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Fusicoccin-activated proton extrusion coupled with K^+ uptake, and its role in the regulation of growth, germination, opening of stomata and mineral nutrition

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Fisiologia vegetale. — *Fusicoccin-activated proton extrusion coupled with* K^+ *uptake, and its role in the regulation of growth, germination, opening of stomata and mineral nutrition* (*). Nota di ERASMO MARRÈ, PIERA LADO, FRANCA RASI CALDOGNO e ROBERTA COLOMBO, presentata (**) dal Corrisp. E. MARRÈ.

RIASSUNTO. — L'esame dei dati disponibili circa gli effetti fisiologici della fusicoccina (FC) nelle piante superiori porta a postulare il seguente gruppo di ipotesi di lavoro:

1) Tutti gli effetti fisiologici noti della FC (quali gli stimoli della crescita per distensione, della traspirazione per apertura degli stomi, della interruzione della dormienza dei semi, della nutrizione minerale) sono riconducibili alla capacità della FC di attivare l'estrusione attiva di protoni e il contemporaneo aumento dell'assunzione di ioni potassio.

2) Gli effetti della FC sull'estrusione di protoni, l'assunzione di potassio–ioni, l'aumento del potenziale elettrico transmembrana sono strettamente intercollegati, e dipendono della attivazione di un singolo meccanismo, presumibilmente localizzato a livello della membrana cellulare.

3) Questo meccanismo è presente in pressochè tutti gli organi della pianta, e in condizioni normali la sua attività è mantenuta su livelli subottimali dall'azione di fattori regolativi interni. L'attivazione di questo meccanismo da parte della FC dipende dalla sua capacità di rimuovere un qualche tipo di controllo negativo fisiologico.

4) Una frazione consistente dell'azione degli ormoni (ed anche di fattori ambientali come quelli coinvolti nel foto– e nel termoperiodismo) sui processi fisiologici sensibili alla FC (crescita per distensione, traspirazione, dormienza e nutrizione minerale) può essere spiegata dalla capacità di regolare, in senso positivo o negativo, l'attività del meccanismo sensibile alla FC e coinvolto nell'estrusione di protoni e risposte associate.

INTRODUCTION

Recent work carried out in this and other laboratories shows that Fusicoccin (FC) a diterpene glucoside produced by *Fusicoccum amygdali Del.*, strongly influences in higher plants several important physiological processes. In fact, FC has been shown: *a*) to promote cell enlargement in stem [19, 20], root [35, 52] and coleoptile sections [52], in isolated cotyledons [33] and in leaf discs [19]; *b*) to strongly stimulate leaf transpiration by inducing opening stomata [10, 27]; *c*) to induce rapid germination of dormant seeds [21]; *d*) to stimulate K^+ [34] and aminoacid active uptake in stem [23] and root segments [41] and in stomata guard cells [49]; *f*) to increase the transmembrane electric potential in stem and root segments and in isolated cotyledons [32].

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Abbreviations: ABA, abscisic acid; FC, fusicoccin; FR, far red light; GA_3, gibberellic acid; IAA, indole–3–acetic acid.

In all cases investigated, the above mentioned responses are accompanied by an increase of the capacity of the FC-treated tissues to increase the acidity of the incubation media [22, 29, 30, 31, 52]: this " proton extrusion " effect has been shown to depend rather specifically on the availability of K^+ in the incubation medium and on the integrity of oxidative-phosphorylative metabolism [34, 31]. The hypothesis has thus been proposed that all of the physiological responses to FC are mediated by its capacity to activate, in the various tissues investigated, a single mechanism, namely the energy dependent exchange of protons (extruded) with K^+ (taken up) at cell membrane level [34].

If this is true, a single FC-sensitive mechanism, characteristic of higher plants, would play a very important role in the control of a wide range of physiological activities of such different organs as leaves, stems, roots, dormant seeds. The interest for the study of this mechanism is emphasized by the fact that: *a*) almost all the physiological processes sensitive to FC are also characteristically influenced by the natural hormones, auxin, gibberellin, cytokinin, and abscisic acid, or by phytochrome-mediated environmental stimula; *b*) in several cases the effects of these natural regulating factors are accompanied by changes of proton extrusion, K^+ uptake and electric potential similar to those induced by FC [33].

Aim of this paper is the attempt to coordinate in a unitary working hypothesis the available evidence on the mechanism of the physiological action of FC. The results reported in the following sections come either from work published or in press, or from unpublished data recently obtained in this laboratory.

