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PHYTOCHROME IN SEEDS OF SOME CUCURBITACEAE: IN VIVO SPECTROPHOTOMETRY

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INTRODUCTION

The study of phytochrome by spectrophotometry *in vivo* in seeds has been undertaken only recently, but the results obtained are encouraging. Phototransformation of the pigment has been demonstrated in a number of seeds (1,2,3,4). It can even be observed in certain non-imbibed seeds (2,3). It proved possible to obtain difference spectra for phototransformation (3,4,6), and to follow the evolution of the pigment content during the imbibition period (3,4,6). A new type of phytochrome reaction has been described by BOISARD *et al* (1): the inverse dark reversion. Together, these findings stress the desirability of continued research in this field and of perfecting the technique involved. The results obtained by SPRUIT and MANCINELLI (3) with *Cucumis* have induced us to examine other seeds of the same family. The first results of these investigations are presented below.

Detection and properties of phytochrome in seeds of gherkin, *Cucumis sativus L., var.* 'Vert de Paris'

The apparatus and techniques used in the detection and measurement of phytochrome in seeds, imbibed or dry, are those described earlier (1,9).

Like in cucumber and melon, the pigment can be found in dry seeds. The values of $\triangle(\triangle O.D)$, between 735 and 807 nm, vary from 4.1 to $7.7.10^{-4}$, depending upon the sample. This is the same order of magnitude as formerly reported for cucumber (3) but considerably larger than the values found in melon (2). The differences between samples are not surprising since the optical density changes are proportional to the light path. In fact, since the seeds are big, the way they are arranged in the cuvette is never the same from one

* Technical assistance: D. Eude

preparation to the other and the effective optical path is likely to vary considerably. In the dry seeds, 66% of the pigment was found in the form P_{fr}. Following actinic irradiation with far red light, P_{fr} reappears during several hours darkness. This is another example of the inverse dark reversion, discovered in lettuce (1) and studied by SPRUIT and MANCINELLI in cucumber (3).

In the imbibed seeds, the values of $\triangle(\triangle O.D)$ are higher and increase with the duration of imbibition. On the other hand, the absolute quantity of P_{fr} does not change in time. Consequently, P_{fr} represents a decreasing fraction of P_{total} (fig. 1). Apparently, phytochrome in the form P_r is produced during the imbibition period preceeding appearance of the radicle. Accordingly, detection of P_{fr} becomes increasingly difficult as imbibition proceeds. We have observed a similar effect in *Nemophila insignis* (7).

Also in the gherkin variety 'Vert de Paris,' the difference spectrum of the phototransformation in imbibed seeds (24 h) is not the same as in dry seeds (fig. 2). There is a slight displacement of the peaks towards longer wavelengths and a shift of 10 nm (from 690 to 700 nm) of the isosbestic point. This confirms the observations made with *Cucumis sativus* 'Pixie' (3).

However, the spectra after 3, 8 and 16 hours of imbibition are not significantly different from those for 24 hours. Therefore, as early as 3 hours after moistening, the characteristics of the absorption spectrum already appear indistinghuishable from those described by different authors for phytochrome in etiolated plants or in imbibed seeds (4,6,9). The differences with respect to the spectrum of dry seeds could result from changes in the molecular structure of the protein part of the pigment, without the need to postulate two different pig-

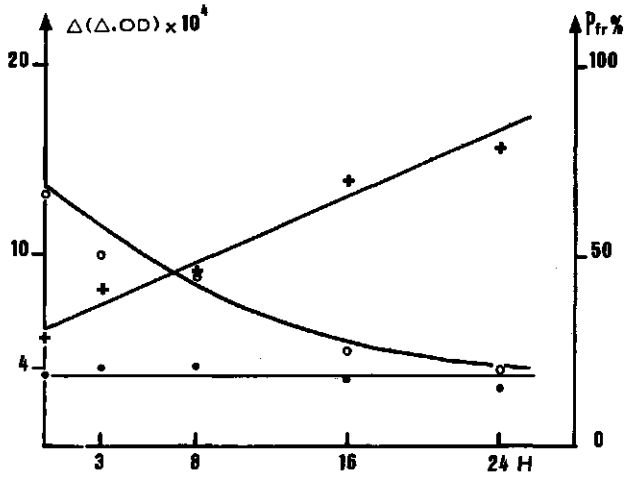


FIG. 1. Time course of phytochrome concentration during imbibition in seeds of *Cucumis sativus* var. 'Vert de Paris' in the dark at 22 °C.
Left hand scale: P_{total} (+) and P_{fr} (●)
Right hand scale: P_{fr} expressed as percent of P_{total} (○)
Sample thickness: 4 mm
$\triangle(\triangle.OD)$ measured between 735 and 807 nm.

ments. These changes could result from conformational changes in the protein during rehydration of the tissues. This hypothesis is not, however, necessarily in disagreement with the idea of the presence in seeds of two types of phytochrome (3): a hypothesis which has been formulated to explain certain results of physiological studies.

Phytochrome in a single isolated embryo of pumpkin, *Cucurbita maxima L . var.* 'Rouge vif d' Etampes'

The measurement of phototransformations of phytochrome *in vivo* in the seeds has, up to now, been done by analysing a certain number of seeds closely packed together in a sample cell.

As far as we know, it is for the first time that measurements have been made on a single seed. This proved possible with large seeds like those of pumpkin. In fact, the analysis was carried out on a single embryo after removal of the thick seed-coat, which hinders the measurement.

