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CONTROL OF THE ACTIVITY OF THE ALEURONE LAYER OF FENUGREEK, TRIGONELLA FOENUM-GRAECUM L.

A. H. G. C. RIJVEN

Division of plant Industry, Commonwealth Scientific and Industrial Research Organization, Canberra, A.C.T., Australia

SUMMARY

A method is described whereby the dissolution of the endosperm of *Trigonella foenum-graecum* L. can be studied in isolation from the embryo, with the only living tissue present in the endosperm fragments being the aleurone layer.

The process of dissolution, when followed for 48 hours, was inhibited by abscisic acid, dinitrophenol, cycloheximide, azetidine-carboxylic acid, 6-methylpurine and actinomycin D, but not by 5-fluorouracil.

The process of dissolution was stimulated by ethylene and carbon dioxide but not by gibberellic acid, 2,4-dichlorophenoxyacetic acid or kinetin.

It is suggested that the dissolution of the endosperm is mediated by synthesis of protein and of RNA, and that these syntheses may be controlled by a balance of natural inhibitors such as abscisic acid, and natural stimulators such as ethylene or carbon dioxide.

1. INTRODUCTION

In many leguminous seeds some endosperm is present. In fenugreek (*Trigonella foenum-graecum* L.) I have estimated it to contribute about 30% to the total dry weight of the seed. During germination this endosperm is consumed. It consists of dead cells filled with galactomannans, surrounded by a monolayer of living cells: the aleurone layer. REID (1971) has shown that the dissolution of the endosperm starts from the aleurone layer, the zone of dissolution enlarging inward with time. He suggested that galactomannan-degrading enzymes (or activators for previously inactive enzymes) are secreted by the aleurone layer rather than by the embryo and noted the analogy to the situation in cereal grains. An interesting aspect of barley aleurone layers is the fact that enzyme release is a hormonal response (PALEG 1964) involving protein and nucleic acid synthesis (FILNER & VARNER 1967; VARNER *et al.* 1965). It seemed therefore worthwhile to investigate to what extent these features were repeated in the fenugreek aleurone system. One of the technical difficulties in attempting to study this question is that the structure of the fenugreek seed does not permit a close imitation of the half-seed approach used with barley. However, guided by our fenugreek cotyledon assay system for cytokinins (RIJVEN & PARKASH 1970) a preparation was arrived at that could be used as a fair approximation.

2. METHODS

Seed, selected for uniformity, was cut in the dry state by two sections made with a single-edged razor blade as indicated in *fig. 1*. Radicle containing fragments

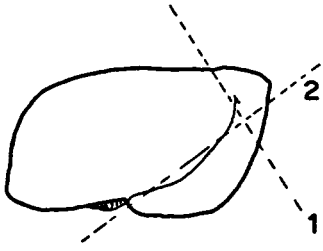

Fig. 1. Cutting of the dry seed in preparation of an experiment.

were discarded and the main cotyledon containing fragments were sterilized for 30 mins with a tenfold diluted, commercial sodium hypochlorite solution, washed and soaked for 6 hr in sterile distilled water. At this stage the cotyledons were removed by pressing with the flat side of a curved, sterilized spatula. The seed-coat-covered endosperm fragments were then distributed over the various treatments. Each treatment was at least in triplicate and each replicate contained 10 endosperm fragments in 4 ml of test solution in a 30 ml conical flask. The basal medium consisted of sterilized 1 mM, pH 5.5, phosphate to which was added 25 μg/ml chloramphenicol whilst other treatments received additions as indicated.

CO_2 treatment was given by incubating the flasks in a desiccator in which CO_2 was generated from sodium bicarbonate with hydrochloric acid. A dish with 5 ml of mercuric oxide dissolved in perchloric acid (YOUNG *et al.* 1951) served the purpose of trapping ethylene. Ethylene treatment was given by adding measured volumes of the gas to a desiccator containing the treatment flasks. In this case, a dish with 5 ml 4N NaOH served the purpose of trapping CO_2. In other than these gas treatments the flasks were capped with parafilm and together with the gas treatments incubated in the dark at 25°C. In connection with the question whether such capped flasks represent fair control gas treatments, it may be noted that in exp. 2 of *table 3* air-control flasks were placed inside replicate desiccators and were not capped with parafilm.

At harvest 10 ml ethanol was added to each flask. This caused the precipitation of a mucous material – presumably unhydrolysed galactomannan – which during incubation had become dispersed through the medium. After storage (at least overnight) the content of a flask was filtered through a membrane filter (oxoid membrane); the mostly fibrous residue and the seed coats together with membrane-filter were dried and weighed. After correction for filter and seed coats, endosperm dissolution was calculated as the percentage decrease in weight from zero time (5.6 mg each). In tables, least significant differences between means (LSD) at the 1% level of probability are given; they are valid for all treatment comparisons within a given experiment.

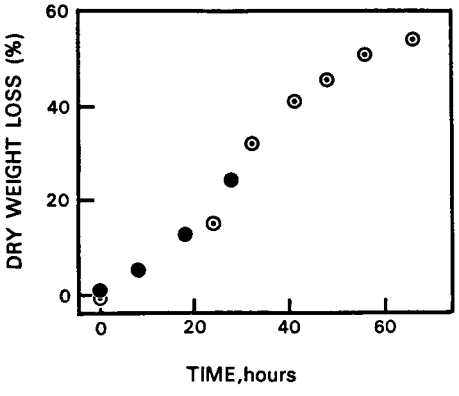

Fig. 2. Progression of dissolution of isolated endosperm fragments in basal medium. Results of two experiments are indicated by the different symbols.

