen phosphate, 1.0; potassium chloride, 0.5; magnesium sulphate, 0.5; ferrous sulphate, 0.01; agar, 20.0 and distilled water up to 1000 ml. It was used for studying the effect of the environmental and cultural conditions on growth and CB production by the experimental fungus.

Cultural conditions

250 ml Erlenmeyer flasks, each containing 50 ml culture broth, were used through the present study. The culture flasks were sterilized in an autoclave at 1.5 times atmospheric pressure for 20 min. Inoculation took place using 6 mm agar disc for each flask. Agar discs were cut from 7 days old culture. Necessary changes were made in the culture conditions to evaluate the effect of different environmental conditions such as shaking, temperature, incubation periods and pH values. Different carbon and nitrogen sources (equimolecular weights) were also tested to select the best ones needed for maximum production of CB by *H. solani*.

Extraction and purification of CB

Mycelial mats were homogenized in methanol and filtered through filter paper. CB was then purified from the methanol extract according to Lipski *et al.* (1987) by filtration the extract through centered glass funnel (G 4) filled with silica gel. CB was then eluted using hexane: tetrahydrofuran (1: 1, V: V).

Qualitative and quantitative determination of CB (Durackova *et al* ., 1976)

Thin layer chromatography (TLC) was used throughout this study using silica gel GF-254. CB samples and standard (Sigma Chemical Co.) were spotted on the chromatograms and developed in the solvent system of toluene: ethyl acetate: formic acid (6: 3: 1; V: V: V). The plates were then sprayed with p-anisaldehyde reagent. CB spots give violet colour under visible light and blue colour under long wavelength ultraviolet rays (366 nm). CB was determined quantitatively according to Scott *et al.* (1975) by mixing a known volume of the purified methanol extract of CB with equal volume of 65 % sulphuric acid and heating the mixture in a boiling water bath for 3- 4 min. After cooling, absorption was then measured using an ultraviolet spectrophotometer at 366 nm.

Effect of CB on mitosis of the cells of onion roots

Seeds of *Allium cepa* L. (Giza 20) were surface sterilized with 0.1 % $HgCl_2$ (w/v) followed by washing with sterilized distilled water. Seeds were germinated in Petri dishes containing CB (30 µg / ml) dissolved in 1 % dimethyl sulphoxide (DMSO) in distilled water. This concentration of CB was previously used by Thomas *et al.* (1973). The seeds were incubated for 5 days at 28 °C. After which, germination ratio and roots length were determined in both treated and control samples. Cytological micro-technique was then carried out using Feulgin-Squash staining method (Darlington & Lacour,1976) with leucobasic fuchsine in its modified formula. One cm sections of root tips were cut, washed and immersed in the fixation solution for 24 hr. Root tips were then hydrolyzed, dehydrated and stained. The cytological preparations were then examined microscopically for observation of mitotic phases and chromosomal abnormalities in the dividing cells. Mitotic index (MI %), mitotic stage index (MSI %) and percentage of abnormalities in a particular phase were then determined.

Results and Discussion

Genus *Helminthosporium* is commonly considered as a contaminant isolated from soil and diseased plants (de Hoog *et al.*, 2000 and Larone, 1995). An isolate of *Helminthosporium* spp. culture was isolated from soil sample collected from Zagazig, Egypt and identified to the species level as *H. solani* according to Barron (1968). Table 1 shows that static conditions gave more growth and CB production by *H. solani* than agitated conditions. Capio *et al.* (2004) found that CB was present at 10.3 and 7.7 mg /g dry weight in the mycelia of *Drechslera wirreganensis* and *D. campanulata*, respectively. Zohri & Saber (1994) screened 100 isolates of 27 species belonging to 13 genera of dematiaceous hyphomycetes for production of cytochalasins A and B. They found that 10 isolates of *Alternaria chlamydospora, Cochliobolus spicifer, Diploococcum spicatum, Phoma herbarum, Phoma multipora* and *Setosphaeria rostrata* were able to produce cytochalasins A and/or B. The optimum temperature for growth and CB production by the experimental fungus was 30 °C (Table 1). The fungus failed to grow at the relatively low (10 and 15 °C) and high temperatures (40 & 45°C). Lopez & Flannigan (1997) found that the fungal biomass of *Aspergillus clavatus* was greater at 25°C than 16°C and cytochalasin E was only detected at 25 °C and not at 16°C. CB biosynthesis was increased gradually with the increase of pH values reaching a maximum at pH 5. 0 and decreased sharply with increasing of the pH values (Table 1). Also, the growth of *H. solani* showed the same response but the optimum pH value was 6.0. The results indicate clearly that the maximum mycelial dry weights of *H. solani* was observed after 6 days of incubation and generally followed by gradual decrease with increase of incubation period, while the maximum amount of CB was determined after 9 days of incubation (Table 1). Larone (1995) reported that *Helminthosporium* spp. colonies grew rapidly and mature in about 5 days.