The usual sample cell was replaced by a support consisting of a small black plastic plate with an oval hole slightly smaller than the embryo (lenth 18 mm, width 10 mm). The latter was fixed upon this hole with modelling clay which also prevents any light leaking around the embryo. The results were very interesting, if compared with those obtained by the classical method using bulk samples (table 1). Detection in the non-imbibed individual embryos proved also possible and gave an average value of 1.6×10^{-4} $\triangle(\triangle$O.D.).

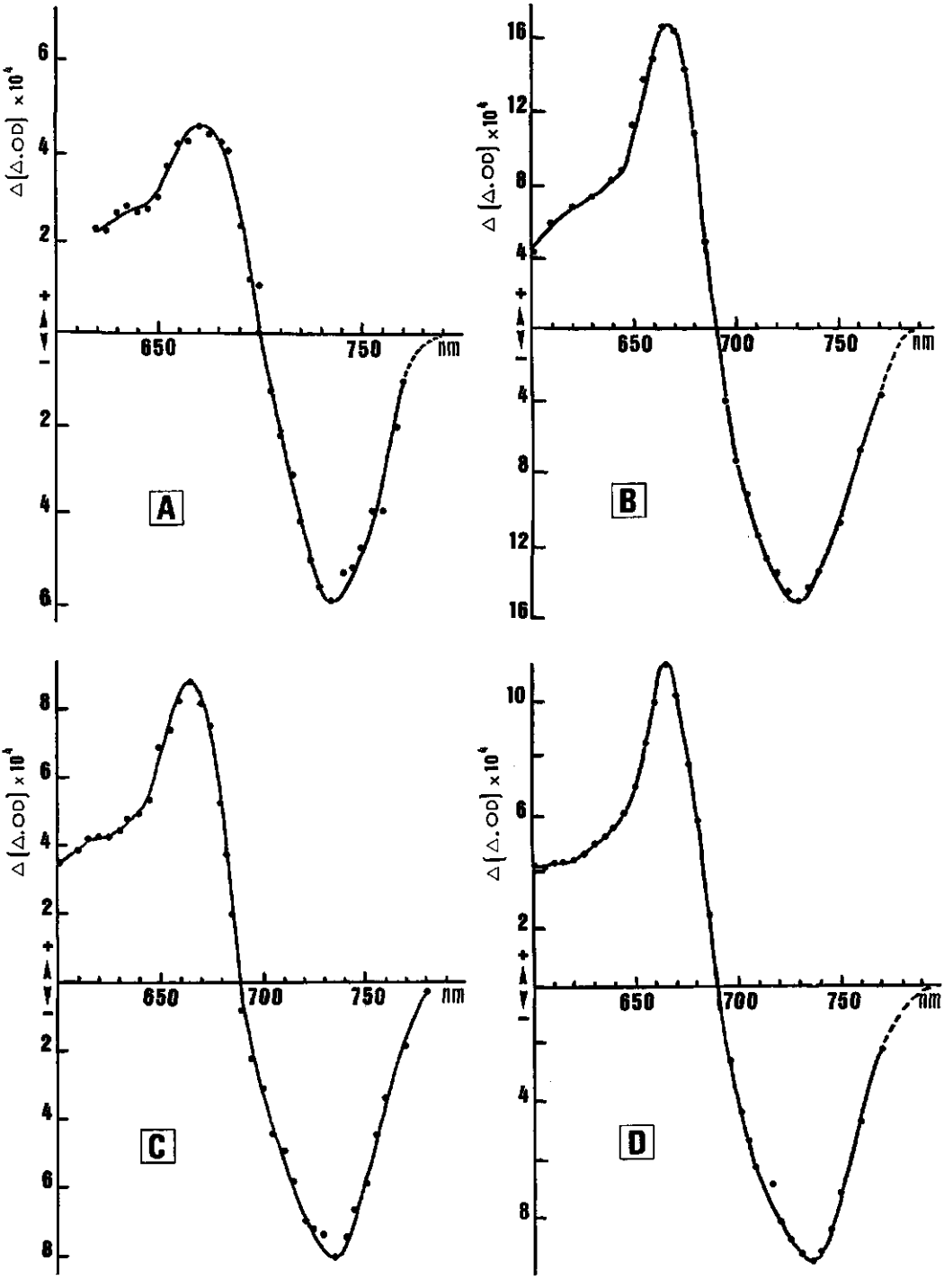
TABLE 1. Phytochrome concentrations in some seeds

Seeds	Time of soaking	$\triangle(\triangle$O.D.) $\times 10^4$	Authors
Phacelia tanacetifolia	2 to 24 h	doubtful	MALCOSTE (2)
Nigella damascena	6 to 48 h	,,	,,
Cucumis melo	0 h	1.2	,,
Nemophila insignis	6 to 30 h	2.7	,,
Sinapis alba	1 to 12 h	5 to 10	,,
Rhaphanus sativus	8 h	10	,,
Lactuca sativa	3 to 16 h	2.5 to 5	BOISARD (1)
Cucumis sativus	6 h	12	SPRUIT (3)
Amaranthus caudatus	4 to 8 h	8 per 200 seeds	KENDRICK (4)
Cucurbita maxima (one embryo)	2 to 15 h	6.4 to 7.6	this study

This technique also allowed us to measure a difference spectrum for the photo-tranformation of phytochrome in a single embryo (fig. 3). It is not significantly different from those of phytochrome in seedlings or imbibed seeds as reported in the literature (4,6,9).

