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OBSERVED IN-VITRO GERMINATION OF OROBANCHACEAE IN THE PRESENCE OF NATURAL COMPOUNDS

I. THE PRESENCE OF STIMULATORY AGENTS IN THE CULTURE LIQUID OF *Centaurea scabiosa* L.

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SUMMARY

A description is given of a non-sterile apparatus for cultivation with automatic irrigation and its possible application for studying root secretions. Two factors, which appeared successively in the culture solution of *Centaurea scabiosa* L., broke the dormancy of seeds of *Orobanche minor* Sutt. One of these factors was equally active on seeds of *Orobanche picridis* Vauch.

INTRODUCTION

We are continuing our work on the germination of the *Orobanchaceae* by studying the activity of the culture liquid of *Centaurea scabiosa* L., *Eryngium campestre* L., *Hedera helix* L., *Picris hieracioides* L., *Trigonella faenum-graecum* L. on the germination of seeds of different species of the *Orobanchaceae* and *Phelipeae*. We observed that the activity was a function of the concentration of the culture liquids and of the time of exposure of the seeds of the parasites to these liquids. This finding enabled us to examine, with precision, the role of these two factors.

We describe in this article the methods used for the cultivation and biological tests. We investigated also the activity of compounds

present in the culture liquids in which Centaurea scabiosa L. had been grown on the germination of seeds of Orobanche minor Sutt. and Orobanche picridis Vauch.

CULTURES

1. Nutrient Solutions

The medium of Tompkins was used at half-strength and supplemented by solution 5 A of Arnon for providing micro elements. Iron was added as ferric chloride 0.026 g/liter. The pH was adjusted to 7.2 with KOH in the first 10 liters used. Later on, pH 7.2-7.5 was maintained without any adjustment, even when fresh solution was added to the apparatus.

2. Apparatus

Crystallizing dishes (cristallisoirs) (A), 12.5 cm in diameter and 6.5 cm deep, served as culture vessels. Glass tubing of variable diameter (4-8 mm) was placed randomly to a depth of 5 cm. Glass beads of different diameters (2-8 mm) were placed into the vessels and formed a layer 1 cm deep. A strip of black paper was fastened on the outside, covered the side wall and prevented the growth of algae. The surface was covered with a circular sheet of black polyethylene (B), provided with parallel slits, about 15 mm apart. A glass tube (C) of 10 mm diameter formed a shaft, free from beads, into which a second tube (D) of smaller diameter was placed, which was connected to the nutrient system.

Six crystallizing dishes were joined together by polyethylene tubes (E) and connected with a main flask (F) which served as a distributor through a syphon. (Fig. 1)

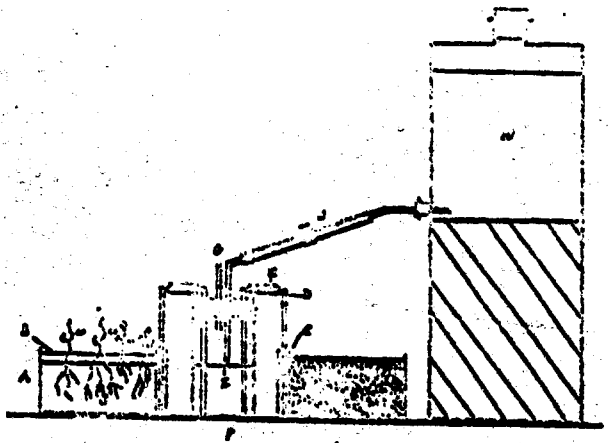

Fig. 1. — Schéma du dispositif de culture.

A tube (G), opening to the air, permitted the equilibration of pressures and levels in the main flask and crystallizing dishes.

The distributor was supplied from an elevated reservoir (H) of 6 liter capacity, connected by a tube with an orifice that was cut at an angle (I) and a rubber connection (J).

The upper opening of the reservoir was carefully stoppered, so that air could enter only through the conduit (I).

When the level of the liquid fell to (F), the orifice of (I) became exposed, air entered and reached the reservoir where it calibrated the pressure and produced bubbles, which agitated the culture liquid and dispersed the precipitate which appeared on account of the elevated pH. Since the liquid descended by gravity, a vacuum was created in the reservoir, and the outflow stopped. When the level of the liquid in F rose, no more air was able to enter conduit (I) and the system was again, momentarily, in equilibrium.