I. PHYSIOLOGICAL RESPONSES TO FC

Table I presents a synthetic view and a roughly quantitative evaluation (where possible) of the most relevant physiological effects of FC. The contents of the Table can be commented as follows:

A) *Growth by cell enlargement.*

In stem and coleoptile segments the effects of FC at optimal concentration (*ca.* 1.5×10^{-5} M) on both elongation and increase in fresh weight are consistently larger (by *ca.* 60 %) than those of natural or synthetic auxins [20]. This growth response is accompanied by changes of cell wall plastic extensibility similar to those induced by auxin [20, 25], and shows a shorter lag phase than that observed with auxin (R. Cleland, personal communication). An important difference is that treatments with superoptimal (up to 10^{-3} M) FC concentrations induce little or no inhibition of growth, and do not promote ethylene production [20], while it is well known that both these effects are characteristic of superoptimal auxin concentrations. The effects of FC and IAA on cell enlargement are additive at the suboptimal concentrations, while with both compounds present together at the optimal concentrations the growth response is equal to that induced by FC alone [20].

TABLE I

Influence of FC *and hormones on various physiological processes.*

Processes	Material	Control	FC	Hormones activating (+) or inhibiting (—) the processes	References
Cell enlargement (data as % increase in length or in fresh weight per hour)	stem (pea)	3.2	11	IAA (+), ABA (—)	[20, 44]
	coleoptile (maize, oats)	2.5	10 (maize)	IAA (+), ABA (—)	[35, 43]
	cotyledons (squash,	1.3	4	cytokinins (+)	[33, 16]
	radish)	1.5	5.6	cytokinins (+)	[33, 15]
	leaves (tomato,	0.5	4		[19]
	clover)	4.4	8.6		
	root segments (pea,	6.0	10 (1)	IAA (+ and —)	[35]
	maize)	6.0	19 (1)		
Opening of stomata (data as mean aperture μm per 3 h)	leaves (Commelina communis,	8.4	10.7	cytokinins (+),	[50]
	bean, dogwood, tobacco,	1.6	9.1 (2) (bean)	ABA (—)	[49]
	barley)	5.2	8.4 (3) (bean)		
Uptake of K^+ (data as μeq × g fr $w^{-1} \times h^{-1}$)	leaves (bean, Commelina communis)	+	+++ (bean)	ABA (—)	[49]
	stem (pea, sunflower)	0.50	1.8 (pea)	IAA (+)	[34]
	coleoptiles (maize, avena)	0.22	0.87 (maize)	IAA (+), ABA (—)	[35, 11, 38]
	root segments (maize)	2.00	4.00		[35]
	germinating seeds (radish)	0.16	0.29		[4]
	cotyledons (squash, sunflower)	0.45	1.75(1)(squash)	cytokinins (+)	[35, 14]
Breaking of seed dormancy (data as % germination)	Radish (14 h)	5	25	GA_3 (+), cytokinins (+)	[21]
	Radish (+ ABA) (20 h)	1	28	ABA (—)	
	Maize (31 h)	28	50		[22]
	Dormant wheat (22 h)	8	44	GA_3 (+)	[21, 8]
	Pea (16 h)	5	25		[35]
	Lettuce (+ FR) (24 h)	0	80	GA_3 (+), cytokinins (+), ABA (—)	[21]
	Tomato (96 h)	7	63		[35]
	Aplopappus (16 h)	12	68		[9]

(1) Data obtained in our laboratory (unpublished); (2) Dark; (3) Light.

In isolated cotyledons, where cell enlargement is promoted by cytokinins, and not by auxins or gibberellins, FC is much more active (by *ca.* 200%) than kinetin or benzyladenine [33]. The response is very rapid and does not involve (as in the case of the cytokinins) stimulation of anthocyanin synthesis [46].

In maize and pea root segments (where cell enlargement is slightly promoted by very low, and inhibited by medium (10^{-7}-10^{-5} M) auxin concentrations) the effect of FC is always stimulatory, an increase by *ca.* 250% of cell enlargement over the controls being induced by the optimal (*ca.* 1.5×10^{-5} M) concentration [35].