Evolution of the phytochrome pool in a pumpkin embryo during imbibition

A special adapter for the spectrophotometer allowed us to follow the evo-


Δ[Δ.OD] x 10⁴
650
700
750
nm
A
B
C
D

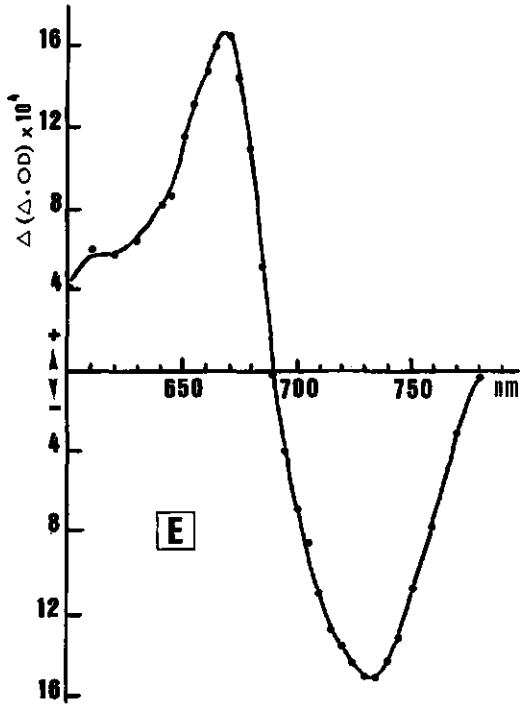


FIG. 2. Difference spectra for phototransformation of phytochrome in gherkin seeds:
A: dry seeds.
B: after 24h imbibition.
C: after 3d imbibition.
D: after 8h imbibition.
E: after 16h imbibition.
Sample thickness: 4 mm.
Temperature of measurement: 0 °C.
Temperature of imbibition: 22 °C.

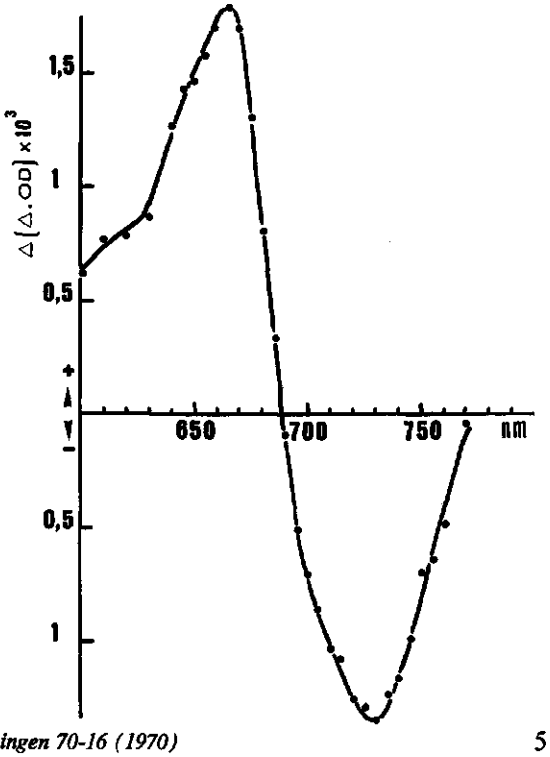


Fig. 3. Difference spectrum for phototransformation of phytochrome in a single pumpkin embryo.
Imbibition 24 h at 22 °C.
Temperature of measurement 22 °C.

lution of the pigment content in an isolated embryo. The embryo was fixed with modelling clay onto a metal plate with an oval hole. This plate was placed in a cell of appropriate size interposed in the measuring beam. Three thin copper tubes were soldered to the plate. One is for taking water in and out of the cell, the other two are for bubbling through air (fig. 4). In this way, the embryo can be continuously supplied with water and oxygen without removing it from the spectrophotometer. The whole arrangement was placed in a transparent enclosure standing in the place usually occupied by the cellholder of the spectrophotometer. This enclosure serves to protect the sample from temperature changes due to the actinic irradiation. During a measurement, the current of air through the cell was stopped temporarily. Under these conditions, the recordings are of excellent quality. The signal to noise ratio is much better than if the imbibed embryo was measured outside this device, in the dry air (fig. 5). In addition, this method allowed us to follow the evolution of phytochrome during imbibition of a single embryo. This gave more consistent values, since it was no longer necessary to make measurements on separate samples, one