Table 2 shows that *H. solani* failed to grow in presence of galactose, glycerol, mannitol, cellulose or caboxymethyl cellulose as a carbon sources. Relatively weak growth was observed on maltose, lactose or mannose as a carbon sources. However, the maximum amount of CB was obtained with glucose or fructose followed by maltose then sucrose as carbon sources. Sucrose produced the maximum amount of mycelial dry weight. Diaz & Bedendo (1999) found that *Helminthosporium oryzae* (*Cochliobolus miyabeanus*) grew well on starch, arabinose, fructose, glucose, lactose, maltose and sorbose. Bouslim *et al.* (1999) studied the effect of carbon sources on growth of *Helmithosporium oryzae* and *Cochliobolus miyabeanus*, Moroccan isolates. They found that monosaccharides (glucose, fructose, galactose and mannose), disaccharides (sucrose, lactose and maltose) as well as soluble starch and mannitol were preferred by the tested isolates. Only the isolate *H. oryzae* was able to metabolize carboxymethyl cellulose. In the present work, *H. solani* failed to grow on carboxymethyl cellulose. Table 2 shows also that the maximum mycelial dry weight of *H. solani* was obtained on yeast extract as a nitrogen source followed by ammonium nitrate, while the minimum dry weight was obtained on urea. The maximum values of CB were produced on sodium nitrate, while potassium nitrate was

inferior to it. Diaz & Bedendo (1999) found that *H. oryzae* (*Cochliobolus miyabeanus*) grew well on asparagine, casein , ammonium chloride , peptone , sodium nitrate , ammonium sulphate and urea. Bouslim *et al.* (1999) studied the effect of nitrogen sources on growth of *H. oryzae* (*Cochliobolus miyabeanus*) Moroccan isolate. They found that sodium nitrate and potassium nitrate induced an excellent growth of *H. oryzae* isolates.

TABLE 1. Mycelial dry weight (g / 50 ml culture medium) and CB amounts (mg / g dry weight) of *Helminthosporium solani* incubated under different environmental conditions .

Environmental Conditions	Treatment	Mycelial dry weights (g/50 ml culture medium)	CB amounts (mg/g dry weight)
Agitation	Static	0.462	5.30
	Shaked	0. 303	2. 60
Temperature (°C)	15	—	—
	25	0. 321	4. 50
	30	0. 492	5. 90
	40	—	—
	45	—	—
pH value	2.0	—	—
	3.0	0.703	0.108
	4.0	0.742	2.810
	5.0	0.764	4.240
	6.0	0.521	5.840
	7.0	0.419	3.020
	8.0	0.346	1.850
Incubation period (day)	2	—	—
	4	0.538	0.363
	6	0.704	0.959.
	7	0.541	2.756
	9	0.501	6.078
	10	0.475	0.370
	12	0.441	0.307
	14	0.426	0.26

Table 3 indicates clearly that CB, at the concentration of 30µg/ml, decreased the percentages of seeds germination of *Allium cepa* L. (Giza 20) by 5.7 % compared with control value. CB decreased the radical length of the germinated onion seeds by 45 % approximately. Capio *et al.* (2004) found that 70 µM of CB in 1 % dimethyl sulphoxide (DMSO) inhibited the germination of wheat grains. Tamagnini *et al.* (1983) reported that the effect of CB on the rate of germination and cell wall extension may be due to an inhibitory effect of CB on reserve substances. Decognet & Citharel (1991) found that 5-50 µg / ml of CB significantly inhibited flax radical growth. Fujii *et al.* (2000) isolated zygosporin D and two new cytochalasins from *Metarrhizium anisopliae* mycelia. Of these

TABLE 2. Mycelial dry weights (g / 50 ml culture medium) and CB amounts (mg /g dry weights) of *Helminthosporium solani* grown on different carbon and nitrogen sources.

