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Effect of zearalenone (F-2) on pea stem, maize root, and rat liver mitochondria

A. Vianello and F. Macrì

Centro di Studio dei Problemi Fitopatologici della Barbabietola da Zucchero, CNR, Istituto di Patologia Vegetale, Università di Padova, I-35100 Padova, Italy

Abstract. At 5 and 10 μg ml^{-1} concentration, zearalenone (F-2), a mycotoxin produced by a number of species of the genus *Fusarium*, causes an inhibition of the oxidative phosphorylation of isolated plant mitochondria, while at 20 and 40 μg ml^{-1} it causes uncoupling. However, when the mitochondria are preincubated for 20 min with F-2, the uncoupling appears to be the prevailing effect. F-2 is also able to inhibit the mitochondrial ATPase activity (Mg^{2+}-dependent). Conversely, F-2 (40 μg ml^{-1}) does not alter the ATP level of maize roots and only slightly affects the ATPase activity of pea stem and maize root microsomal fractions. In addition, F-2 (10-40 μg ml^{-1}) inhibits ATP synthesis catalyzed by rat liver mitochondria. It is suggested that the phytotoxicity of F-2, also known for its ability to collapse the transmembrane electric potential of maize roots, may be mainly linked to its ability to increase the proton permeability of the cell, similar to the common uncouplers.

Key words: ATPase – Mitochondria – Mycotoxin – *Pisum* – *Zea* – Zearalenone.

Introduction

Zearalenone (6-(10-hydroxy-6-oxo-trans-1-undecenyl)-β-resorcylic acid lactone; F-2), a mycotoxin causing an estrogenic syndrome on several animal species (Mirocha et al. 1971), is synthesized by a number of species of the genus *Fusarium* both in infected maize plants in the field and in stored maize grains (Mirocha et al. 1971; Caldwell and Tuite 1974).

In a previous work, we have found that F-2 inhibits an H^+/K^+ electrogenic ion pump in maize roots and the activity of a K^+-Mg^{2+}-dependent microsomal ATPase of maize coleoptiles (Vianello and Macrì 1978). All observed effects imply some interference at the level of the energy transduction and therefore a direct check of the mitochondrial effects of zearalenone was necessary.

The experiments described in the present paper were aimed at testing the effect of F-2 on the synthesis of ATP catalyzed by isolated pea stem, maize root, and rat liver mitochondria and on the ATP level of maize roots. The reason for using mitochondria from animal tissues as the testing material is related to the mycotoxic nature of the compound. In fact its more relevant toxic effects are exerted on animal organisms (Mirocha et al. 1971).

Materials and methods

Pea (*Pisum sativum* L., cv. Alaska) and maize (*Zea mays* L., cv. Dekalb XL 342) seeds were germinated and grown in the dark at 25° C for 5–6 d over aerated 0.5 mM $CaCl_2$.

Pea stem and maize root mitochondria were fractionated as previously described (Macrì et al. 1980). Rat liver mitochondria were isolated in 0.125 M sucrose buffered with 3 mM Tris-HCl at pH 7.4, as indicated by Myers and Slater (1957).

Oxygen uptake was monitored with a platinum electrode assembly of the Clark type (Estabrook 1967). Acid-base titration was performed according to the Mitchell and Moyle procedure (1967).

Mitochondrial ATPase activity was assayed in a final volume of 1 ml of a medium composed of 50 mM KCl, 1 mM $MgCl_2$, 20 mM 2[N-morpholino]ethane sulfonic acid (MES)-Tris (pH 8.7), and 100 μl of diluted pea stem (approx. 40 μg protein) or maize root (approx. 300 μg protein) mitochondrial suspension. The samples were preincubated in an ice bath for 10 min. The reaction was started by adding 1 mM ATP and carried out at 27° C for 30 min with plant mitochondria and for 10 min with rat liver mitochondria. Pi released was determined by the Fiske and Subbarow procedure (1925), modified according to Cross et al. (1978).

Microsomes were obtained from both etiolated pea stem and maize root ground in 50 mM Tris-HCl containing 0.1 mM $MgCl_2$, 1 mM ethylene-diamine-tetraacetic acid (EDTA), 0.25 M sucrose, pH 8, and fractionated following the procedure of Hodges and Leonard (1974).

ATPase activity of the microsomal fraction was measured in a 1-ml vol. containing 1 mM $MgCl_2$, 5 or 50 mM KCl, pH 6, and 100 μg of microsomal protein. The mixtures were pre-incubated for 10 min in an ice bath and then incubated at 35° C for 10 min.

Abbreviations: F-2 = zearalenone; DCCD = N,N′-dicyclohexylcarbodiimide; FCCP = carbonyl cyanide, p-trifluoromethoxiphenylhydrazone; CBT = *Cercospora beticola* toxin

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The reaction was started by adding 1 mM ATP. Pi released was determined as for mitochondrial ATPase.