Finally, in leaf discs or fragments (where cell enlargement is insensitive to auxin, cytokinins and ABA) FC induces an early increase of fresh weight by *ca.* 100–200% larger than that of the controls in water. This effect is accompanied by a corresponding decrease of the osmotic pressure of the cell sap, and seems therefore to depend on a cell wall loosening effect similar to that shown in FC treated stem tissues [19]. No effects of FC are detected in these materials on senescence symptoms such as chlorophyll decrease and proteolysis, two processes which are known to be strongly inhibited by cytokinins and variously affected by other plant hormones.

These data show that FC stimulates cell enlargement in a quite large variety of plant tissues, independently of their capacity to give or not a similar response to one or the other of the natural growth promoting substances. Moreover, the available data are in agreement with the view that in all cases the effect of FC on cell enlargement is mediated by its capacity to increase the plastic extensibility of the cell wall.

B) *Control of stomata opening and traspiration.*

FC strongly stimulates leaf transpiration and induces leaf wilting, as a consequence of its capacity to increase turgor in the stomata guard cells, thus promoting stomata opening [1, 10, 39, 50]. Recent work shows that FC-induced opening of stomata is accompanied by a very large accumulation of K^+ in the guard cells [49]. This suggests that the mechanism of action of FC is fundamentally the same operating in the physiological control of stomata by light. In fact, recent evidence indicates that ligth-induced opening of stomata depends on an increase of osmotic pressure in the guard cells consequent to the activation of K^+ uptake accompanied by active proton extrusion [40].

C) *Control of mineral nutrition and active uptake.*

This physiologically relevant aspect of FC action is still very little explored. However, the available evidence strongly indicates that the glucoside markedly influences some key point of ion and solute active uptake and traslocation. In fact, a marked stimulation of active K^+ uptake by FC has been found in stomata guard cells [49], stem [34] and coleoptile sections, isolated cotyledons [35], germinating seeds [4] and root segments [35]. The stimulation of K^+ uptake is accompanied (as further discussed in greater detail) by

acidification of the medium. It is well known that active K^+ uptake and acidification of the environment are very important components of the whole process of mineral nutrition at the level of the root system [15].

Evidence is also accumulating that a proton gradient across the cell membrane built up by means of K^+/H^+ exchange can be utilized for the active transport of other (not necessarily charged) solutes, such as aminoacids and sugars [6, 28, 37].

The finding is of interest in this regard that FC significantly increases the active (uphill) uptake of leucine in the pea internode segments [23] and in the isolated root tips [41].

D) *Breaking of seed dormancy and germination.*

FC at concentration between 10^{-6} and 10^{-5} M accelerates germination of several seeds. This promotion effect is most evident when seed germination is inhibited because of either physiological dormancy or treatment with inhibitors such as abscisic acid (ABA) or (as in the photosensitive lettuce seeds) far red light. Under these conditions FC appears much more active than the natural germination promoting hormones gibberellic acid and cytokinins. In far-red treated photosensitive lettuce seeds and in recently harvested wheat seeds FC completely substitutes red light or, respectively, vernalization in inducing maximum germination [21].

A possibility of interpreting these effects of FC as mediated by the same mechanism as in the case of its action on cell enlargement and stomata opening comes from the data [2, 12] suggesting that seed dormancy depends on a mechanical limitation of embryo growth by the surrounding tissues. If this is true, it seems reasonable to hypothesize that the primary effect of FC in the seed is the stimulation of water uptake and cell enlargement of the embryo tissues, consequent to acidification and thus to the loosening of the cell wall (as in the promotion of cell enlargement in stems), and/or to the increase of intracellular osmatic pressure (as in the effect on stomata opening). In fact, Lado *et al.* [22] have shown that the promoting effect FC on germination is preceeded or accompanied by acid secretion, just as in the case of the promotion of growth of stem and coleoptile segments, and unpublished data by Cocucci *et al.* [4] indicate that FC stimulates the development of active K^+ uptake in the early phase of seed germination.

II. Mechanism of action of fusicoccin. Relationship between the effects on proton extrusion, potassium uptake and transmembrane electric potential

The various physiological effects of FC appear constantly accompanied by a marked increase of energy- dependent acid secretion and K^+ uptake (Table II). Moreover, the proton extrusion effect is strongly and specifically stimulated by the presence of K^+ in the extracellular medium, other mono-

valent ions appearing ineffective [34]. The K^+-requirement of the proton extruding mechanism might well be very strict, as the residual acid secretion observed when K^+ is not added to the medium might depend on the presence of low but still significant levels of this ion in the cell wall space, due to either leakage from the cells or acidification-induced cation release from the Donnan free space.

