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Inhibition of Plant Cell Membrane Transport Phenomena Induced by Zearalenone (F-2)

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Abstract. Zearalenone (F-2), an estrogenic factor produced by a number of *Fusarium* spp., stimulates a leakage of electrolytes, β-cyanin and aminoacids from three plant tissues. F-2 inhibits rubidium uptake in roots of *Zea mays* L. and *Beta vulgaris* L. var. rubra. However the effect in the latter tissue is evident after long-term treatments with the toxin. Rubidium uptake is not affected in *Solanum tuberosum* L. var. Bintje. The toxin also causes inhibition of H^+ extrusion, of root elongation, of ATPase activity of plasmalemma-enriched fractions and depolarization of transmembrane potentials of corn roots. The evidence presented supports the hypothesis that F-2 affects plasma membranes of several plant species.

Key words: Membrane potential – Membrane transport – Plasmalemma – Transport (membrane) – Zearalenone.

Introduction

The mycotoxin zearalenone (F-2) is an estrogenic secondary metabolite produced by a number of species of *Fusarium* colonizing corn and other cereals. The estrogenic compound has been found in commercial feed in sufficient amounts to cause the estrogenic syndrome in animals which were fed by such feed (Mirocha et al., 1971). Chemically zearalenone is a 6-(10-hydroxy-6-oxo-trans-1-undecenyl) β-resorcylic acid lactone with a molecular weight of 318 which fluoresces under ultraviolet (UV) excitation (Mirocha et al., 1971).

Hitherto the effect of zearalenone on plants has not been studied in sufficient detail. It was only observed that toxins produced by *Fusaria*, which cause an estrogenic effect in animals, kill young plants (Joffe, 1971) and inhibit seed germination and embryo growth (Brodnik, 1975).

In this work we have focussed our attention on the effect of the mycotoxin F-2 on plasma membrane activities of three plant species.

Abbreviations: F-2 = Zearalenone, PD = Potential difference, FC = Fusicoccin, DCCD = Dicyclohexylcarbodiimide

Materials and Methods

Chemicals

All chemicals were reagent grade. $^{86}RbCl$ was purchased from Radiochemical, Amersham, England. Zearalenone was generously given to us by Prof. C.J. Mirocha, University of Minnesota, and IMC Chemical Group, Terre Haute, USA. Fusicoccin (FC) was a gift of Prof. E. Marré, University of Milan. F-2 was dissolved in ethanol to give a 2 mg/ml stock solution and this added to incubation solutions to give a final F-2 concentration of 10 μg/ml. The controls contained an equal amount of ethanol. ATP (Na^+ salt) was purchased from Boehringer GmbH, Mannheim, Germany. ATP (K^+ salt) was prepared with the aid of H^+ Dowex column.

Plant Material

Potato (*Solanum tuberosum* L. var. Bintje) and red beet (*Beta vulgaris* L. var. rubra) were obtained from local commercial sources. Disks of these plant materials with a diameter of 8 mm and 4 mm thickness for leakage experiments and of 6 mm in diameter and 2 mm thickness for rubidium transport were obtained by a cork borer. Corn grains (*Zea mays* L.) were surface sterilized with 1% sodium hypochlorite and germinated on stainless steel mesh above aerated 0.5 mM $CaCl_2$ at 25° C in the dark. Roots of 6 to 7-day-old were cut into 2 cm subapical segments; the apical 10 mm have been removed. For potential difference (PD) measurements corn seeds were pre-germinated for 60 to 70 h on wet filter paper in the dark at 26° C and then germinated in 0.5 mM $CaSO_4$ solution constantly aerated.

