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Differential susceptibility of *Sclerotium cepivorum* Berk. to some synthesized visnagin sulfonamide derivates

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Abstract

Twenty-five visnagin sulfonamide derivatives were tested *in vitro* against sclerotial germination, growth and cellulolytic activity of *Sclerotium cepivorum* Berk. The effectiveness of the derivatives depends on the concentration and the substituent introduced to the title compound. The introduction of SO_2Cl_2 to C_9 of visnagin induced high toxicity than introducing SO_2NH_2. Compounds with sulfonyl piperidine or sulfonyl morpholine gave small toxicity only at 30 and 75 $\mu g\ cm^{-3}$. Addition of N-aryl ring to visnagin-9-sulfonamide rendered the title compound to be more toxic. The substitution of the N-aryl ring by *m*-CH_3, *m*-Cl or *p*-Cl enhanced the toxicity, while its substitution with *o*-CH_3, *p*-CH_3, *p*-Br, *o*-OCH_3 or *m*-OCH_3 caused a drop in the toxicity as compared to compounds with unsubstituted aryl ring. Visnagin sulfonamide derivatives having azole rings were strongly inhibitory for sclerotial germination, growth, sclerotial formation and cellulolytic activity, even when applied at 4 $\mu g\ cm^{-3}$. The most toxic one was that having dimethyl isoxazole. The cleavage of γ-pyrone ring led to a decline in the toxicity as compared with the other sulfonamide derivatives.

Introduction

Sclerotium cepivorum Berk. is a soilborne fungus which causes the white rot disease of onion. Primary inoculum of the pathogen results from spherical small black sclerotia. Sclerotia, which are formed by many other fungi, play a vital role in life cycle because they are the structures by which these fungi survive long period in unfavourable conditions in the soil (Adams and Papavizas 1971, Willetts 1971).

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Although several heterocyclic compounds were tried against the sclerotium-forming fungi, however, the antifungal activity of these compounds depends on their chemical structure. The structure activity relationship was demonstrated by many workers (Christias 1975, Maneva *et al.* 1987, Le Tourneau *et al.* 1957, Ismail *et al.* 1984, Ouf and Sherif 1993). Attempts have been made to increase the toxicity of biologically active compounds through introducing efficient functional group(s). Fungicides containing sulfonamides as active ingredient were reported (Hatsuta *et al.* 1988, Kato and Igami 1989).

Visnagin is an important adjunct in the furochromone constituents of *Ammi visnaga* L. fruits grown locally (Bencze *et al.* 1954). It has been reported that visnagin possesses bioactivity against some viral diseases (Hudson *et al.* 1988). We synthesized some visnagin sulfonamide derivatives and evaluated their bioactivity against the mycelial growth, sclerotial formation and cellulolytic activity of *Sclerotium cepivorum.*

Material and methods

Sclerotium cepivorum Berk. was isolated from an infected onion bulb (*Allium cepa*). The sclerotia were surface sterilized in 0.5 % sodium hypochlorite for 20 min and then transferred dry to plates containing fresh potato-dextrose agar (PDA) medium. The plates were incubated at 20 °C for 30 d after which the sclerotia were individually transferred to the experimental media.

Visnagin sulfonamide compounds: Twenty-five visnagin sulfonamide derivatives were used, each in four concentrations (namely 4, 12, 30 and 75 $\mu g\ cm^{-3}$). For each treatment three Erlenmeyer flasks (250 cm^3), each containing 90 cm^3 of previously prepared PDA medium were melted and cooled to about 50 °C. Each of the three flasks received 10 cm^3 of previously prepared stock solutions of visnagin sulfonamide compounds. Control flask lacked the tested compounds. Suitable aliquot of each mixture was then poured into each of six sterile plates (9 cm diameter) to form upon solidification, a thin layer at the bottom. Thirty-d-old sclerotia were then individually transferred under aseptic conditions to each of three plates for estimation of percentage germination. The other three plates of each treatment were used for estimation of mycelial growth and sclerotial formation.

The used visnagin sulfonamide derivatives (Scheme 1) were synthesized by the Laboratory of Natural Products, National Research Centre, Cairo, Egypt (El Gamal *et al.* 1987 and 1989).

Sclerotial germination: Ali *et al.* (1987) found that the germination of sclerotia of *S. cepivorum* was about 50 % after 44 h when grown on PDA medium. This estimated time interval was taken as a limit at which the variously treated plates were examined. Two PDA blocks from each sulfonamide treated plate were removed on labelled slides and transferred to a dissicator with a vapour of formalin to stop the

sclerotial germination. Four microscopic fields per block were then examined at random for their content of germinated sclerotia (*i.e* .24 readings for each treatment).