The orifice of tube (I) was placed 4 cm above the bottom of the distributor.

The set-up was primed by gravity; the upper stopper of the reservoir was lifted; tube (G) was closed.

When the nutrient solution was renewed, it became necessary to refill the reservoir to not more than 4/5th of its capacity in order to have the system function properly.

Discontinuous feeding permitted partial aeration of the roots. A very important aeration was done once a week: the draining of the solution was interrupted by pinching tube (J) with a pinch-cock.

3. Planting Seedlings

Seeds were germinated in Petri dishes on sheets of filter paper, lying on top of hydrophilic cotton, saturated with water. The seedlings that were obtained were transplanted in the cotyledonous stage, using 30 plants per crystallizing dish for Centaurea scabiosa L., Picris hieracioides L., Trigonella faenum-graecum L. and 24 plants for Eryngium campestre L. and Hedera helix L. The nutrient solution was raised to the level of the circular polyethylene cover.

After several days, the normal level of the solution was reached and the irrigation was started.

The cultures were placed near a glass window and exposed to "daylight" type fluorescent strips, 40 watts, at a distance of 10 cm, operated for 16 hours in 24.

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4. Comments

a) These cultures were not sterile. The compounds that were isolated were those excreted by the roots, those produced by metabolism and those obtained from the destruction of microorganisms which were present in the crystallizing dishes.

In order to complete these initial investigations, aseptic cultivation is indispensible. However, the method used was not suitable for this purpose.

b) Although the seedlings were of the same age when transplanted, finished plants showed considerable differences, because some were dominant, while others seemed dominated. For example, it was frequently observed with Picris hieracioides L., grown side by side, that after six months of cultivation some plants flowered, while others had only a few leaves.

c) Nevertheless, the method has the advantage of providing a relatively large crop of active biological material. Also, it indicates qualitative variations in root excretions during the first two months of cultivation, i.e. as a function of the physiological age of the plants studied.

ISOLATION AND DETERMINATION OF STIMULATORY COMPOUNDS

1. Isolation

Five days before introducing the nutrient liquid, the plants received only distilled water which tended to lower the concentration of the mineral salts in the crystallizing dishes.

The same culture was sampled in intervals which were at least three weeks apart.

The culture solution was filtered, evaporated to dryness under a partial vacuum at a temperature below 40°C. The residue was dissolved in 2 ml of water and used immediately or stored at -4°C.

2. Setting up Different Tests

Agar 0.8% in water was poured into polyethylene capsules (which were stoppered with hemolysis tubes). A Durieux Filter No. 268 (12 mm diameter) was placed on the agar. This kept the humidity constant and supported the seeds that were tested (Fig. 2).

The capsules, prepared as described, were placed into the holes of a leucoflex disc. The entire setup was then placed into a Petri dish of 15 cm diameter. Ten ml of distilled water was added to maintain a saturated atmosphere (Fig. 3).

Fig. 2. A. Capsule filled with agar in distilled water
B. Durieux Filter No. 266 on which the seeds of the Orobanchaceae were placed
C. Complete unit seen from above

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Fig. 3. Petri dish with the proper arrangement, ready to receive pieces of the chromatogram to be tested

We prefered this method over the drop method of Brown et al. (1,2) and over the concavity sheet method of Sunderland (6).

Our setup could be equally well used for similar studies pertaining to the germination of spores, pollen grains or hatching of nematode cysts. These problems are very similar to our problem, i.e. the breaking of dormancy by chemical compounds produced by the host organism. In some cases, the dormant material, expected to react to the stimulants, consisted of tiny cuttings.

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3. Effect of Concentration and Exposure Time on the Rate of Germination (% G)

a) Experimental Data

Seeds of the different Orobanchaceae under investigation were stored in the cold at +4°C.

One month before each test, a sufficient quantity of seed was removed and placed between two sheets of filter paper, on top of hydrophilic moistened cotton, in a Petri dish.

When they were needed, the seeds were washed on a Swinny Filter by a technique described earlier (3).

The crude concentrated solution, obtained as described above, was diluted in a series of hemolysis tubes. Approximately the same amount of washed seeds was placed into each tube, stoppered with a polyethylene capsule and placed among the samples. Samples were collected with a curette containing 100-300 seeds.