Net Measurements of Electrolyte, β-Cyanin and Aminoacids Leakage

Disks of red beet and potato tuber were washed in tap water for 3 h before use. Aliquots of 1 g (5 disks) were incubated in

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15 ml of aqueous toxin solution (10 µg/ml) at 30° C with constant shaking in the dark. Ten segments of corn roots (0.2 g) were washed for 30 min in tap water and incubated in 6 ml of toxin solution at the conditions specified above. Loss of electrolytes from all tissues was measured as increase of conductivity of the bathing solution with a Philips conductivity meter, model PW 9501, using a dip type electrode cell (cell constant 0.77). From potato disks the leakage of aminoacids was measured by the ninhydrin method of Moore (1968). Loss of β-cyanin from beet disks was determined by measuring the increase in absorbance of the incubation solution at 530 nm.

Toxin uptake

Thirty-five disks of potato and red beet (6 mm in diameter and with 2 mm thickness) were incubated in 6 ml of aqueous toxin solution (10 µg/ml), at 30° C with shaking. Since the aqueous toxin solution strongly absorbs at 236 nm, toxin uptake was evaluated as decrease of absorbance of the solution at such wavelength. After 18 min of incubation, the toxin penetrated was extracted from the tissues by homogenizing the disks in 20 ml of benzene. The homogenate was filtered through paper and the toxin concentration in the benzene fraction was evaluated from the increase in absorbance at 236 nm with respect to the untreated tissues.

Rubidium Uptake Experiments

Disks of potato and red beet (6 mm in diameter and 2 mm thickness) were activated for 24 h and 72 h, respectively, in aerated 0.5 mM $CaCl_2$. Batches of 20 activated potato and beet disks or 40 corn root segments were pre-incubated in 20 ml of toxin solution (10 µg/ml) at 30° C in the dark. At 1, 3, 6 h intervals, groups of 5 disks of potato or 20 corn root segments were rinsed in water and incubated in 20 ml of 0.5 mM $CaCl_2$ plus 0.1 mM RbCl labeled with 0.06 µCi/ml of ^{86}Rb. The initial pH of the solutions was 5.6 and the temperature throughout the experiments was maintained at 25° C. At the same intervals, groups of 5 beet disks were also rinsed with water and incubated in 20 ml of 0.2 M Tris-HCl buffer, pH 7.8, plus 0.5 mM $CaCl_2$ and 0.1 mM RbCl labeled with 0.06 µCi/ml of ^{86}Rb. After an uptake period of 30 min, all tissues were rinsed with about 200 ml of cold unlabeled 10 mM KCl for a total of 30 min to remove free space contamination. The material was finally digested at 70° C in vials containing H_2O_2. The radioactivity was measured after adding 1 ml of water and 5 ml of scintillation cocktail (7 g PPO, 0.3 g dimethyl POPOP, 100 g naphthalene per 1,000 ml of 1,4 dioxane)[1] with a Packard Tri Carb spectrometer, model 3320.

Elongation Test and H^+ Extrusion from Corn Roots

Batches of 40 apical segments (10 mm long, tip included) were pre-incubated for 30 min in a 0.5 mM $CaCl_2$ plus 0.25 mM $MgCl_2$ solution and then transferred in 8 ml of the same medium containing 10 µg/ml of F-2 at 30° C for 3 h in the dark.

Twenty root segments (0.4 g), washed for 30 min in distilled water, were incubated in 8 ml of unbuffered toxin solution (10 µg/ml). Measurement of pH was performed with a Corning Eel pH meter, model 109. The incubation was carried out in the dark at 30° C with shaking.

[1] PPO, 2,5-diphenyloxazole; POPOP, p-bis-(2-(5-phenyloxazolyl)) benzene

Transmembrane Potential Measurement in Apical Corn Root Segments

Apical root segments (1 cm long) were pre-incubated for 30 min in 10 ml of an aerated 0.5 mM phosphate buffer pH 6.2, containing 0.5 mM $CaCl_2$ and 0.125 mM $MgSO_4$ at 26°C in the dark. The segments were then incubated in a lucite cuvette of 1.5 ml volume, under continuous flow 3 ml/min of the aerated, thermostatized (26° C) medium containing 10 µg/ml of toxin. After 60 min, using the same roots, fusicoccin (10^{-5} M) was added to the incubation medium. Changes in the electric transmembrane potential were measured according to the conventional procedure described by Cocucci et al. (1976).