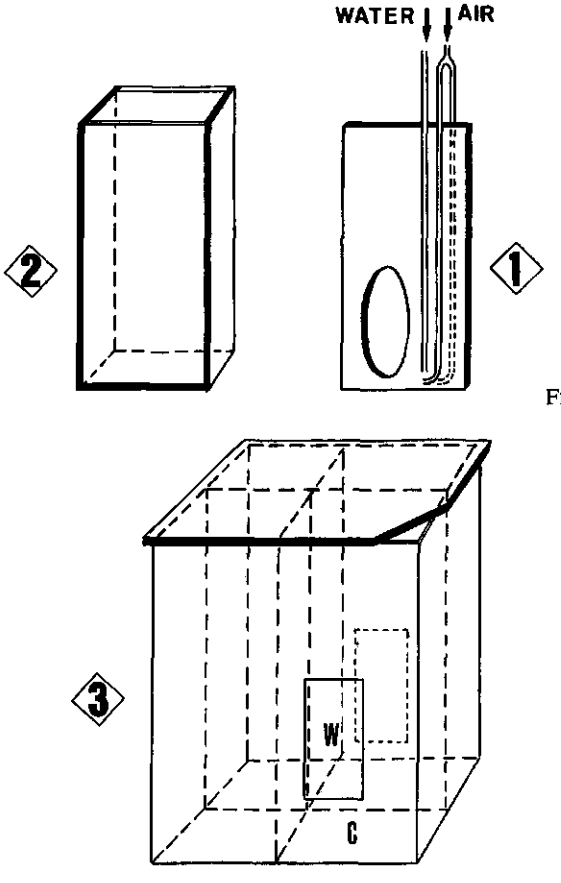


FIG. 4. Diagram of the special sample holder for measurements of $\Delta(\Delta.OD)$ in one isolated embryo during continuous imbibition.
(1) holder for the embryo (brass, painted black)
(2) special cell containing the holder (made of black and transparent plexiglass)
(3) adjustable enclosure (black plexiglass with transparent windows).
The sample cell is put in part C, tubes for air and water passing through an opening in the corner of the lid.
The light passes through the windows W.

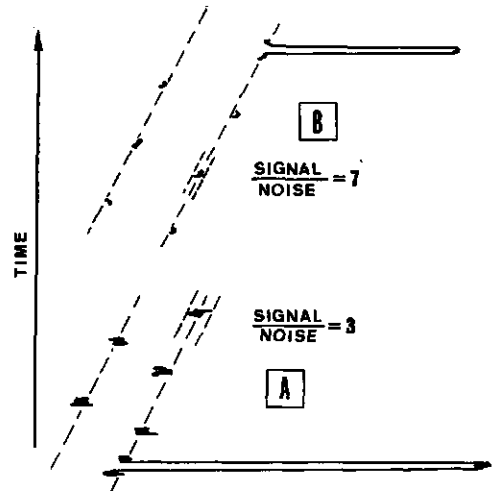


FIG. 5. Copies of original recordings showing improved signal to noise ratio, due to immersion of the sample in water.
A: measurement in air.
B: measurement in water.

for each period of imbibition. Such samples cannot easily be compared since it is difficult to make all the samples even approximately identical. Also, part of the scattered light is eliminated, thanks to the immersion of the seed in water. The fact that the embryo germinates in the apparatus shows that it is undamaged. This method, therefore, represents a real case of spectrophotometry '*in vivo*'.

The curve obtained in this way for evolution of the pigment content in the course of time (fig. 6), shows the different phases described by BOISARD in lettuce (fig. 7), viz.:

1. a rehydration phase stretching over a period of about three hours after sowing. It should be pointed out that during this period, it is not possible to measure the $\triangle(\triangle O.D)$ as the recordings show too much drift. This is probably due to initial rapid changes in the optical properties of the tissue, caused by the swelling of the embryo. When this period is over, we find a value of $\triangle(\triangle O.D)$ higher than that in a non-hydrated embryo. This may point to synthesis of pigment but it is more likely that rehydration of an inactive pigment form is responsible for the increase in $\triangle(\triangle O.D)$.
2. a phase when the phytochrome content is almost constant, lasting for 16–17 hours. The length of the corresponding phase in lettuce was 12 hours and 20–22 hours in *Nemophila*. On the other hand, KENDRICK *et al.* (4) found the duration of this phase in *Amaranthus* to last only about 6 hours, whereas SPRUIT and MANCINELLI observed no plateau in *C. sativus* 'Pixie', the phytochrome content rising slowly but steadily during the first 30 hours following imbibition.
3. a phase when $\triangle(\triangle O.D)$ increases, beginning 20 hours after sowing (15–16 hours in lettuce and 24 hours in *Nemophila*). This increase precedes the sprouting of the radicle which takes place around the 38th hour in pumpkin, the 24th hour in lettuce and the 48th hour in *Nemophila*.

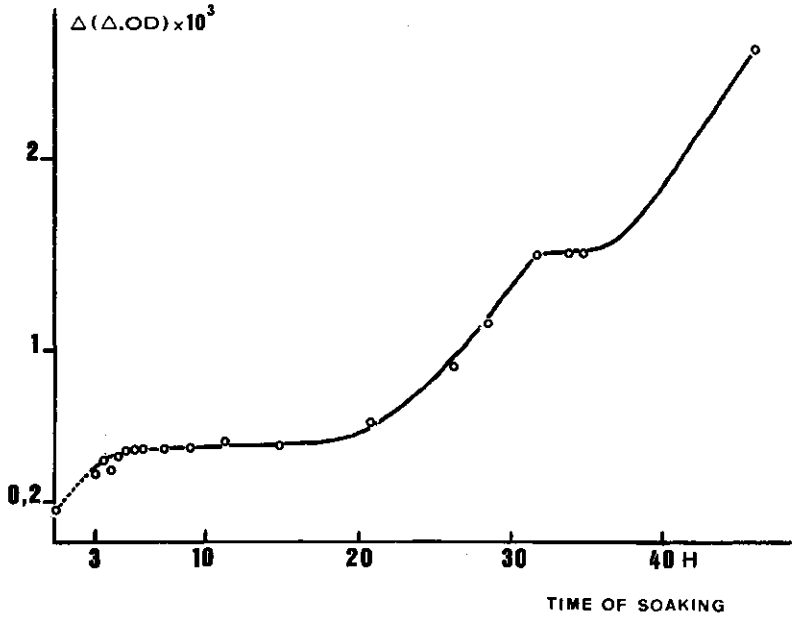


FIG. 6. Time course of phytochrome concentration upon imbibition of a single excised embryo of pumpkin. Measurements were made during continuous hydration in the apparatus described in fig. 4. Temperature 22°C.

