The effect of S-(ferrocenylmethyl)-thiosalicylic acid sodium salt on the germination and growth of cereal grains and seedlings and on the development of pathogenic fungi

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Abstract

The sodium salt of S-(ferrocenylmethyl)-thiosalicylic acid was studied in the context of its possible use as a systemic fungicide and, concurrently, as a source of physiologically active iron for crop plants. It was found that this metallocene was taken up by maize seedlings growing in liquid mediums, and used for chlorophyll synthesis, in a concentration range as low as $0.05\text{--}0.08\text{ mM}\cdot\text{dm}^{-3}$. In the concentration range of $0.05\text{--}1.5\text{ mM}\cdot\text{dm}^{-3}$, it inhibited germination, seedling growth and α -amylase activity while it stimulated the activities of proteinases, catalase and peroxidase. When sprayed on cereal leaves at a concentration of $1.0\text{--}2.0\text{ mM}\cdot\text{dm}^{-3}$, it exhibited fungicidal properties: inhibition of fungus development without harming cereal plant leaves and stimulated chlorophyll synthesis.

Key words: fungicides, ferrocene derivatives, rye, maize, fungi

INTRODUCTION

Intensive studies have been undertaken during recent years on new compounds exhibiting fungicidal properties. From the information available (Pierevalova et al. 1983, Japanese patent 1983), it can be seen that new types of fungicides are also being sought from among synthetic organo-metallic compounds, also called metallocenes. The ability of these compounds to exchange electrons with the cell metalloproteins, the ease with which they are labeled with γ -radioisotopes, their inhibitory or stimulatory effect on several enzymes as well as their cytostatic and mycostatic properties point to their possible use in studies on biological processes and in applied science. The abundant amount of literature already available on this subject also points to this conclusion (Taylor and Wenzel 1977, Hanzlik and

Soine 1978, Wenzel et al. 1979, Hanzlik et al. 1980, Hetnarski et al. 1980, Pladziewicz and Carney 1982, Chao et al. 1983, Karlsson et al. 1984).

In this study, one of the ferrocene derivatives, S-(ferrocenylmethyl)-thiosalicylic acid sodium salt, was examined. The aim was to determine if this compound exhibited fungicidal properties and if it could be used simultaneously as the source of physiologically active iron for crop plants.

MATERIALS AND METHODS

The sodium salt of S-(ferrocenylmethyl)-thiosalicylic acid (Fig. 1) is a solid, yellow substance, well dissolvable in water. This metallocene contains iron with an oxidation number of 2, and has a molecular weight of 347 daltons. The physiological activity of this compound was tested on rye grains of the "Dankowskie Złote" (original class II) variety and on maize seedlings, KB-DC-270 (original class I 2173) obtained from the Seed Qualification District Inspectorate in Olsztyn. The grains were germinated

Fig. 1. The structure of the sodium salt of S-(ferrocenylmethyl)-thiosalicylic acid

at 20 C in Petri dishes lined with three layers of filter paper (disinfected in an autoclave) moistened with 10 cm³ of the tested salt at the following concentrations, expressed in mM·dm⁻³: 0.05, 0.15, 0.30, 0.70, 1.00, 1.50. A double control was used: 1) grain germinated in distilled water only, 2) grain germinated in 1.5 or 2.0 mM·dm⁻³ FeSO₄. After 4 days of germination, the following were determined: number of germinating grains, growth of roots and sprouts, total protein content, level of free amino acids and α-amylase, proteinase, catalase and peroxidase activities. The total protein content was determined in homogenates obtained by triturating 10 germinating grains with 10 cm³ of 7% trichloroacetic acid (TCA). The homogenate was centrifuged at 3000 × g for 10 min, the supernatant discarded and the sediment dissolved in 30 cm³ 1 N NaOH and placed in a water

bath at 38 C for 24 hrs. After centrifugation, the protein in this extract was determined by the method of Lowry et al. (1951).

Extracts of free amino acids were obtained by triturating 10 germinating grains with 30 cm³ of 80% ethanol. The homogenate was filtered through 6 layers of gauze, transferred to a separatory funnel and treated with 60 cm³ of chloroform. This mixture was left for 12 hrs. until the layers separated. The lower layer was discarded, the upper evaporated and the residue dissolved in 2 cm³ of 10% propanol. The amino acid content was determined in this extract by the ninhydrin method as described by Kłyszajko-Stefanowicz (1972).