Membrane ATPase Activity

"Crude membrane" fraction from etiolated corn coleoptiles was prepared according to the method described by Beffagna et al. (1977). ATPase activity was measured at 26° C in a 1 ml volume containing an aliquot of membrane preparation (30 µg protein), 1 mM ATP (K^+ salt), 1 mM $MgCl_2$ and the desired amounts of KCl and toxin concentration at pH 6. The reaction was started by addition of ATP. Pi released was determined after 120 min of incubation by the method of Fiske and Subbarow (1925). Substrate blanks were subtracted to calculated enzyme activities.

Protein was estimated by the method of Lowry et al. (1951). All experiments were repeated at least three times.

Results

Effect of F-2 on Loss of Organic Metabolites and Ions from Plant Tissues

Figure 1 shows the loss of electrolytes and β-cyanin from beet disks stimulated by F-2. The effect was evident after 30 min of incubation and progressively increased with time. Similar effects were also induced on ion and aminoacid leakage from potato (Fig. 2) and on loss of ions from corn roots (Fig. 3). Figure 4 shows the concentration dependence of the F-2 effect expressed as loss of β-cyanin and electrolytes from beet and potato disks, respectively. The effect of the toxin was linear and did not reach a tendency to saturation within the range of concentrations used. The dosage-response was similar in potato and beet root. The experiment carried out to test the effect of pH on F-2 activity was run in buffered medium because of the instability of the toxin at pH far from neutrality. The activity of F-2, measured as loss of β-cyanin from beet, was strongly dependent upon the external pH (Fig. 5). The maximum loss of β-cyanin was observed at pH 6.0, whereas the activity dropped rapidly at pH 4.0 and 8.0.

F-2 Uptake in Potato and Beet Disks

Figure 6 shows F-2 uptake evaluated as diminished absorbance of the aqueous toxin solution at 236 nm.

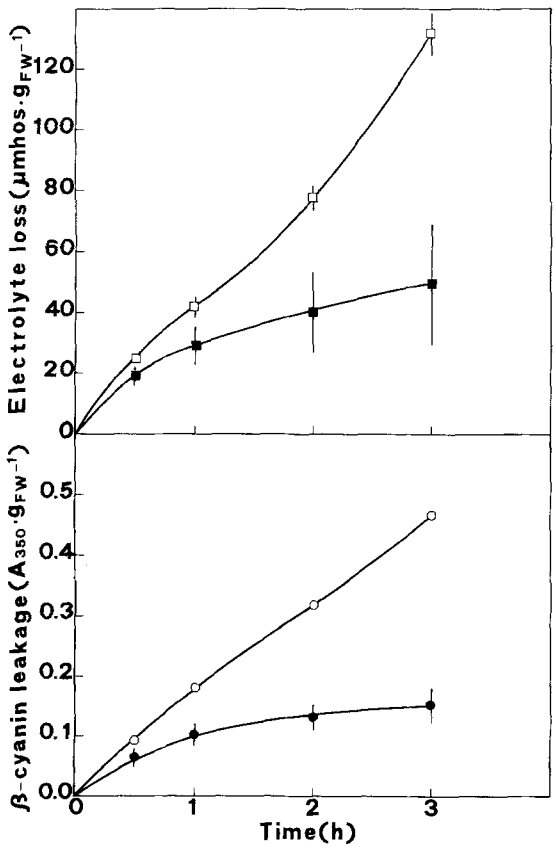

Fig. 1. Effect of F-2 on the leakage of ions and β-cyanin from beet root disks. Ion loss from F-2-treated (□) and untreated tissues (■); loss of β-cyanin from F-2-treated (○) and untreated tissues (●). The data are the mean of 5 replicates. Vertical bars represent standard deviation