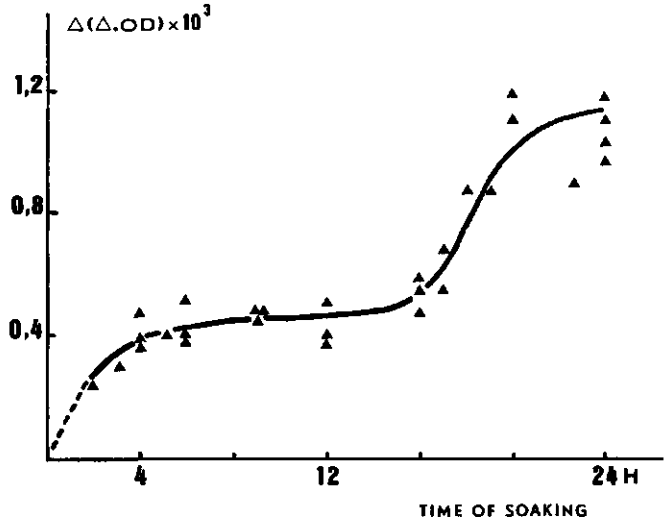


FIG. 7. Time course of phytochrome concentration during imbibition of lettuce seeds (after BOISARD, 6). Measurements were made with different samples of seeds packed in cuvettes. Note the large scatter of the measurements, as compared with fig. 6.

4. a new phase when the pigment content increases and which can be linked with the growing of the vegetative axis. A measurement made with a seed, imbibed during 48 hours, but which happened not to germinate, gave a value identical to that at the end of the 3rd phase in germinating seeds.

Although these different phases are not as clear-cut in cucumber (fig. 1), and *Amaranthus caudatus* (4), it is becoming increasingly clear that in many seeds a considerable increase in phytochrome may take place before appearance of the radicle.

Phytochrome concentrations in different areas of the gourd embryo, *Cucurbita pepo L. var.* 'Black Beauty'

The isolated embryo of the gourd seed is fairly oval, the length being 12-14 mm and the width 8–9 mm. As with pumpkin, it proved possible to measure the $\triangle(\triangle O.D)$ in a single embryo fixed with putty onto an appropriate support. Since this type of embryo is very large, we considered measuring the changes in optical density in a part of the embryo only. Fig. 8 shows that this is indeed possible. The major part of the embryo was hidden by a mask. Measurements were only taken on the radicle end, through a hole, 3 mm in diameter. This result induced us to use various forms of masks in order to explore limited parts of the embryo.

Use of masks

The embryo was fixed onto a plate with 20 holes, 1.2 mm in diameter, 1 mm apart and distributed according to the shape of the embryo. To limit the areas chosen, a number of holes were covered. The measurements point to a slightly higher concentration in the region of the vegetative axis (fig. 9).

Another type of mask with 3 holes, 2 mm in diameter and 3 mm apart allowed us to select more limited areas by covering up two of the holes (fig. 10). The values measured along the large axis do not show any notable differences. They cannot be compared directly, however, as the thickness of the sample is not the same in the three areas, the radicle area being 1 mm thick and the central area 3 mm. It is likely, therefore, that the phytochrome concentration is highest in the region of the vegetative axis. We have attempted to verify this

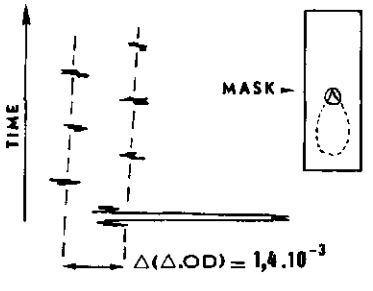


FIG. 8. Copy of an original recording, using a mask of black plastic (left). This recording shows that phytochrome detection is possible in a localised part of the embryo.

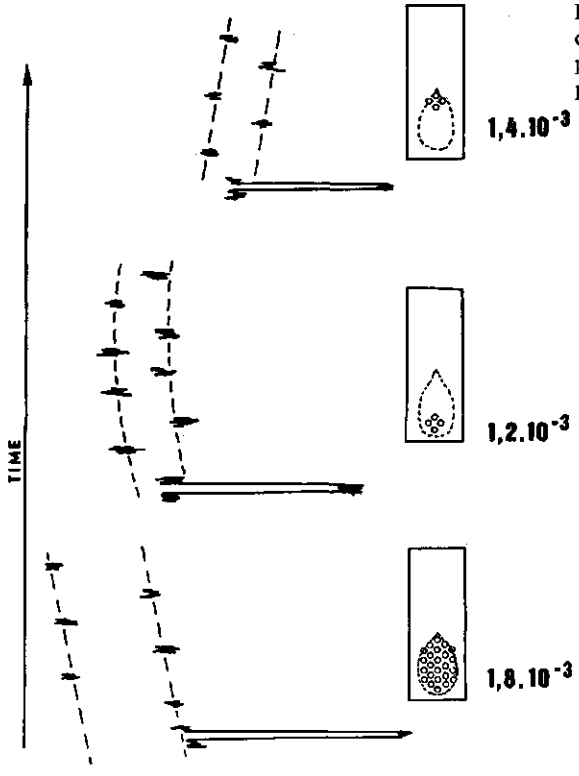


FIG. 9. $\Delta(\Delta O.D)$ values in different parts of the embryo of pumpkin seed using masks. Copies of original recordings (left).

hypothesis by resorting again to the method of measuring dissected parts of the embryo in a sample cell of fixed depth.