Proteinase activity was assayed by the Anson method as given by Angel et al. (1969). The enzyme extract was obtained by triturating 50 grains which had been germinating for 4 days, with 10 cm³ of 0.9% NaCl solution. The reaction medium was a buffered, 1% casein solution (pH 5.9). The results of the colorimetric readings were expressed as μ moles of tyrosine released during 24 hrs by the enzymes obtained from 100 grains. The activity of α -amylase was determined by the method of Shuster and Gifford (1962). The amylolytic reaction was carried out in a boiled, then cooled reaction medium containing 150 mg insoluble starch, 600 mg KH₂PO₄ and 30 mg CaCl₂ and 100 cm³ water. The following volumes were used in the assay: 1 cm³ starch solution, 0.8 cm³ water and 0.2 cm³ enzyme extract. The reaction was stopped after 1 min. by adding 1 cm³ of iodine reagent, obtained by dissolving 60 μ g I₂ and 600 μ g KI in 100 cm³ 0.05 N HCl, after which 5 cm³ water were added and the extinction measured at 620 nm against water. From the differences in extinction between the samples with enzyme and control (reagents, without enzyme), the enzyme activity was determined, taking as one arbitrary unit the amount of enzyme which under the conditions of the assay, lowered the absorption by 0.1 unit. The enzyme activity was also calculated per mg of enzymic protein.

The catalase activity was determined by the method of Bach and Oparin as given by Bielozyerski and Proskuriakov (1954), based on the titration with 0.1 N KMnO₄ of the H₂O₂ used as the substrate, remaining in the incubation medium. The activity of this enzyme was expressed in mg H₂O₂ decomposed in 6 min. by the extract obtained from 100 grains. The result was then multiplied by a factor of 1.7, equivalent to 1 cm³ 0.1 N KMnO₄ under the conditions of this experiment.

The peroxidase activity was assayed by the method of Rychter and Lewak (1971). The reaction mixture contained 1 cm³ 0.1 M acetate buffer, pH 5.0, 0.25 mm³ enzyme extract and 1 cm³ 2 mM benzidine. The increase in extinction (1 cm/590 nm) was determined after 1 minute of incubation at room temperature. A unit of peroxidase activity was taken as the amount of enzyme which caused an increase in extinction of 1 unit in 1 minute under conditions of the experiment.

Water cultures. Maize kernels were germinated for 7 days in Petri dishes lined with moistened filter paper. The seedlings so obtained were transferred to 11 jars containing Hoagland's medium, pH 5.6, with the studied metallocene in an amount equivalent to $0.05 \text{ mM} \cdot \text{dm}^{-3}$ instead of the usual iron compound. The nutrient without any iron and another variant, with $0.05 \text{ mM} \cdot \text{dm}^{-3} \text{ FeSO}_4$, served as double controls. After 10 weeks of vegetation of the seedlings in the medium, their root system was appraised and the chlorophyll content of the leaves (Arnon 1949) determined.

Pathogenic fungi were isolated from rye grains by the washing-out method (Naumova 1973). The species which were isolated (*Mortierella camerdensis*, *M. humilis* and *Fusarium avenaceum*) were then transferred to Petri dishes containing agar-glucose-potato medium to which $1 \text{ mM} \cdot \text{dm}^{-3} (\text{Fe}_1)$ or $1.5 \text{ mM} \cdot \text{dm}^{-3} (\text{Fe}_2)$ of the studied compound had been added. Isolates on the same mediums but without the metallocene, served as controls. Another control variant was made up by the isolates on mediums containing $250 \text{ mg} \cdot \text{dm}^{-3}$ of the fungicidal drug, N-methylglucosamine-N-glucosylpolyfungine (N-MGP). According to its manufacturer (TZF "Polfa"), 1 mg of this drug has an activity of 2000 international units. The growth and development of the fungi were appraised after 5 days. All of the experiments, both with the cereals and with the fungi, were done in triplicate.

RESULTS AND DISCUSSION

The results of the experiment show that the control grain, that is, in the solutions without metallocene or in those with different FeSO_4 concentrations, germinated the best. The grain which imbibed in solutions of the sodium salt of S-(ferrocenylmethyl)-thiosalicylic acid germinated poorly and their sprouts grew more weakly (Table 1). The inhibition of germination and seedling growth increased with the increase of the concentration of the added compound. Maize seedlings growing on Hoagland's medium in the presence of the studied compound had a highly reduced root system. Similarly as with the inhibition of rye grain germination, this reaction intensified with the increase in the concentration of this substance (Figs. 2-5). At the same time, the leaves of these seedlings contained more chlorophyll than the control plants growing on FeSO_4 -containing mediums and those without iron (Table 2). It should be noted that maize seedlings were much more sensitive to the ferrocene derivative than rye grain. In the case of the seedlings, concentrations of this compound exceeding $0.08 \text{ mM} \cdot \text{dm}^{-3}$ were lethal, whereas rye grain germinated in the concentration range of $0.05\text{-}1.5 \text{ mM} \cdot \text{dm}^{-3}$ ($17\text{-}520 \text{ mg} \cdot \text{dm}^{-3}$). It was also found that this