Mycelial growth and sclerotial formation: Preliminary experiments indicated that the mycelium was failed to form sclerotia on using 12 μg cm^{-3} of the tested sulfonamide compounds. For this reason, the mycelial growth, sclerotial formation and cellulolytic activity were studied only at 4 μg cm^{-3}.

Each of the prepared plates received, on the centre, an agar disc (5 mm diameter) covered with fungal mycelium. The discs were cut by a sterile cork borer from the margin of 5-d-old colony grown on PDA at 20 °C. The inoculated plates were incubated at 20 °C. Linear growth rate was determined for 10 d through the measurement of the colony diameter every 2 d. Mature sclerotia were counted after 30 d. The count of sclerotia was performed by cutting 4 discs (5 mm area) from the margin and the number of mature sclerotia in each disc was counted under a dissecting microscope and the results were averaged. The time required for first formed sclerotia in each treatment was recorded.

Cellulase activity: The potato broth-carboxymethyl cellulose (CMC) medium was used for this study. The medium was dispensed into 250 cm^3 Erlenmyer flasks, each was supplemented with 4 μg cm^{-3} of visnagin sulfonamide derivatives. Three replicate flasks per treatment were used. Each flask was inoculated with 5 mm disc covered with fungal mycelium from the margin of actively growing colony on PDA medium. Then the flasks were incubated for 10 d.

The cellulolytic activity in the culture filtrate was determined by the viscosimetric method (Abdel-Razik 1970). It was expressed as percentage decrease in viscosity of 1.2 % CMC using the following equation:

$$[\%] \text{ activity} = \frac{T_b - T_s}{T_b - T_w} \times 100$$

where T_b = time of flow of blank, T_s = time of flow of the sample, T_w = time of flow of distilled water.

Data of all experiments were subjected to analysis by least significant difference test (L.S.D.).

Result and discussion

Sclerotial germination: Most of the visnagin derivatives were inhibitory at 4 μg cm^{-3} for the sclerotial germination of *Sclerotium cepivorum* and the inhibition increased as the concentration was elevated (Table 1). At 4 μg cm^{-3}, the derivatives 2f and 2g were lethal for the sclerotial germination. Some derivatives of compounds 1 and 2 were ineffective or slightly effective as fungitoxicant for sclerotial germination at this concentration *e.g.* 1d, 1e, 2d and 2e. Increasing the concentration of the derivatives

of compound 1 induces a slight increase in inhibition for sclerotial germination as compared to the title compound (1a). This increase was continued on elevating the concentration of these derivatives where they were highly inhibitory at 75 μg cm^{-3} for derivatives 1b, 1d, and 1e and were completely toxic for derivatives 1c. At 12 μg cm^{-3}, the derivatives 2c and all of compound 3 caused complete inhibition to sclerotial germination.

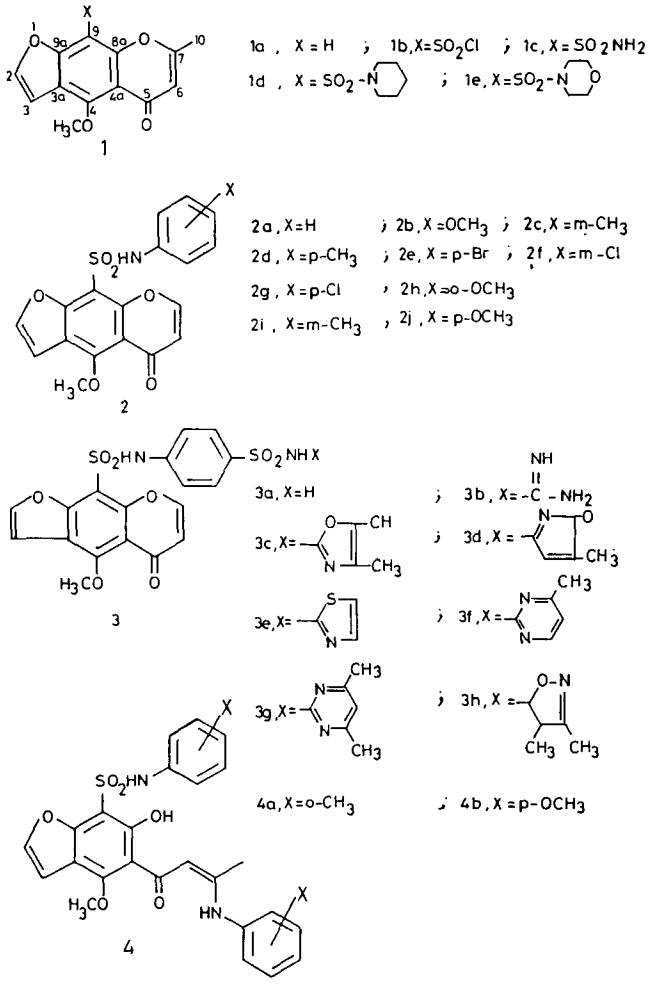
Table 1. Effect of different concentrations of visnagin sulfonamide derivatives after 44 h on percentage inhibition of gemination of *Slerotium cepivorum*. The activity of each derivative was compared to the title compound 1a (visnagin).