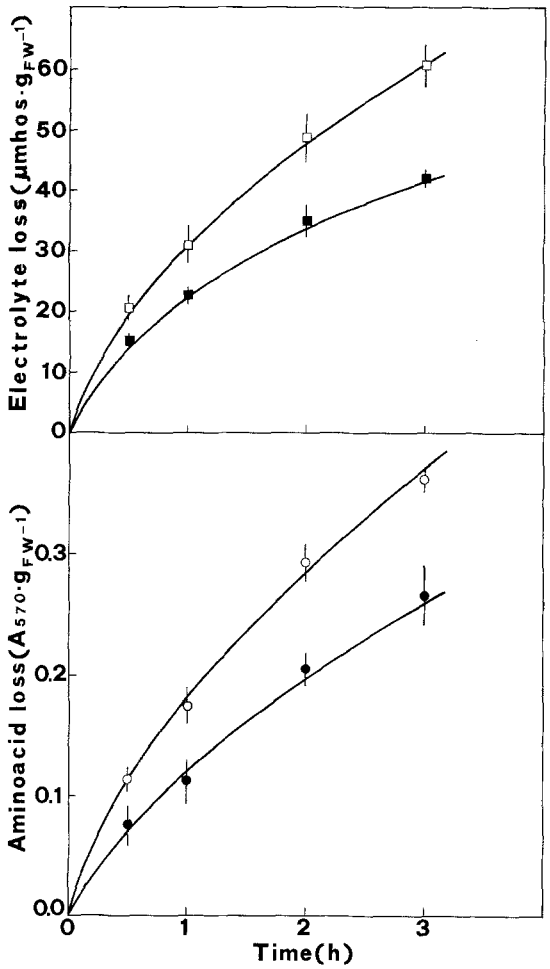

Fig. 2. Effect of F-2 on loss of electrolytes and aminoacids from potato disks. Ion leakage from F-2-treated (□) and untreated controls (■); aminoacid leakage from F-2-treated (○) and untreated tissues (●). Data are the average of 5 replicates. Variability is given as standard deviation

F-2 penetrated in both tissues. The uptake was very rapid in the early period of treatment and decreased rapidly with the increase of the incubation time. The absorbed toxin was also readly extracted from the treated tissues by treatment with benzene.

Inhibition of Rubidium Uptake

F-2 inhibited rubidium uptake in corn roots by 30 to 40% after 1 h of pre-incubation and also completely inhibited the "augmentation" of rubidium uptake observed in the controls and that reached its maximum value after 6 h of pre-incubation (70% inhibition of uptake) (Fig. 7a). This "augmentation" seems to be due to aging of the tissue as previously observed in low salt corn root segments (Laties, 1969; Anderson, 1972; Leonard and Hanson,

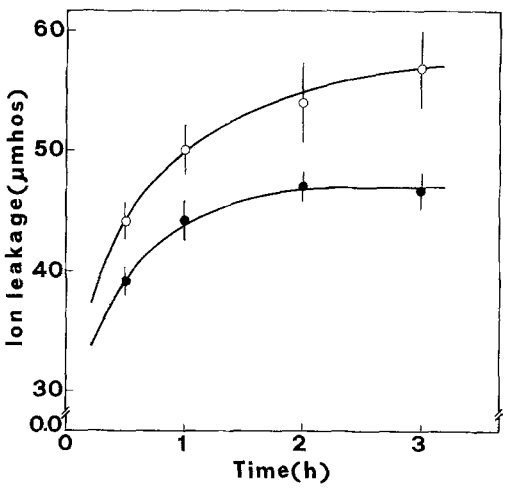

Fig. 3. Effect of F-2 on ion leakage from corn roots. F-2-treated, (○); untreated, (●). Data are the mean of 3 replicates. Variability is given as standard deviation