For ATP level measurement, 0.5 g maize roots were incubated in 8 ml 0.5 mM $CaCl_2$ in the presence or absence of F-2 (40 μg ml^{-1}) for 2 h at 28° C, while being shaken. The tissue was homogenized with 8 N perchloric acid by an Ultra-Turrax apparatus in an ice bath. The homogenate was centrifuged at 20,000 *g* for 10 min and the supernatant was neutralized with KOH buffered 1.2 M triethanolamine-HCl at pH 7.6 and re-centrifuged. The enzymic assay of ATP was performed on the supernatant according to Lamprecht and Trautschold (1974), with minor changes. The protein content was estimated by the biuret method (Gornall et al. 1949).

Tris-ATP, ADP, oligomycin, carbonyl cyanide, p-trifluoromethoxiphenyl-hydrazone (FCCP), N,N′-dicyclohexylcarbodiimide (DCCD), NADH, NADP, and succinate were obtained from Sigma Co., St. Louis, MO, USA. Glucose-6-phosphate dehydrogenase and hexokinase were purchased from Boehringer GmbH, Mannheim, FRG. F-2 was a gift of IMC Chemical Group, Terre Haute, USA.

Results

Figure 1 shows the effect of F-2 (40 μg ml^{-1}) on isolated pea stem (traces A, B and C) and maize root (traces D, E and F) mitochondria. Traces A and D show basal and coupled oxygen uptake in control pea stem and maize root mitochondria, respectively. F-2, added before the substrate (NADH), caused a marked increase in electron flow (traces B and E). ADP addition did not cause any appreciable increase in oxygen uptake rates. F-2 was also capable of releasing the inhibition of oxygen uptake caused by oligomycin (2.5 μg ml^{-1}) during oxidative phosphorylation (traces C and F). The dose-response relationship between F-2 and oxygen uptake (Table 1), shows that the mycotoxin induced a moderate inhibition of the oxidative phosphorylation at 5 and 10 μg ml^{-1} concentration, while at 20 and 40 μg ml^{-1} the uncoupling was the prevailing effect. However, when the mitochondria were pre-incubated with F-2 for 20 min, the uncoupling was evidently already at 5 and 10 μg ml^{-1}.

F-2 inhibited oxygen uptake of rat liver mitochondria coupled to phosphorylation, with succinate as the substrate; the effect was dependent of F-2 concentration in the range from 10 to 40 μg ml^{-1} (Fig. 2, traces A, B and C), and such an inhibition was reversed by adding 1 μmol FCCP (trace C). F-2, instead, did not influence the basal electron flow (trace D). The inhibition observed at 40 μg ml^{-1} F-2 was comparable to that of oligomycin at 2.5 μg ml^{-1} concentration (trace E).

Table 2 shows the effect of F-2 on ATPase activity of pea stem, maize root, and rat liver mitochondria. F-2 inhibited ATPase activity of about 35% in plant mitochondria at 40 μg ml^{-1} concentration, whereas

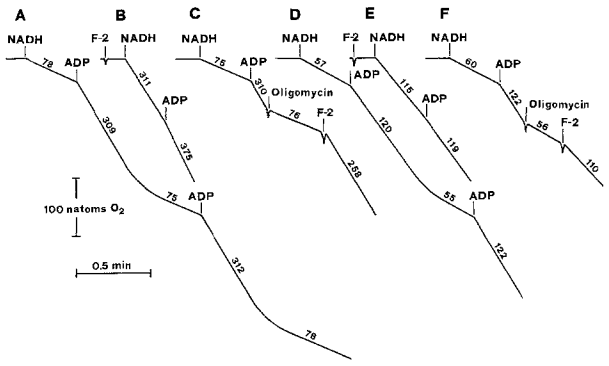

Fig. 1. Effect of F-2 on basal and coupled electron transport in pea stem (traces A, B and C) and maize root (traces D, E and F) mitochondria. The figures next to each trace are expressed in natoms O_2 mg^{-1} protein min^{-1}. The reaction medium contained 20 mM HEPES, 0.4 M sucrose, 5 mM Na-K-phosphate, 5 mM $MgCl_2$ and 1.5 mg (traces A, B and C) or 2.5 mg (traces D, E and F) of mitochondrial protein in a final volume of 2 ml (pH 7.4). The substrate was 4 μmol of NADH. State 3 rates were obtained by adding 200 nmol ADP. F-2 and oligomycin were 40 and 2.5 μg ml^{-1}, respectively

Table 1. Effect of F-2 concentration on respiratory chain and oxidative phosphorylation of isolated pea stem mitochondria