Maize seedlings grown for 10 weeks on Hoagland's mediums. Fig. 2—without iron, Fig. 3—with $0.08 \text{ mM} \cdot \text{dm}^{-3} \text{ FeSO}_4$, Fig. 4—with $0.05 \text{ mM} \cdot \text{dm}^{-3} \text{ S-(ferrocenylmethyl)-thiosalicylic acid sodium salt}$, Fig. 5—with $0.08 \text{ mM} \cdot \text{dm}^{-3} \text{ S-(ferrocenylmethyl)-thiosalicylic acid sodium salt}$. $\times 3.75$

The effect of S-(ferrocenylmethyl)-thiosalicylic acid sodium salt on the growth and development of the pathogenic fungi: *Mortierella comerdensis* (Fig. 6), *M. humilis* (Fig. 7), *Fusarium avenaceum* (Fig. 8). $\times 3.75$. K—control (medium without metalocene), Fe₁—medium with $1.0 \text{ mM} \cdot \text{dm}^{-3}$ metalocene, Fe₂—medium with $1.5 \text{ mM} \cdot \text{dm}^{-3}$ metalocene, P—medium with 250000 units ($130 \text{ mg} \cdot \text{dm}^{-3}$) N-methylglucamine-N-glucosylpolyfungine

Table 1

The effect of different concentrations of the sodium salt of S-(ferrocenylmethyl)-thiosalicylic acid on the germination of rye grain

Concentration of the compound used in the germination of rye grain, mM · dm ⁻³	% Grain germinating after 4 days	Analysis of seedling growth	
		coleoptyle length, mm	root length, mm
Water	94 ± 4.8	48 ± 6.2	103 ± 5.2
FeSO ₄ — 1.0	90 ± 4.6	46 ± 3.7	95 ± 5.0
FeSO ₄ — 2.0	91 ± 3.9	39 ± 4.0	75 ± 4.0
Fe ₁ — 0.05	88 ± 4.0	30 ± 3.7	43 ± 5.0
Fe ₂ — 0.15	83 ± 2.2	28 ± 5.0	35 ± 4.0
Fe ₃ — 0.30	79 ± 2.0	24 ± 1.4	28 ± 2.2
Fe ₄ — 0.70	77 ± 1.3	20 ± 3.1	26 ± 2.0
Fe ₅ — 1.50	75 ± 1.3	17 ± 3.4	16 ± 2.3

Fe₁–Fe₅ — different concentrations of the S-(ferrocenylmethyl)-thiosalicylic acid sodium salt.

Table 2

The effect of the sodium salt of S-(ferrocenylmethyl)-thiosalicylic acid and FeSO₄ in Hoagland's medium on the chlorophyll content in maize seedlings

Concentration of the compound used in Hoagland's medium as the iron source, mM · dm ⁻³	Chlorophyll a content in leaves, mg · 100 g ⁻¹ fresh weight	Chlorophyll b content in leaves, mg · 100 g ⁻¹ fresh weight
Water	263	100
FeSO ₄ — 0.05	374	155
Fe ₁ — 0.05	600	250
Fe ₂ — 0.07	870	350

Fe₁ and Fe₂ — different concentrations of S-(ferrocenylmethyl)-thiosalicylic acid sodium salt.

metallocene, sprayed on maize seedling leaves (at the 4-5 leaf stage) had a stimulatory effect and increased their green pigmentation. This preparation, then, removed signs of chlorosis. This points then to the possibility of using the S-(ferrocenylmethyl)-thiosalicylic acid sodium salt as a foliar nutrient for plants, in respect to iron.

This compound also had an effect on the content of basic organic

Table 3

The total protein content, the level of free amino acids and activities of some hydrolytic enzymes in the rye grain germinated in solutions of S-(ferrocenylmethyl)-thiosalicylic acid sodium salt at various concentrations

Concentration of the compound used in the germination of rye grain, $\text{mM} \cdot \text{dm}^{-3}$	Total content, mg per 100 grains	Level of free amino acids, mg α -amino nitrogen per 100 grains	Proteinase activity				α -Amylase activity					
			embryos		endosperm		embryos			endosperm		
			μmoles tyrosine per 100 grains	% activity of control	μmoles tyrosine per 100 grains	% activity of control	in arbitrary units per 100 grains	in units of activity per 1 mg protein	% activity of control	in arbitrary units per 100 grains	in units of activity per 1 mg protein	% activity of control
Water	420	56	55.6	100	68.3	100	500	38.4	100	3300	253	100
Fe_4 — 0.7	336	64	80.0	144	84.0	123	460	37.4	92	2640	217	80
Fe_5 — 1.5	300	92	81.0	145	87.2	127	400	35.0	80	2280	200	70