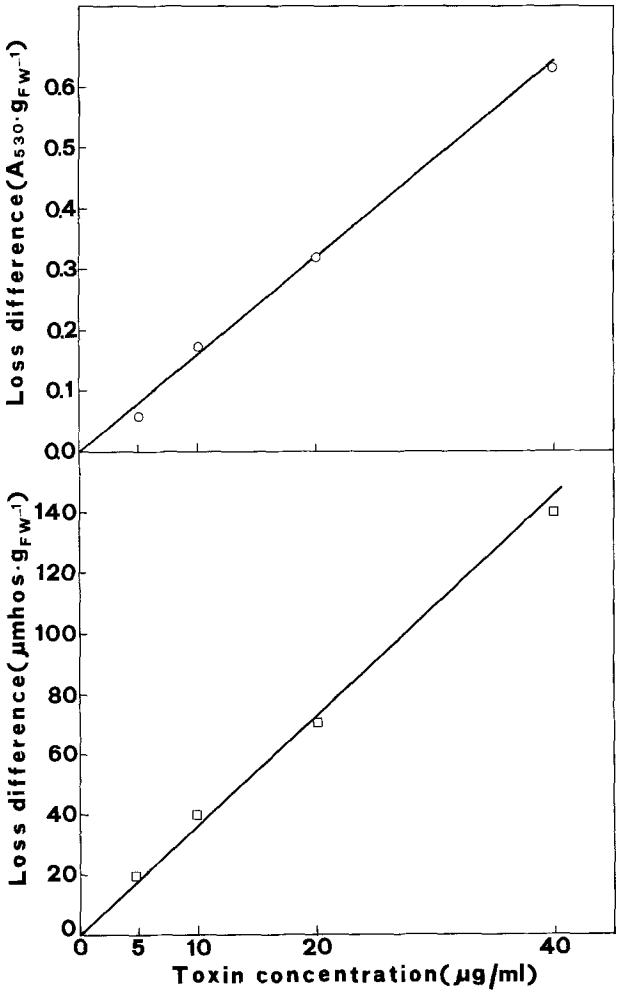

Fig. 4. Effect of F-2 concentration on F-2-induced leakage of β-cyanin (above) and ions (below) from beet and potato disks, respectively. The effect was evaluated as difference between the values from F-2-treated and untreated. Data are the mean of 5 replicates

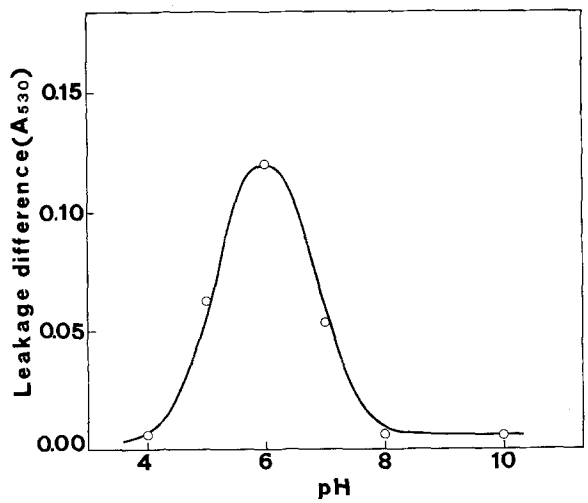

Fig. 5. Effect of pH on F-2-induced β-cyanin loss. The effect was determined as difference between the values from F-2-treated and untreated. The data are the mean of 5 replicates