	Additions	Natoms O_2 mg^{-1} protein min^{-1}		RCR
		State 4	State 3	
Without pre-incubation	Control	71	214	3.0
	F-2: 5 μg ml^{-1}	66	168	2.5
	10 μg ml^{-1}	71	163	2.3
	20 μg ml^{-1}	175	203	1.2
	40 μg ml^{-1}	174	191	1.1
With 20 min pre-incubation	Control	66	205	3.1
	F-2: 5 μg ml^{-1}	88	176	2.0
	10 μg ml^{-1}	100	150	1.5
	20 μg ml^{-1}	128	141	1.1
	40 μg ml^{-1}	141	141	1.0

Conditions as in Fig. 1. RCR = respiratory control ratio. State 4 indicates the rate of oxygen consumption without added ADP. State 3 indicates the rate of oxygen consumption after addition of 300 nmol of ADP

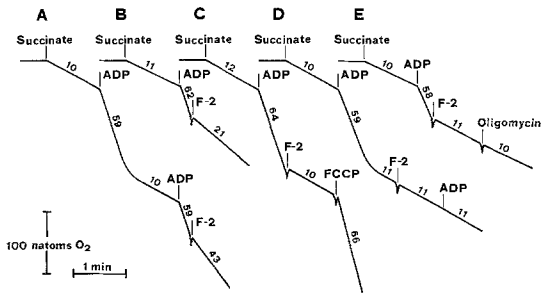

Fig. 2. Effect of F-2 on basal and coupled electron flow in rat liver mitochondria. The figures next to each trace are expressed in natoms O_2 mg^{-1} protein min^{-1}. The reaction medium contained 3 mM HEPES, 0.25 M sucrose, 5 mM Na-K-phosphate, 5 mM $MgCl_2$ and 5 mg of mitochondrial protein in a final volume of 2 ml (pH 7.4). Succinate, 10 μmol; ADP, 300 nmol; F-2, 10 (trace A), 20 (trace B) and 40 μg ml^{-1} (traces C, D and E); FCCP, 1 nmol; oligomycin, 2.5 μg ml^{-1}

Table 2. Effect of F-2 on ATPase activity of pea stem, maize root and rat liver mitochondria

	ATPase activity (nmol Pi mg^{-1} protein min^{-1})					
	Pea stem	% inhibit.	Maize root	% inhibit.	Rat liver	% inhibit.
Control	186±2.5		42±3.1		72±3.1	
F-2: 5 μg ml^{-1}	150±1.7	19	36±2.1	14	66±1.7	8
20 μg ml^{-1}	123±1.2	34	30±1.7	29	60±2.6	17
40 μg ml^{-1}	117±0.6	37	28±0.2	33	57±1.0	21
Oligomycin (2.5 μg ml^{-1})	75±0.5	60	15±0.1	64	15±0.6	79
+F-2 (40 μg ml^{-1})	78±0.2	60	15±0.1	64	18±2.2	75
CBT (20 μg ml^{-1})	80±0.3	57	20±2.1	52	24±3.2	67
+F-2 (40 μg ml^{-1})	73±0.6	60	21±1.0	52	18±2.1	75

Data are the mean of three replicates. Variability is expressed as standard deviation

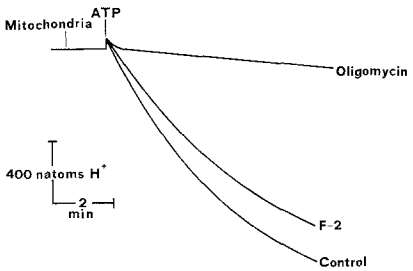

Fig. 3. Effect of F-2 on ATP hydrolysis catalyzed by rat liver mitochondria. The reaction medium was as in Fig. 2. ATP added was 500 nmol. ATP hydrolysis was monitored by measuring the acidification of the medium with a glass electrode pH meter (Orion Research, model 701 A). F-2, 20 μg ml^{-1}; oligomycin, 2 μg ml^{-1}

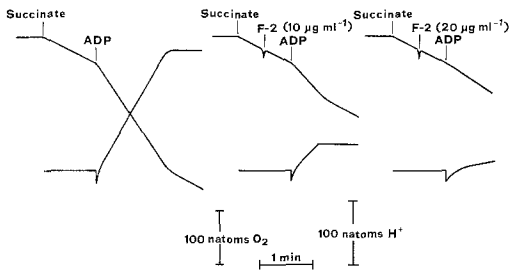

Fig. 4. Effect of F-2 on ATP synthesis catalyzed by rat liver mitochondria. The reaction medium was as in Fig. 2. ATP synthesis was continuously monitored both as changes in oxygen uptake and alkalinization of the incubation medium by mean of an oxygen electrode of the Clark type and a glass electrode (Orion Reserch pH meter, model 701 A) inserted in the same vessel and connected to a double pen recorder. Succinate, 10 μmol, ADP, 300 nmol

the activity of rat liver mitochondria was only 17–21% inhibited. In all the mitochondrial preparations, the inhibition of ATPase activity by F-2 was dependent on the concentration, although the inhibition tended to reach a saturation at higher concentrations of mycotoxin. The effect of F-2 did not increase in the presence of oligomycin or *Cercospora beticola* toxin (CBT), a phytotoxin inhibiting mitochondrial ATPase activity (Macrì et al. 1980).