ANALYSIS OF FRAGMENTS OF EMBRYOS

About 50 seeds were removed from their coats, 6 hours after sowing. A central strip, 3 mm wide, was cut out parallel to the large axis. This strip was then divided into 4 parts. The first one contains the vegetative axis, the three others correspond to three different areas of the cotyledons. Four cells, each of 4 mm depth were prepared. The analysis of these samples shows the existence of a concentration gradient decreasing from the region of the vegetative axis towards the cotyledons (fig. 11), confirming the preliminary conclusion, drawn from the experiments with masks.

THE PROBLEM OF 'INVERSE DARK REVERSION'

While studying phytochrome by spectrophotometry in lettuce seeds var. 'Reine de Mai', BOISARD *et al.* (1) observed a spontaneous transformation of P_r to P_{fr} in darkness. This reaction was called 'inverse dark reversion' since

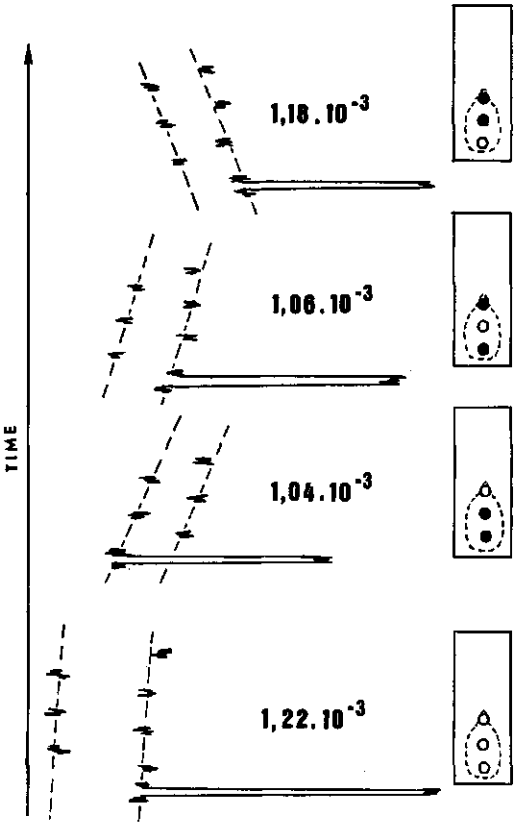


FIG. 10. △(△.OD) values in different parts of the embryo of gourd.

it appears to be the opposite of the well-known dark reversion of P_{fr} to P_r.

This reversion may explain the need for long FR irradiations to prevent germination in seeds that germinate spontaneously in darkness. Unfortunately, it is not always observed, and the conditions for its occurence are not yet completely clear. In *Nemophila insignis* seeds, another species germinating in darkness, no definite proof of its existence could be obtained, mainly as a consequence of the low pigment content (7). In *Sinapis alba*, it appears to occur only irregularly (1). On the other hand, its existence in cucumber is beyound doubt and a kinetic study has been made (3). Also in *Amaranthus* (4), the experiments seem to point to the presence of a fraction of the pigment, possessing this property.

We have looked for this reaction also in some large seeds with a relatively high pigment content, like gourd, pumpkin and colocynth (*Citrullus colocynthis L.*). It should be noted that the physiology of germination of these seeds is not known. However, it appeared desirable to study the pigment reactions, awaiting a closer examination of the physiology of their germination. This might aid to avoid the unpleasant experience of inconclusive spectrophotometric measurements with a seed the physiology of which is already known, such as *Nemophila*