3. RESULTS AND DISCUSSION

Results given in *Fig. 2* show that dissolution of the isolated endosperm fragments proceeded in the control, basal medium for 48 hrs.

Tetrazolium testing (GRABE 1970) suggested the single-cell aleurone layer to be the only living tissue in the endosperm fragments.

Treatments in the experiment of *table 1* included a number of inhibitors and amongst these abscisic acid, dinitrophenol, cycloheximide, azetidine-2-carboxylic acid and 6-methylpurine proved to be very effective inhibitors. Actinomycin D at 16 μg/ml was not effective but substantial and statistically significant inhibition ($P < 1\%$) was observed in another experiment in which actinomycin D was applied at 50 μg/ml. It is therefore suggested that both protein and nucleic acid synthesis are involved in the dissolution process. However, 5-fluorouracil, which inhibits kinetin-induced RNA increase in isolated fenugreek cotyledons (RIJVEN 1972, in press) was ineffective as an inhibitor of the dissolution process as it was also ineffective in inhibiting kinetin-induced expansion of fenugreek cotyledons. With reference to the specificity of fluorouracil action, i.e. inhibiting only rRNA and tRNA synthesis (KEY & INGLE 1968) the data seem to suggest a

Table 1. Effect of inhibitors on endosperm dissolution (48 hrs).

		Decrease in dry weight %
Control		38.7
Abscisic acid	5.10^{-6}M	1.3
Dinitrophenol	5.10^{-5}M	3.7
Cycloheximide	10 μg/ml	2.0
Azetidine-2-COOH	2.10^{-3}M	6.0
6-Methylpurine	1.10^{-4}M	0.7
Actinomycin D	16 μg/ml	33.0
5-Fluorouracil	5.10^{-4}M	40.0
LSD at $P < 1\%$		11.8

Table 2. Effect of abscisic acid on endosperm dissolution (48 hrs) in absence and presence of ethylene.

	Decrease in dry weight (%) Ethylene	
	Absent (Exp. 1)	Present (Exp. 2)
Control	45.3	50.7*
Abscisic acid 5.10^{-9}M	37.0	48.0
Abscisic acid 5.10^{-8}M	21.0	38.3
Abscisic acid 5.10^{-7}M	11.0	30.7
Abscisic acid 5.10^{-6}M	6.0	13.3
LSD at $P < 1\%$	6.5	13.2

* Control in absence of ethylene in this experiment was 36.7.

requirement for the synthesis of mRNA. It is noteworthy that a similar conclusion was reached by ZWAR & JACOBSEN (1972) concerning the gibberellic acid-induced production of amylase in the barley aleurone layers using the same inhibitors. The agreement with the barley aleurone layer system extends to the effectiveness of abscisic acid as an inhibitor (CHRISPEELS & VARNER 1966).

The effects of a concentration range of abscisic acid on isolated endosperm fragments of fenugreek are shown in *table 2*. In the presence of ethylene this inhibition appeared to be less severe. Ethylene appeared also to be stimulating in the absence of abscisic acid, an observation that was confirmed in every experiment in which it was applied (see also *table 4*).

Carbon dioxide was tested as it has been shown to be effective in removing dormancy in legume seeds (BALLARD 1958), also in those of fenugreek (RIJVEN & PARKASH 1970). The seeds under testing, however, were not dormant. Experiments using an ethylene trap did not always give significant carbon dioxide responses. *Table 3* gives results of two experiments in which highly significant stimulatory responses to carbon dioxide were obtained in the absence of an ethylene trap.

Table 3. Effect of carbon dioxide on endosperm dissolution (29 hr) in absence and presence of abscisic acid.

	Decrease in dry weight %	
	(Exp. 1)	(Exp. 2)
Control-air	29.8	31.8
Control + abscisic acid 10^{-8}M	18.2	26.6
Carbondioxide 2.5%	38.0	40.7
Carbondioxide + abscisic acid 10^{-8}M	29.2	35.8
LSD at $P < 1\%$.	8.3	5.7

Table 4. Effect of growth regulators on endosperm dissolution in four experiments.

		Decrease in dry weight (%)			
			Abscisic acid		
	M	Nil 24 hr	Nil 28 hr	Nil 28 hr	5.10^{-8}M 48 hr
Control		13.3	24.0	27.0	28.7
Gibberellic acid	1.10^{-8}	–	–	25.4	–
	1.10^{-7}	–	28.4	27.4	–
	5.10^{-7}	9.7	–	–	23.3
2,4-dichlorophenoxy-acetic acid	1.10^{-8}	–	–	26.6	–
	1.10^{-7}	–	30.0	21.6	–
	1.10^{-6}	7.0	–	–	25.0
Kinetin	1.10^{-7}	–	–	28.6	–
	1.10^{-6}	–	27.0	23.8	–
	5.10^{-5}	10.3	–	–	25.7
Ethylene, 3 ppm		26.3	33.6	–	51.3
LSD at $P \leqslant 1\%$		12.1	8.0	7.3	10.8

Table 4 summarizes results of four experiments attempting to establish the possible activity of the plant growth regulators, gibberellic acid, 2,4–dichlorophenoxyacetic acid, kinetin, and ethylene. Except for the latter no consistently significant effects were observed. In contrast with ethylene and carbon dioxide (*table 3*), gibberellic acid, 2,4-dichlorophenoxyacetic acid and kinetin (*table 4*, last column) were unable to counteract an intermediate inhibition by abscisic acid.

A possible stimulatory role of the embryo on the dissolution process by the production of ethylene and carbon dioxide may be considered. However, so far I have not been able to establish such activity by adding isolated germinating embryos to incubates of endosperm fragments.

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