TABLE II

Effects of FC *on proton extrusion,* K^+ *uptake and transmembrane potential* (V_m).

MATERIAL	H^+ extrusion ($\mu eq \times g\ fr\ w^{-1} \times h^{-1}$)		K^+ uptake ($\mu eq \times g\ fr\ w^{-1} \times h^{-1}$)		V_m (after hour) (*m*V)	
	— FC	+ FC	— FC	+ FC	— FC	+ FC
Pea stem	0.03 (*b*)	1.80 (*a*) 1.30 (*b*)	0.5 (*b*)	5.05 (*a*) 1.80 (*b*)	— 60	— 83
Maize coleoptiles	0.10	0.45	0.22	0.87		
Maize roots	0.0	1.70	2.00	4.00	— 90	— 120
Squash cotyledons	0.0	0.25	0.45	1.75	— 74	— 97
Germinating radish seeds	0.01	0.08	0.16	0.29 (*c*)	—	—

(*a*) Pretreated with FC; (*b*) Pretreated with acid; (*c*) Rate of K^+ absorption between the 4th and the 15th h of germination.

An interesting phenomenon accompanying FC-induced proton extrusion and K^+ uptake is a consistent increase (ranging from 20 to 40 mV) of the negative electric transmembrane potential (Table II). This hyperpolarization effect is depressed (together with proton extrusion, growth and the other physiological responses) by respiration inhibitors and by phosphorylation uncouplers, and strongly reduced by protein synthesis inhibitors [32]. The increase of negative intracellular potential in the FC treated tissues augments the steepness of the electrochemical gradient against which protons have to be extruded.

The most likely interpretation of the hyperpolarization effect of FC is that it depends on the activation of proton extrusion by an energy-requiring electrogenic mechanism. The electrogenic (rather that diffusion-depending) nature of this mechanism is suggested by: *a*) the magnitude of the response to FC (often up to 40 mV); *b*) its earlyness (practically no lag phase, and maximum change reached in less than 8 minutes, under optimal conditions); *c*) the important finding that FC-induced hyperpolarization is relatively little affected by changes of external $[K^+]$ in the range between 10^{-4} to 10^{-2} M, while in the controls the potential difference steadily decreases with the increase of K^+ in the medium [4].

The mechanism of the stimulation of K^+ net uptake by FC is open to investigation. It might be interpreted either as due to an H^+—K^+ exchange mediated by a single mechanism, with a stoichiometry favouring H^+ extrusion in order to account electrogenesis, or as a consequence of the increase of intracellular negative potential. A difficulty which arises here is that the available data indicate a consistent excess of net K^+ uptake on net H^+ extrusion. Thus, some of the K^+ taken up should be accounted by the parallel net increase of negative charges in the cells.

More extended data on the stoichiometry of the effects of FC on fluxes of H^+, K^+ and other ions are obviously necessary to attempt a reasonable hypothesis about the nature of this mechanism.

At the light of the present knowledge, the finding that FC promotes together these 3 apparently interlinked responses: H^+ extrusion, K^+ uptake and cell hyperpolarization is relevant from two different points of view. First, it opens a new approach to the investigation of various aspects of the mechanism of these important membrane functions. Secondly, it emphasizes the complexity of the physiological implications of the stimulation of the proton extruding mechanism; in fact, while acidification of the cell wall space is probably important in controlling plastic extensibility of the wall, also the two other associated responses, namely the increase of the capacity for K^+ uptake and the increase of intracellular potential, must lead to relevant changes of the cell membrane activity in translocating solutes, of the ionic state of the cell wall compartment, etcetera.

III. Relationship between the mechanism of action of FC and the one of plant hormones

The evidence presented above points to the conclusion that the physiological effects of FC on cell enlargement, seed germination, opening of stomata and ion translocation are mediated by the activation of a single mechanism, involved in proton extrusion, K^+ uptake and electrogenesis, and depending for its activity on metabolic energy. The magnitude of the responses induced by FC indicates that all of the above mentioned physiological processes are strongly limited by the rate of operation of this mechanism. On the other hand, the fact that this operation rate is much lower in the absence than in the presence of FC suggests that it is normally maintained on a relatively low level by some physiological regulating factor(s). The question thus arises whether and to what extent the effects of plant hormones on the FC-sensitive processes depend on their capacity to control the mechanism mediating FC action.