The seeds were withdrawn from the tubes in five-minute intervals during the first half hour, then after 1,2,4,8,16,32,48,72 and 96 hours.

After sampling, the seeds were again washed in a Swinny Filter with 80 ml distilled water and were spread out with a flattened needle on one of the supporting discs.

The Petri dishes were incubated in darkness at 21°C. Germinated seeds were counted after 15 days.

In every case, the percentage of germinated seeds was calculated and tabulated. Curves were drawn which represent the variation in the rate of germination as a function of concentration and exposure time (duration of contact).

Two experiments will be described, conducted on September 16 and October 10, 1964. They corresponded, roughly, to the two- and six-leaf stage of Centaurea scabiosa L. This plant does not seem to break the dormancy of the seeds of O. picridis Vauch. until it reaches the 5-leaf stage. It was of interest to us to study the culture liquids after that stage.

b) Results

First experiment of September 16, 1964. The following dilutions were utilized: 1, 1/5, 1/25, 1/125, 1/625.

O. picridis did not germinate in any concentration.

O. minor did not start to germinate until after two hours of exposure time.

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Results are shown in Table I.

Table I

Each column (1), (2), (3), (4) and (5) was used to draw a curve of equal concentration, carrying a number corresponding to the same number shown in Fig. 4. The figures in the table indicate the percentage of germinated seeds of *G. minor* (% G).

Dilutions	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Concentration	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Exposure Time / N°	[illegible]	(4)	(3)	(2)	(1)
4 h.	[illegible]	2	0	0	0
8 h.	1	1,5	0	0	0
16 h.	1,2	5	6	2	1
32 h.	1	38	19	4	1,5
48 h.	1,2	75	33	4	2
72 h.	2,5	66	51	8	10
96 h.	6	54	47	11	15

In order to establish curves corresponding to equal exposure times it seemed best to show on the graph the concentrations rather than the dilutions. The greatest dilution 1/625 was assigned as basis 1.

Second experiment of October 10, 1964. The following dilutions were utilized: 1/1, 1/2, 1/10, 1/20, 1/100 and 1/200.

In order to provide more information, more subgroups were formed than in the preceding experiment.

Two series of curves were established. The first one (Fig. 5) was for the time between 2 and 96 hours of exposure; the second one (Fig. 6) was for an exposure time between 5 minutes and 2 hours. This was done in order to study the breaking of dormancy during short exposure times to the stimulatory solutions.

The greatest dilution 1/200 was assigned as basis 1, for studying the variation in the rate of germination as a function of the concentration. Results are shown in Table II.

Table II

Each number of column corresponds to a curve in Figs. 5 and 6 carrying the same number. The figures at the table [illegible] G. picridis and G. minor received identical treatments: same exposure times, same dilutions and the same amounts of liquid.

Exposure Time	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Concentration	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	(5)	(4)	(3)	(2)	[illegible]	[illegible]	
5"	1.4	4.2	1.3	0	0	0	0	0
[illegible]	61	14	0	0	0	0	[illegible]	[illegible]
15"	36	24	5.5	1	0	1	0	0
20"	52	38	1.3	1.6	1	0	0	0
25"	55	41.5	6	1	0	1	0	0
30"	48	33	6	3.6	3.4	4	0	0
1 h.	51	20	8	1	0.6	3	1	0
2 h.	71	48	17	1	0	3	1	0
4 h.	80	38	14	1	1.4	3.3	18	0.5
8 h.	76	63	15	14	4	4.8	36	2
16 h.	71	48	13	6	4	2.2	24	0
32 h.	8[illegible]	73	62	21	10	3	1.5	2
48 h.	82	80	67	27	4	4	0.5	0
72 h.	88	86	78	52	3	6.3	0	0.5
96 h.	68	77	66	46	6.6	5	1.5	1