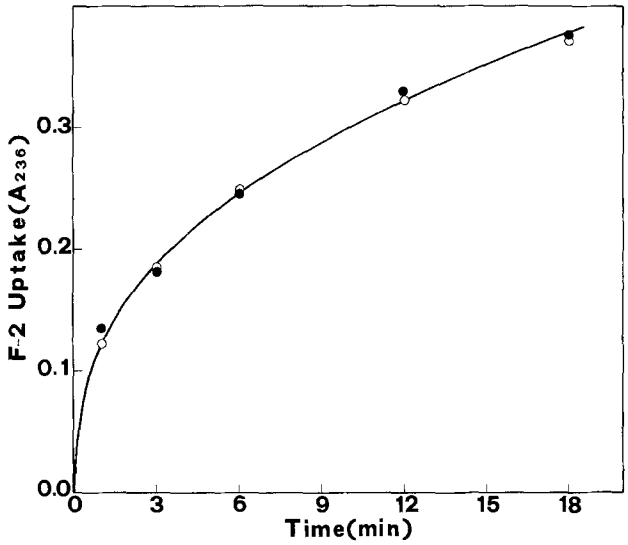

Fig. 6. F-2 uptake in potato (●) and beet (○) disks. Uptake was evaluated as decreased absorbance at 236 nm of the bathing solution. Data are the mean of 5 replicates

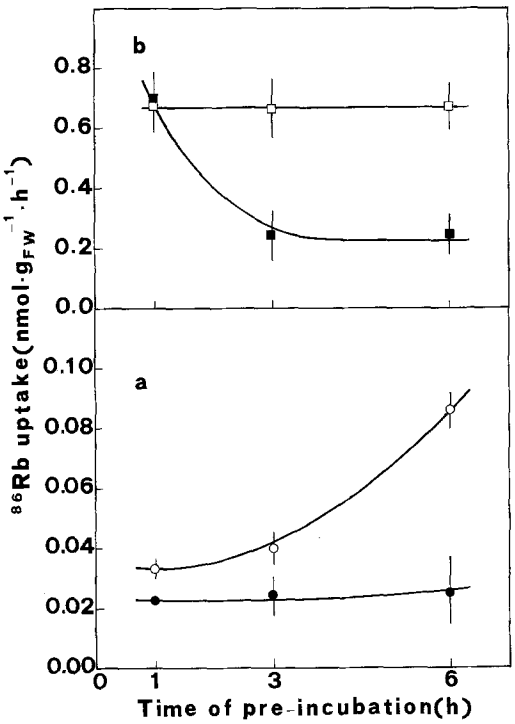

Fig. 7a and b. Effect of F-2 on ^{86}Rb uptake in red beet (**b**) and corn roots (**a**). F-2-treated beet, (■) and corn, (●); untreated beet, (□) and corn, (○). Data are the mean of 3 replicates. Variability is given as standard deviation

1972). The F-2 also affected the uptake of rubidium in activated beet disks (Fig. 7b). In this case, however, the inhibitory effect was evident only after 3 h of pre-incubation and remained constant at longer times of pre-treatment. The rubidium transport tested in activated potato disks was not affected by the toxin (Table 1).

Table 1. Effect of F-2 on ^{86}Rb uptake in activated potato disks. Data are expressed in nmol $g_{FW}^{-1} h^{-1}$

	Time of pre-incubation (h)		
	1	3	6
Control	4.2±0.2	4.0±0.7	4.0±0.4
F-2-treated (10 µg/ml)	3.7±0.3	3.9±0.4	4.0±0.3

Twenty disks of potato were pre-incubated in 20 ml of F-2 (10 µg/ml). At 1, 3, 6 h intervals, groups of 5 disks were transferred in 20 ml of 0.1 mM RbCl labeled with 0.06 µCi/ml ^{86}Rb plus 0.5 mM $CaCl_2$ for 30 min at 25° C. Data are the mean of 4 replicates. Variability is given as standard deviation

Effects of F-2 on H^+ Extrusion and on Corn Root Elongation

The data in Figure 8 show the effect of F-2 on the acidification of the incubation medium. Under the conditions of this experiment, the acidification in controls was marked during the first 4 h while pH in the subsequent period tended to stabilize at ca. 5.7 value. In toxin-treated roots H^+ extrusion was completely inhibited. The block of acidification of the medium caused by F-2 corresponded to a marked inhibition of root elongation in apical corn segments. Growth of control in 3 h was 17%, and F-2 inhibited it by 23%.