Compound no.	Concentration [μg cm^{-3}] 4	12	30	75
1a	—	—	—	—
1b	30.6	36.5	44.1	60.1
1c	22.9	23.8	35.0	100.0
1d	7.3	8.2	12.1	49.2
1e	7.3	11.9	26.5	65.7
2a	43.9	56.7	100.0	100.0
2b	31.0	40.1	79.8	100.0
2c	68.5	100.0	100.0	100.0
2d	8.0	28.1	31.8	73.4
2e	5.1	33.9	38.8	72.4
2f	00.0	100.0	100.0	100.0
2g	00.0	100.0	100.0	100.0
2h	26.6	49.7	100.0	100.0
2i	23.5	44.1	49.0	100.0
2j	20.8	44.3	41.8	48.0
3a	79.2	100.0	100.0	100.0
3b	83.4	100.0	100.0	100.0
3c	61.7	100.0	100.0	100.0
3d	84.7	100.0	100.0	100.0
3e	84.3	100.0	100.0	100.0
3f	67.6	100.0	100.0	100.0
3g	78.1	100.0	100.0	100.0
3h	88.2	100.0	100.0	100.0
4a	1.5	3.6	1.9	10.2
4b	1.9	4.7	6.6	9.2

L.S.D. at 5 % between treatments 11.9
within treatment 8.2

It appears from the results that the effectiveness of the synthesized visnagin derivatives depends on the concentration and the substituent introduced to the title compound. Introduction of SO_2Cl_2 to carbon number 9 of visnagin induced higher toxicity than introducing SO_2NH_2. Hatsuta *et al.* (1988) prepared fungicides containing sulfonamide as active ingredient. They found that aqueous application of the prepared fungicides at 10 μl l^{-1} controlled 95 - 99% of *Pyricularia oryzae* in rice

seedlings. In the present work the addition of sulfonyl piperidine or sulfonyl morpholine produces 1d and 1e, respectively, which gave a small but significant increase in toxicity of the visnagin only when applied at 30 and 75 μg cm^{-3} Morpholine derivatives as agrochemical fungicides were prepared and gave successful control, when used at 100 μg cm^{-3} as a foliar spray, to *Erysiphe graminis* on barley and *Cercospora arachidicola* on peanut (Anthony *et al.* 1988).



Scheme 1. The tested visnagin sulfonamide derivates.

Addition of N-aryl ring to visnagin-9-sulfonamide renders the title compound to be more toxic. The toxicity could be further increased when the N-aryl ring was substituted by *m*-CH_3 (2c), *m*-Cl (2f) or *p*-Cl (2g), while its substitution with *o*-CH_3 (2b), *p*-CH_3 (2d), *p*-Br (2e), *o*-OCH_3 (2h), *m*-OCH_3 (2i) or *p*-OCH_3 (2j) caused a drop in toxicity as compared to the unsubstituted aryl ring. Le Tourneau (1984) showed that the addition of N-aryl groups to phenylthiourea further enhance the inhibition of sclerotium formation of *Sclerotium rolfsii* and, in some cases, resulted in complete inhibition of growth. Ismail *et al.* (1984) proved that the toxicity of phenolic compounds against *Aspergillus fumigatus* and *Fusarium oxysporum* f.sp. *lycopersici* depends on the nature and position of the linked group in the benzene nucleus. They showed that phenolics having NO_2 or CH_3 group were more toxic than those having OH group, also addition of the active group in o-position was more toxic than in *m*- or *p*-position. In the present research, the reduced toxicity of the derivative having *o*-CH_3 group attached to the N-aryl ring, although its efficiency in m-position may be due to the different orientation of the phenyl rings by steric interference of CH_3 and the visnagin moiety.

Visnagin sulfonamide derivatives having azole rings caused strong inhibition for sclerotial germination. The most toxic derivative was that having dimethyl isoxazole (3h) while the compound having dimethyl oxazole (3c) showed least toxicity. This is due to the relative stability of the former compound which was proved by El Gamal *et al.* (1989). Fungicides containing azole as active ingredient have been used to control some pathogens as *Botrytis cinerea* (Schade *et al.* 1990) *Phytophthora infestans* (Parsons *et al.* 1990) and *Pyricularia oryzae* (Seele *et al.* 1990). Ouf and Sherif (1993) showed that introduction of thiazole ring together with two aryl groups to 2-aminopyrimidine induced drastic toxicity for growth, sporulation and nucleic acids of *Fusarium oxysporum* f.sp. *lycopersici* and *Helminthosporium oryzae* which are pathogenic fungi to tomato and rice respectively. Also they added that the toxicity was decreased when the compounds were free from thiazole ring.