Fe_4 and Fe_5 — different concentrations of S-(ferrocenylmethyl)-thiosalicylic acid sodium salt.

substances in germinating rye grain. The grain germinating for 3 days in solutions of this metallocene exhibited a lowered protein content and elevated free amino acid level (Table 3). These effects were accompanied by an increase in proteinase activity and a decrease in α -amylase activity. These changes can be explained by stimulation of proteolytic processes. Catalase and peroxidase also exhibited increased activities (Table 4). The role of

Table 4

Changes in the activities of oxidases in rye grains germinated in solutions of S-(ferrocenylmethyl)-thiosalicylic acid sodium salt and FeSO_4

Concentration of the compound used in germination of grain, $\text{mM} \cdot \text{dm}^{-3}$	Catalase activity		Peroxidase activity	
	in arbitrary units per 100 grains	% of control activity	in $\text{mg H}_2\text{O}_2$ per 100 grains in 9 min	% of control activity
Water	288	100.0	16.4	100
FeSO_4 — 1.0	272	94.3	17.0	103
Fe_4 — 1.0	376	130.0	26.0	158
Fe_5 — 1.5	480	166.0	36.0	219

Fe_4 and Fe_5 — different concentrations of S-(ferrocenylmethyl)-thiosalicylic acid sodium salt.

these iron porphyrins is connected (Briggs and Ray 1956) with the oxidative inactivation of indoleacetic acid, and hence with the processes of shoot and root growth. It is also accepted that the increased tissue iron ion level stimulates these oxidases (Machold 1968).

In light of the data presented above, it seems that the sodium salt of S-(ferrocenylmethyl)-thiosalicylic acid may constitute a source of iron for chlorophyll synthesis in the green parts of plants. This compound has a less favorable effect on young seedlings and germinating grain because it inhibits the development of their vegetative organs. In this context, the mycostatic properties of this compound should be considered favorable. The experiments done on fungi (*Mortierella camerdensis*, *M. humilis* and *Fusarium avenaceum*) (Figs. 6-8) showed that concentrations of this preparation between 1.0 - $2.0 \text{ mM} \cdot \text{dm}^{-3}$ in agar-glucose-potato mediums inhibited the growth and development of these organisms. The mycostatic activity of the compound under study was of a similar order as that of N-methylglucamine-N-glucosylpolyfungine. The latter is an antifungal drug used also for surface sterilization of seeds used in physiological studies (Michalczyk and Pawlicka 1984).

The fungicidal effects seen after spraying maize seedlings with 1.0 - $2.0 \text{ mM} \cdot \text{dm}^{-3}$ solutions of S-(ferrocenylmethyl)-thiosalicylic acid sodium salt have been attributed to this compound. The positive influence on chlorophyll synthesis, lack of inhibition of growth processes and the mycostatic action of this compound, in the studied concentration range, on pathogenic fungi,

should be acknowledged as effects of this compound. Its fungicidal properties are not a unique phenomenon among metallocenes. The authors of the Japanese patent (1983) cited in the Introduction, suggest that very many more ferrocene derivatives will show this property when tested on the fungus, *Spherotheca fullinea*, which attacks cucumbers. The selective reaction of different crop plants to different fungicides from this group of compounds is also interesting.

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Wpływ soli sodowej kwasu S-(ferrocenylometylo)-salicylowego na kiełkowanie ziarniaków i wzrost siewek zbóż oraz na rozwój grzybów patogenicznych

Streszczenie

Badano możliwości wykorzystania związku pochodnego ferrocenu, soli sodowej kwasu S-(ferrocenylometylo)-tiosalicylowego jako źródła fizjologicznie aktywnego żelaza przez kiełkujące ziarniaki żyta i siewki kukurydzy. Stwierdzono, że zastosowany metalocen w stężeniach $0,15\text{--}1,5\text{ mM}\cdot\text{dm}^{-3}$ obniżał żywotność ziarniaków żyta, hamował aktywność ich α -amylazy oraz stymulował aktywność proteinaz, katalazy i peroksydazy. Obserwacje rozwoju siewek kukurydzy w kulturach wodnych wykazały inhibicyjny wpływ badanego związku na wzrost i rozwój korzeni oraz syntezę chlorofilu w częściach nadziemnych. W stężeniach nieszkodliwych dla opryskiwanych liści preparat ten przejawiał silne działanie mykostatyczne w stosunku do grzybów pasożytniczych: *Fusarium avenaceum*, *Mortierella camerdensis* oraz *Mortierella humilis*.