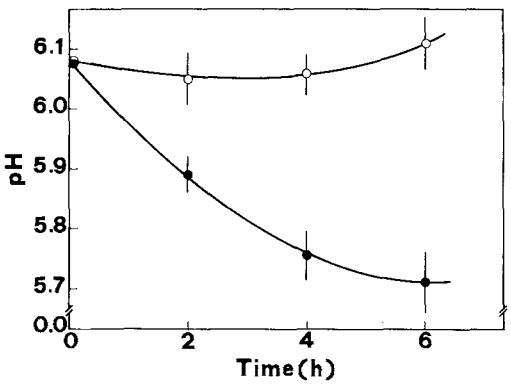

Fig. 8. H^+ extrusion in F-2-treated corn roots. H^+ extrusion was measured as increase of pH of the bathing solution. F-2-treated, (○); untreated, (●). The data are the mean of 5 replicates. Vertical bars represent standard deviation

Kinetic of F-2 Effect on Cell PD

F-2 caused a rapid depolarization of PD in cortical cells of corn roots (Fig. 9). Maximum depolarization was observed after 10 min from the beginning of the experiment. PD value fell from −98 to −75 mV and then remained constant in the next period. The very rapid response to the toxin might be interpreted as an indication of its action at cell membrane level. When FC, a known hyperpolarizing compound (Cocucci et al., 1976), was added to the F-2 treated roots, a rapid increase of PD was observed. The effect on the PD took 20 min ca. to reach its maximum value (−110 mV). This value did not correspond to those usually observed in FC-treated tissues (−130 mV) (Cocucci et al., 1976). However the increase of PD from −87 to −110 mV was of the same magnitude.

Effect of F-2 Treatment on ATPase of Enriched-Membrane Preparation

The addition of F-2 to a plasmalemma-enriched membrane fraction, prepared from untreated corn coleoptiles, induced a decrease of ATPase activity (Table 2a, b). The inhibition was 12 and 27% respectively at 30 and 60 µg/ml of toxin concentration, while F-2 did not affect the ATPase activity at a concentration of 10 µg/ml. When KCl concentration in the medium was increased from 4 to 30 mM the inhibitory effect of F-2 increased if expressed as absolute value, but slightly diminished if expressed as a percentage. The

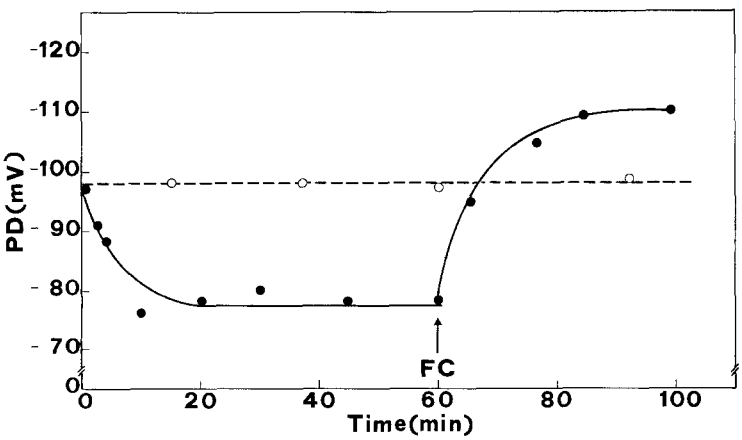

Fig. 9. Effect of F-2 on PD as a function of time. FC (10^{-5} M) was added at the time indicated by arrow. F-2-treated, (●); untreated, (○). Data are the mean of 3 replicates

Table 2a. Effect of F-2 and DCCD on the ATPase activity of a plasmalemma-enriched preparation at low and high KCl concentration