The available data concerning this possibility can be resumed as follows:

Auxins. Strong evidence indicates that the mechanism of natural and synthetic auxins in stimulating cell enlargement is, in its final steps, the same as that of FC [20]. In fact, the effects of auxin on proton extrusion [18],

increase of K^+ uptake and hyperpolarization [34, 35] are qualitatively similar to those induced by FC. The magnitude of the 3 responses is markedly greater with FC, but this is also true for the effect on cell enlargement. Moreover, auxin and FC influence in the same way the plastic extensibility of the cell wall [52] and some important metabolic parameters, such as respiratory O_2 uptake [29], pyruvate level [17] and the activity of the oxidative part of the pentose phosphate cycle [28].

On the other hand, FC is active on several tissues insensitive to auxins; FC at high concentrations does not induce ethylene synthesis, while auxin does; no competition is observed, in cell free preparation, between labelled FC and auxin (Rayle, Dormann, personal communication). These and also other indications (cfr. Masuda [32]) point to the conclusion that the two substances primarily act on different receptor sites, and that the first part of the chain of reactions mediating auxin action is different, and probably more complex, than in the case of FC.

Cytokinins. These hormones promote cell enlargement [16], proton extrusion [33], K^+ uptake [35] and hyperpolarization in isolated cotyledons [32], a material practically insensitive to other hormones. Here again, all of the effects of the cytokinins are much lower that those of FC. Moreover, cytokinins stimulate in this material anthocyanin synthesis and the development of phenylalanine–ammonia lyase, while FC does not [46, 48].

On the other hand, FC is able to induce cell enlargement and proton extrusion in a number of tissues which do not give this response to cytokinin, in spite of the fact that the activity of the latter is clearly detectable, as in the case of leaf fragments, where FC stimulates cell enlargement and does not influence chlorophyll and protein breakdown, while the reverse is true for cytokinins [5, 19].

These results suggest that in the isolated cotyledons the effect of cytokinins on cell enlargement is probably mediated, in its final steps, by the same mechanism involved in FC action. However, the same data also indicate that the primary receptors for FC and for cytokinins must be different, and that a large part of the multifarious physiological activity of cytokinins is mediated by mechanisms different from the one influenced by FC.

Gibberellic acid. Gibberellic acid (as also, in some cases, cytokinin) is often able to promote germination of dormant seeds or to reverse germination inibition induced by far red light. In the materials so far investigated, FC always appeared to act on seed germination in the same sense, but more efficiently than gibberellic acid. Recent work carried out in this laboratory by Galli and Sparvoli and by Bellini [9, 48] shows that early DNA and enzyme synthesis in the germinating seeds are much less influenced by FC than by gibberellic acid. This indicates that here again, as in the case of cytokinins, only part of the physiological effects of the hormone (probably those directly connected with K^+ uptake and proton extrusion) can be mediated by the mechanism mediating the activity of FC.

Abscisic acid. Abscisic acid is known to induce closing of stomata [36], and to inhibit seed germination and, at least in some materials, cell enlargement [44] and K^+ uptake [38, 43]. The effects of ABA on these processes is thus opposite to that of FC. In some cases, as those of stomata behaviour and seed germination, the effect of ABA can be completely reversed by treatment with FC [21, 47].

This suggests that at least some of the important physiological properties of this hormone depend on its capacity to inhibit the mechanism activated by FC. Further work on the interaction between ABA and FC, and more detailed data on their effects on the various processes influenced by the two compounds are required to elucidate this interesting possibility.

Conclusions

On the basis of the results discussed above we propose the following group of working hypotheses:

I) All known physiological effects of FC (such as stimulation of growth by cell enlargement, transpiration due to stomata opening, breaking of dormancy and mineral nutrition) can be interpreted as consequences of the capacity of the drug to activate proton extrusion and K^+ uptake.

II) The effects of FC on proton extrusion, K^+ uptake and transmembrane electric potential are strictly interlinked and depend on the activation of a single mechanism, presumably located at cell membrane level.

III) This mechanism is present in almost all plant organs, where, under physiological conditions, its activity is regulated on a suboptimal level by internal regulating factors. The activation of this mechanism by FC depends on the capacity of the drug to remove some kind of negative physiological control.

IV) A consistent fraction of the action of plant hormones (and also environmental factors such as those involved in photo– and thermoperiodism) on FC–sensitive physiological processes such as extension growth, transpiration and ion translocation depends on their capacity to regulate the operation of the FC-sensitive system involved in proton extrusion associated to K^+ uptake and increase of the electric potential.

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