Table 3. Effect of F-2 on ATPase activity of pea stem and maize root microsomes

	ATPase activity (μmol Pi mg^{-1} protein h^{-1})	
	Maize root	Pea stem
5 mM KCl	2.05±0.32	10.1±1.1
50 mM KCl	3.14±0.10	18.4±0.7
+F-2 (40 μg ml^{-1})	2.65±0.20	15.2±1.8
+DCCD (40 μg ml^{-1})	2.10±0.16	13.5±2.5
+DCCD+F-2	2.12±0.41	13.8±3.1

Data are the mean of three replicates. Variability is expressed as standard deviation

Figure 3 shows the effect of F-2 on ATP hydrolysis catalyzed by a rat liver mitochondrial preparation and measured as acidification of the incubation medium. Conversely to oligomycin, F-2 was much less active in inhibiting an ATP-driven reaction. F-2, instead, markedly inhibited the ATP synthesis, measured both as changes of oxygen uptake and alkalinization of the medium (Fig. 4). The effect of F-2, compared to the control (trace A), increased with the increasing of mycotoxin concentration (traces B and C).

Table 3 shows the effect of F-2 on the ATPase activity of maize root and pea stem microsomes. Only at 40 μg ml^{-1} concentration did F-2 slightly inhibit such an activity and the inhibitory effect disappeared in the presence of DCCD, a specific inhibitor of K^+-dependent ATPase activity.

F-2 (40 μg ml^{-1}) did not alter the ATP level of maize roots after 2 h of incubation. The amount of ATP recovered was 76.1±3.0 nmol ATP g_{FW}^{-1} in the control and 82.8±4.5 nmol ATP g_{FW}^{-1} in F-2 treated roots, respectively.

Discussion

In an our previous paper we reported that F-2 inhibits H^+ extrusion and K^+ uptake, almost immediately

collapses the transmembrane electric potential of maize root, and inhibits the ATPase activity of a microsomal fraction of maize coleoptiles (Vianello and Macrì 1978). The present results indicate that F-2 interacts with isolated pea stem and maize root mitochondria by a dual mechanism: at high concentrations (20–40 μg ml^{-1}) F-2 causes uncoupling, while at low concentrations (5–10 μg ml^{-1}) inhibition of the oxidative phosphorylation occurs. The latter effect is linked to an increasing inhibition of ATPase activity in the range from 5 to 20 μg ml^{-1} of F-2. The mode of action of F-2 is very similar to that postulated for most herbicides termed by Moreland (1980) "inhibitory uncouplers" and known for exhibiting multiple types of responses. In any case, when the mitochondria are pre-incubated with F-2 for about 20 min, the uncoupling effect occurs even at low concentrations of F-2. Hence, the uncoupling appears to be the main effect caused by F-2 on isolated plant mitochondria.

In addition, we have observed that F-2 affects only slightly the ATPase activity of pea stem and maize root microsomal fractions, and does not affect the ATP level of maize roots, indicating that F-2 does not alter the functionality of plant mitochondria in vivo.

The previously shown effects of F-2 on the transmembrane electric potential and thus on related transport phenomena (Vianello and Macrì 1978), in light of the present data, can be interpreted as having been caused mainly by its ability to increase the proton permeability of the plasmalemma, similar to the common uncouplers.

From the reported data, it appears that F-2 inhibits the oxidative phosphorylation of rat liver mitochondria, but only slightly affects their ATPase activity, measured as ATP hydrolysis, this activity being only 17–21% inhibited even at high F-2 concentrations.

These results can be interpreted on the basis of a prevailing effect of the mycotoxin on the synthesis of ATP rather than on its hydrolysis. Inhibition of ATPase activity was observed with other mycotoxins (Wallace 1978) and aflatoxins in particular (Desaiah et al. 1979); however the mechanism of action of F-2 on rat liver mitochondria seems quite similar to that of the antibiotic aurovertin (Lenaz 1965; Lee and Ernster 1968; Roberton et al. 1968).

There is no contradiction between the results obtained with animal mitochondria and those obtained with plant mitochondria. In fact, it is well known that plant mitochondria exhibit several similarities to animal mitochondria (Palmer 1976), but also important differences, mainly related to sensitivity to inhibitors.

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