	ATPase activity (nmol Pi g_{FW}^{-1} 120 min^{-1})		
	DCCD KCl 4 mM	KCl 4 mM	KCl 30 mM
Control	1.01 ± 0.13	2.60 ± 0.13	3.66 ± 0.37
F-2 (60 µg/ml)	1.05 ± 0.11	1.69 ± 0.11	2.75 ± 0.16
F-2 effect (as difference)	–	0.91	1.01
% effect of F-2	–	35	27

Experimental conditions are specified in Materials and Methods section. DCCD at final concentration 3×10^{-4}M was added 10 min before starting the reaction with ATP. The ethanol used to dissolve F-2 and DCCD did not exceed the concentration of 2% in the incubation mixture. Controls also contained ethanol at the same concentration. Data are the mean of 3 replicates. Variability is expressed as standard deviation

Table 2b. Effect of F-2 concentration on the ATPase activity of a plasmalemma-enriched preparation at high KCl concentration (30 mM)

	F-2 concentration (µg/ml)			
	0	10	30	60
ATPase activity (nmol Pig_{FW}^{-1} 120 min^{-1})	3.66 ± 0.37	3.79 ± 0.46	3.31 ± 0.05	2.75 ± 0.16
F-2 effect (as difference from control)	–	–	0.46	1.02
% F-2 effect	–	–	12	27

Experimental conditions as in Table 2a

effect of F-2 disappeared when the activity was tested in the presence of the K^+-dependent ATPase inhibitor DCCD (dicyclohexylcarbodiimide). Thus suggesting that only DCCD-sensitive ATPase activity is inhibited by the toxin.

Discussion

The data reported in the present paper indicate that the estrogenic factor zearalenone is also strongly phytotoxic and that it alters membrane permeability of some plant tissues. In fact changes in cell permeability, detected as loss of ions, β-cyanin from beet and aminoacids from potato, were observed in all the plant materials studied. Since β-cyanin is a vacuolar compound, we suggest that the toxin can alter both the plasmalemma and the tonoplast. The effects were somewhat rapid and marked after few hours from the beginning of the experiment.

F-2 also inhibited rubidium uptake in corn and activated beet roots, whereas transport in activated potato disks was unaffected even after a long-term pre-treatment with the toxin. The effect on corn roots was more rapid than in beet. In the latter tissue inhibition was observed only after 3 h of pre-incubation. The lack or the delayed effect of F-2 on rubidium transport of activated storage tissues and the observation that the toxin inhibited the enhanced transport rate, shown to occur only in aged low salt corn segments (Laties, 1969; Anderson, 1972; Leonard and Hanson 1972), might suggest that F-2 acts especially on this "augmented" uptake. A similar effect was also observed for other phytotoxins which were shown to inhibit ion uptake (Frick et al., 1976; Vianello et al., 1976).

The association among H^+ extrusion, ion uptake, transmembrane potential and plasma membrane ATPase activity is well established (Higinbotham and Anderson, 1974; Haschke and Lüttge, 1975; Marrè, 1977). The inhibition of rubidium uptake that we have observed in excised corn roots was associated with inhibition of H^+ extrusion, with an almost immediate (no detectable lag period) depolarization of transmembrane potential, and with the inhibition of the ATPase activity of plasmalemma-enriched membrane preparations. Hence, F-2 seems to depress an electrogenic mechanism of H^+/K^+ cation exchange. From these results it appears that F-2 acts in a similar manner to that observed for the host selective toxins known to induce necrosis an reduction of growth on sensitive plants (Mertz and Arntzen, 1973; Novacky and Hanchey, 1974; Gardner et al., 1974; Van Sambeeck et al., 1975). This legitimates the hypothesis that the damages induced by *Fusarium* toxins on young plants (Joffe, 1971) and the inhibition of seed germination and embryo growth (Brodnik, 1975) may depend on the capacity of F-2 to inhibit some fundamental cell membrane functions.

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