Carbon and nitrogen sources	Mycelial dry weights (g / 50 ml culture medium)	CB amounts (mg / g dry weight)
Glucose	0.427	8.21
Fructose	0.410	8.21
Galactose	—	—
Mannose	0.395	2.00
Sucrose	0.568	5.35
Lactose	0.22	1.63
Maltose	0.189	6.25
Glycerol	—	—
Mannitol	—	—
Cellulose	—	—
Carboxymethyl cellulose	—	—
Sodium nitrate	0.422	7.57
Potassium nitrate	0.429	6.86
Ammonium nitrate	0.497	4.23
Ammonium chloride	0.454	5.20
Ammonium sulphate	0.460	4.07
Urea	0.272	4.40
Yeast extract	0.542	2.59
Peptone	0.374	0.68

cytochalasins, only zyogsporin D was an effective inhibitor of shoot elongation of rice seedlings. The results in Table 3 and Fig. 1 show that different types of chromosomal abnormalities were observed as a result of the germination of the onion seeds in the presence of 30µg / ml of CB. These abnormalities were stickiness, un-oriented chromosomes, chromosome bridge, free chromosome and disturbed spindles. The percentages of mitotic phases (MSI%) were largely altered, where a decrease in the percentages of prophase and metaphase was observed, while a slightly increase in anaphase and telophase was recorded. CB

TABLE 3. Effect of CB (30µg/ml) on the percentages of phases (M.S.I %), number and percentages of abnormalities in each phase and mitotic index (MI %).

Treatment	No. of examined cells	No. of divided cells	Prophase				Metaphase				Ana-telo				No. of abn. cells					MI%
			No.	M.S.I %	No. of abn.	% of abn.	No.	M.S.I %	No. of abn.	% of abn.	No.	M.S.I %	No. of abn.	% of abn.	Stick	Unori.	Bridge	Free	Dist.	
Control	2446	206	98.00	4.00	0.00	0.00	35.00	1.43	0.00	0.00	73.00	2.98	0.00	0.00	0.00	0.00	0.00	0.00	0.00	8.42
Sample (CB at 30µg/ml)	2834	137	17	0.599	2.00	11.7	26.00	0.91	18.00	69.2	94.00	3.31	57.00	60.6	66.00	4.00	3.00	2.00	2.00	4.83

Character	Control	Treated
Radical length (cm)	4.0	2.2
Percentage of germination (%)	100	94. 91

No = Number, M.S.I. = Mitotic Stage Index, abn. = Abnormal, Stick = Stickiness, Unori = Unoriented, Dist. = Disturbed