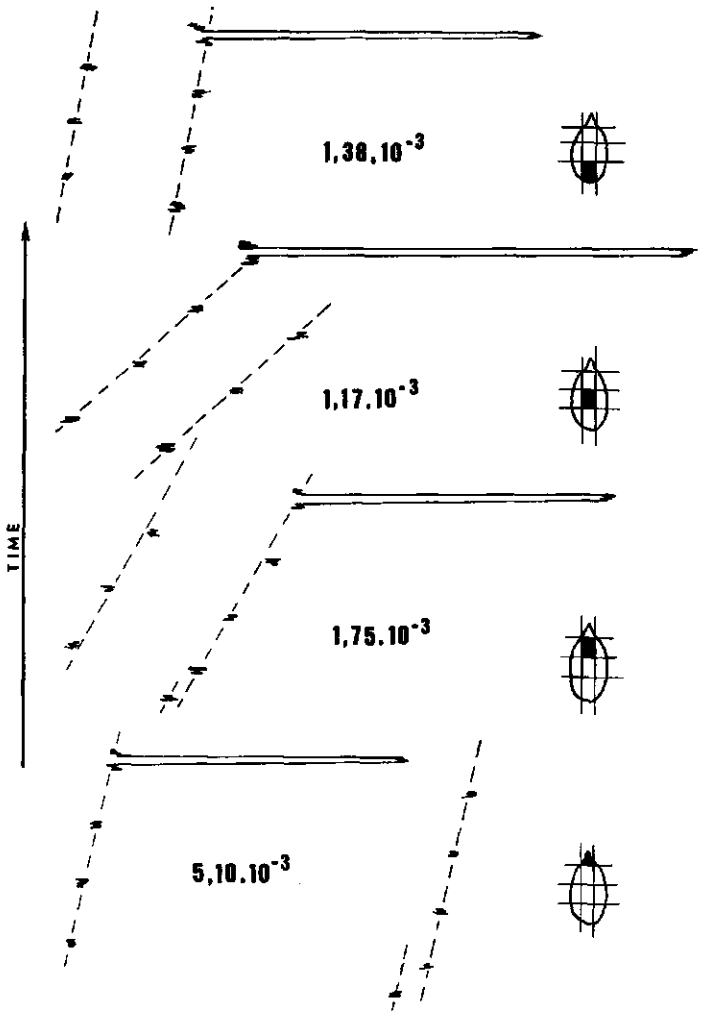


FIG. 11. Copy of original recordings obtained by dissecting different parts of the embryo of gourd and packing them in sample cells.
The diagrams of embryos show the part which is analysed.
Thickness of the sample: 4 mm.
Preparation and measurement at 0 °C.

(8). Since detection and measurement are fairly easy in seeds of Cucurbitaceae, the study of the physiology of their germination was immediately undertaken and is being actively pursued by us. Only some results of the spectrophotometric study will be reported here.

The phenomenon of inverse reversion appears to be accompanied by the presence in the seeds, prior to the first actinic irradiation, of a certain quantity of P_{fr}. The presence of this form of the pigment follows from the change in

$\triangle$O.D. upon irradiation with far red light. The way in which the relative amount of P_{fr} as well as the occurrence of inverse reversion are determined, are illustrated in fig. 12.

The three types of seeds, mentioned above were examined in this way, yielding the following results.

a. Gherkin. The presence of P_{fr} in the seeds before any treatment with light is evident as shown in table 2. As we have already pointed out, the quantity of P_{fr} does not increase during imbibition. Table 3 shows that the inverse dark reversion occurs (even in non imbibed tissues), provided the dark period following FR irradiation is sufficiently long.

b. Gourd. Photo-active phytochrome is also present in the seeds, all of it in the P_{fr} form. During the later stages of imbibition, this form does not represent more than a small percentage of the total pool, the increase of which is due to the synthesis of P_r (table 4). Inverse reversion can be demonstrated regularly in the imbibed seeds as shown in table 5.

c. Colocynth. The analysis of seeds and embryos shows the presence of a photo-active form of phytochrome, both in the dry tissues as well as after a period of soaking, table 6. It should be pointed out that, here as well, the quantity of P_{fr} represents a high percentage of the total pool of pigment in the dry

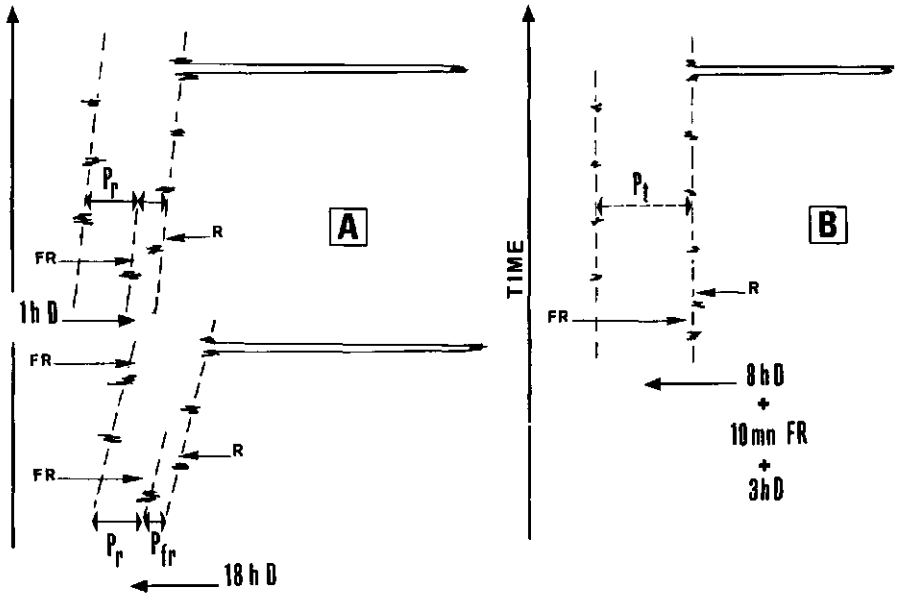


FIG. 12. Copies of original recordings.
A: gourd (whole seeds). P_{fr} in imbibed seeds prior to irradiation, and inverse dark reversion.
B: colocynth embryos. No inverse dark reversion.
P_{fr} = active form of phytochrome
P_r = inactive form
$P_t = P_{fr} + P_r$

TABLE 2. Percentage of P_{fr} present in seeds of gherkin, prior to any irradiation

Soaking (in darkness)	P_t $\triangle(\triangle.OD) \times 10^4$	P_{fr} $\triangle\triangle O.D \times 10^4$	P_{fr} / P_t %
0 h	5.8	3.5	60
3 h	8.2	3.9	47
8 h	6.3	3.7	59
16 h	15.4	3.1	23
24 h	13.8	3.2	23

TABLE 3. The inverse dark reversion in seeds of gherkin

Soaking (in darkness) before treatment	Treatment	Darkness after treatment	P_{fr} / P_t %
0 h	FR	8 h	33
3 h	FR	15 min.	0
5 h	FR	10 min.	0
12 h	FR	6 h	24
24 h	FR	10 min.	0
24 h	FR	12 h	10

TABLE 4. Percentage of P_{fr} present in seeds of gourd.