The cleavage of the γ-pyrone ring (4a and 4b) led to a declivity in the toxicity as compared with the other sulfonamide derivatives and this indicates the importance of this ring in the establishment of the toxicity. Coumarins (heterocycles having pyrone ring) have high activity against some pathogenic fungi as *Plasmopara viticola* (Pearson *et al.* 1990) and *Trichophyton mentagrophytes* (Agrawal *et al.* 1981).

Mycelial growth and sclerotial formation: The mycelial growth rate of *S. cepivorum* was inhibited at 4 μg cm^{-3} on application of all tested derivatives of 1,2 and 3 (Table 2). As compared to the control, the least inhibition was manifested by the derivatives 1d, 2d and 2e although they were not inhibitory when compared to 1 a (the title compound). All the derivatives of 3 strongly inhibited the rate of mycelial growth in the range of 2.31 to 2.95 as compared with 12.60 for the control or with 8.73 mm d^{-1} for 1a. Failure of mycelial growth was observed on applying the derivatives 2f and 2g.

The sclerotial formation was blocked by all the derivatives of 1,2 and 3 except those of 1a, 1d, 1e, 2d and 2e. Whenever formed, a clear delay in their formation and their number were observed as compared with the control. Yet the two derivatives of

compound 4 have insignificant effect, when compared to 1a, on the rate of mycelial growth and the time of formation of mature sclerotia as well as their number.

The role of sulphur-containing compounds has been implicated on growth and sclerotium formation of *Sclerotium rolfsii*. Christias (1975) working on some sulphur containing compounds reported that sclerotium formation in *S. rolfsii* was completely inhibited by 2-mercaptoethanol at a concentration of 2 - 4 mM without any adverse effect on mycelium growth. Le Tournea (1984) showed that 1,3-dibutyl-2- thiourea inhibited the growth and sclerotium formation of *S. rolfsii*.

Table 2. Effect of 4 μg cm-3 of visnagin sulfonamide derivatives on growth rate, time of formation of first slerotium, number of sclerotia and cellulolytic activity (expressed as loss in viscosity) of *Sclerotium cepivorum*

Compound no	Growth rate [mm d^{-1}]	Time of formation of first sclerotium [d]	Number of sclerotia per 19.6 mm^2	Cellulolytic activity
Control	12.60	10	10.22	88.20
1a	8.73	22	8.33	61.07
1b	7.92	—	—	54.37
1c	7.22	—	—	51.36
1d	9.11	28	4.14	62.55
1e	8.16	28	3.33	62.46
2a	6.35	—	—	49.12
2b	6.67	—	—	56.11
2c	5.99	—	—	55.07
2d	9.73	23	8.50	60.90
2e	9.32	21	8.45	60.34
2f	0.00	—	—	00.00
2g	0.00	—	—	00.00
2h	7.50	—	—	51.16
2i	8.23	—	—	56.54
2j	6.71	—	—	50.91
3a	2.70	—	—	12.66
3b	2.53	—	—	00.00
3c	2.95	—	—	00.00
3d	2.44	—	—	12.36
3e	2.46	—	—	15.15
3f	2.79	—	—	18.32
3g	2.66	—	—	14.40
3h	2.31	—	—	00.00
4a	10.57	18	8.95	64.11
4b	10.20	17	8.10	66.22
L.S.D. at 5 %	3.10	6	1.83	7.42

Cellulolytic activity: Since the cellulases are ones of the recoverable enzymes in extracts of infected plant tissues and are considered to be primarily responsible for

pathogenesis, so it is desirable to study the role of the synthesized sulfonamide derivatives on the cellulolytic activity of *S. cepivorum.* All the visnagin derivatives inhibited the cellulolytic activity of the test fungus (Table 2). However on comparing the effect of the derivatives 1b-e, 2a-j and 4a-b to the title compound (1a), the derivatives 1c, 2a, 2h and 2j significantly inhibited the cellulolytic activity. The derivatives containing azole ring (3a-h) inhibited the enzyme activity more than did the azole-free ones. The cellulolytic activity was completely inhibited on using 3b and 3c. In this connection our results are coupled with those of Viswanathan and Nagrayanasamy (1991) working on *Pyricularia oryzae.* They reported that at 200 μg cm^{-3}, tricyclazole reduced the activity of the pectinolytic enzymes endopolygalacturonase, polygalacturonate - transeliminase and exopolygalacturonase by 47, 57 and 94 % respectively and of the cellulolytic enzymes C_1 and C_x by 45 and 72 % respectively.

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