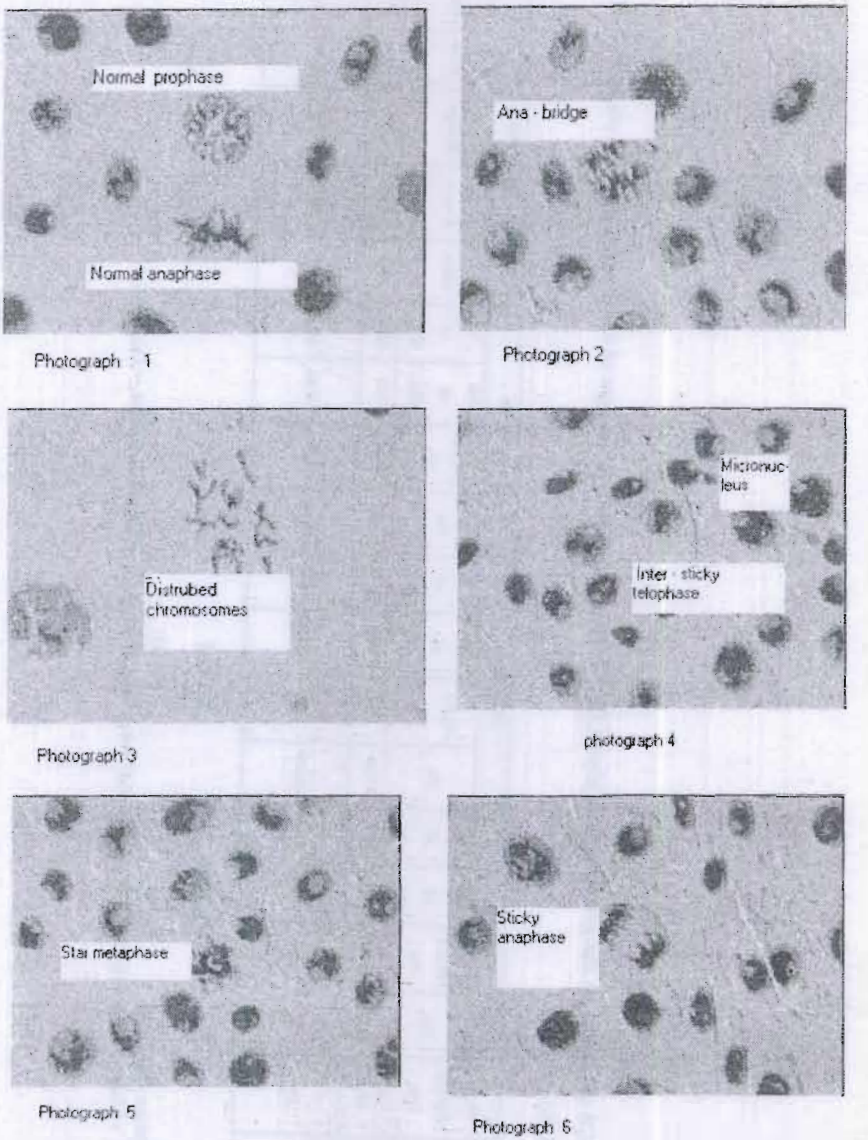

Fig. 1. Photographs of normal and different types of chromosomes abnormalities observed as a result of germination of *Allium cepa* L. (onion) seeds in the presence of 30 µg / ml of CB.

induced an alteration in the mitotic index (MI%) of onion seeds, where it was decreased from 8.42 % in the control seeds to 4.83 % in the treated seeds. It is clear also that the percentages of chromosomal abnormalities were increased in metaphase followed by anaphase but it was decreased largely in the prophase. CB induced an increase in the number of stickiness, un-oriented, bridge, free and disturbed chromosomes aberrations. On the other hand, star-chromosomes and micro nucleated cells were rarely occurred. Capio *et al.* (2004) found that CB induced the formation of binucleated cells in barley root tips. Kamulu *et al.* (1990) found that the addition of CB as a sequential treatment to amiprophos-methyl (amiprofos-methyl) resulted in an enhancement of frequencies of metaphases or micro nucleated cells in potato. Eleftheriu & Palevitz (1992) reported that cytochalasin D induced morphogenesis effects and affected the preprophase band organization in root tip cells of *Allium cepa* L. CB has been found to have a morphogenetic effect on *Allium cepa* L. roots by producing a reversible inhibition of mitosis and axis elongation (Thomas *et al.*, 1973; Eleftheriou & Palevitz, 1992 and Behboodi & Samadi, 2002). Cytochalasins were found to induce morphological and cytogenetic effects on other plants such as maize (Zholkevich & Chugunova, 1995), flax (Decogent & Citharel, 1991) and cotton (Seagull, 1998). Recently, Zhou *et al.* (2004) found that three new cytochalasins, namely, aspochalasins I, J, and K, and four known cytochalasins, aspochalasins C, D, and E and TMC-169 exhibited weak to moderate cytotoxicity against cancer cell lines.

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إنتاج السم الفطرى السيتوكلازين ب بواسطة فطرة *الهلمنثوسبوريوم سولانى* تحت ظروف مزرعية مختلفة وتأثيره علي الإنقسام الميتوزي لخلايا جذور نبات البصل

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قسم النبات - كلية العلوم - جامعة الزقازيق – الزقازيق – مصر.

اتضح أن درجة الحرارة المثلى كانت ٣٠ درجة مئوية لإنتاج أقصى كمية من السيتوكلازين ب بواسطة فطر *الهلمنثوسبوريوم سولانى* ، وكان الرقم الهيدروجينى الأمثل يتراوح ما بين ٥,٥ و ٦ ، وفترة التحضين المثلى هي ٩ أيام . و إتضح أن الجلوكوز و نترات الصوديوم هما أنسب المصادر الكربونية و النيتروجينية ، على الترتيب. واستطاع الفطر إنتاج قدر اكبر من السم الفطري على المزارع الثابتة مقارنة بالمزارع المهتزة .

تم اختبار تأثير تركيز ٣٠ ميكرو جرام لكل ملليلتر من السم الفطري سيتوكلازين ب على مراحل الإنقسام الميتوزي لجذور نبات البصل (جيزة ٢٠) ، وأدى هذا التركيز إلى خفض معامل الإنقسام الميتوزي من ٨,٤٢ إلي ٤,٨٣ % ، و قلت نسبة إنبات البذور حوالي ٥,٧ % عند هذا التركيز ، و لوحظ نقص طول جذور البصل مقارنة بالعينات القياسية الغير معاملة .

وعلاوة علي ذلك ، فقد أدى هذا التركيز إلي ظهور العديد من ظواهر الشذوذ الكروموسومى في المراحل المختلفة للإنقسام الميتوزى لجذور البصل مثل التصاق الكروموسومات ، وعدم إنتظام توزيع الكروميتدات مع خيوط المغزل ، وتشتيت كروموسومات فردية . ولوحظ أيضا عدم ظهور خلايا تحتوى على العديد من الأنوية كنوع من أنواع الشذوذ الكروموسومى في طور الانقسام النهائى .