Soaking (in darkness)	P_t $\triangle(\triangle.OD) \times 10^4$	P_{fr} $\triangle(\triangle.OD) \times 10^4$	P_{fr} / P_t %
0* h	4.8	4.8	100
6* h	9.1	3.0	30
18** h	11.1	3.3	30

* without seed coats (embroys)
** whole seeds

TABLE 5. The inverse dark reversion in gourd embryos

Soaking (in darkness) before treatment	Treatment	Darkness after treatment	P_{fr} / P_t %
3 h	FR	8 h	20
12 h	FR	24 h	26
18 h	FR	1 h	36

TABLE 6. Percentage of P_{fr} in seeds of colocynth

Soaking (in darkness)	P_t $\triangle(\triangle.OD) \times 10^4$	P_{fr} $\triangle(\triangle.OD) \times 10^4$	P_{fr} / P_t %
0* h	2.9	2.2	75
4** h	6.0	3.4	57
4** h	10.4	5.2	50
6* h	11.8	3.8	33
12** h	9.5	3.2	33

* embryos
** whole seeds

seeds. As far as the inverse reversion is concerned, table 7 shows that we have not found this reaction in isolated embryos, even after a period of several hours in darkness. It occurs, however, in whole seeds after only 15 min. of darkness. The significance of this phenomenon is not clear and will have to be investigated further.

d. Pumpkin. The occurrence of inverse dark reversion has been followed throughout the imbibition period with the same embryo, in the apparatus described above. Results are given in Table 8. This shows that the inverse reversion can be observed around the 7th hour and disappears again around the 26th hour. It is to be noted that the first five measurements were carried out after only 20 min. of darkness. If the period of darkness were increased, we

TABLE 7. The inverse dark reversion in seeds of colocynth

Soaking (in darkness) before treatment	Treatment	Darkness after treatment	P_{fr} / P_t %
3* h	FR	8 h	0
3* h	FR	8 h	0
4* h	FR	5 h	0
10* h	FR	9 h	0
3** h	FR	8 h	55
11** h	FR	15 min.	60
11** h	FR	45 min.	33

* embryos
** whole seeds

TABLE 8. The inverse dark reversion in the embryo of pumpkin

Soaking (in dark) (hours)	Darkness after FR (hours)	P_t $\Delta(\Delta.OD) \times 10^4$	Inverse dark reversion
3	0.20	4.4	−
3.30	0.20	3.7	−
4	0.20	4.5	−
4.30	0.20	4.8	−
5.30	0.20	4.8	−
7	1.20	4.9	+
8.50	1.40	4.9	++
11	0.50	5.3	+
14.30	3.20	5.1	+
20.30	5.50	6.3	+
26	5.20	9.3	++
28.10	2.00	11.5	−
31.10	3.00	15.1	−
33.30	1.40	15.2	−
35	1.00	15.2	−
46	11.00	26.0	−

− not detectable
\+ 1.2 to 2.4 $\times 10^{-4}$
++ 2.4 $\times 10^{-4}$

could expect reversion also here. After 26 hours imbibition and a sufficient dark period following the far red irradiation, we could not demonstrate the phenomenon. If we suppose that only the P_{fr} present at the start of the imbibition shows this reaction, the increase of the total pool, by 'diluting' the initial P_{fr}, should be accompanied by a gradual disappearance of the inverse reversion reaction.

Whatever the case, the existence of this particular phytochrome reaction appears more and more as a general phenomenon, at least in those seeds germinating spontaneously in darkness. The first results of our physiological study have shown this to be the case in these seeds. It will have to be kept in mind in explaining the physiology of this type of germination.

Summary

Phytochrome phototransformations were studied in seeds of four species belonging to the Cucurbitaceae (gherkin, gourd, pumpkin and colocynth). For the first time, measurements of optical density changes were made in a single seed, imbibed in the cell compartment of the spectrophotometer. This method allows making spectrophotometry actually *in vivo* since no signs of damage to the seed were observed. Difference spectra and curves for the evolution of pigment concentration during the period of imbibition could be made in this way in a single seed.

A pigment concentration gradient, decreasing from the radicle towards the opposite end was demonstrated by two different methods in the isolated gourd embryo.

A study of the so called 'inverse dark reversion' has been undertaken in these large seeds, confirming the existence of this reaction also here. This reaction appears to be a property of a form of the pigment, occuring in the dry state, and surviving a relatively restricted period following the initial moistening of the seeds. It appears most likely at the time that this gradual disappearance of the inverse reversion reaction is due to dilution of the 'seed-phytochrome' by newly formed 'classical' (= 'seedling') phytochrome. However, still other factors, as yet not understood, may be involved as well.

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