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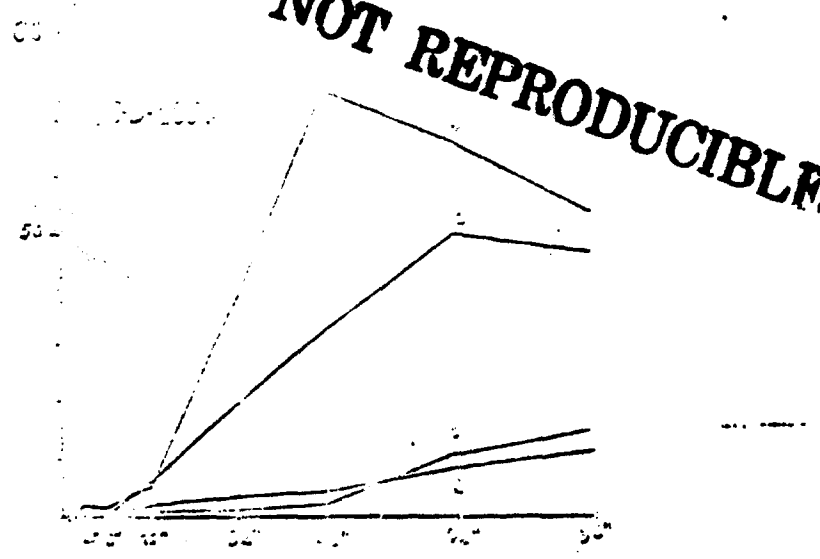

Fig. 4. Variation of the rate of germination (% G) of seeds of O. minor Sutt. as a function of the exposure time to test solutions (Exp. September 16, 1964). The number of each curve corresponds to the same number over the columns in Table I.

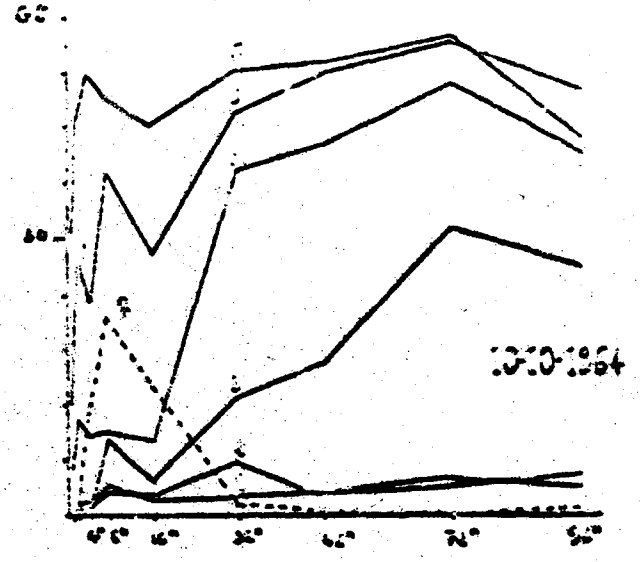

Fig. 5. Variation of the rate of germination (% G) of seeds of O. minor Sutt. ———— and O. picridis Vauch. ----- as a function of the exposure time to test solutions (Exp. October 10, 1964). The number of each curve corresponds to the same number over the columns in Table II.

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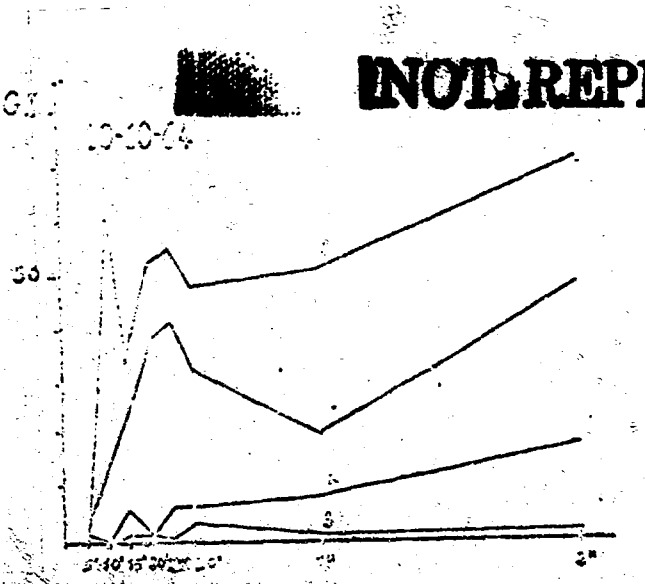

Fig. 6. Variation of the rate of germination (% G) of seeds of O. minor Sutt. as a function of the exposure time to test solutions (Exp. October 10, 1964). Curves show the first two hours of exposure time in the solutions. The number of each curve corresponds to the same number over the columns of Table I.

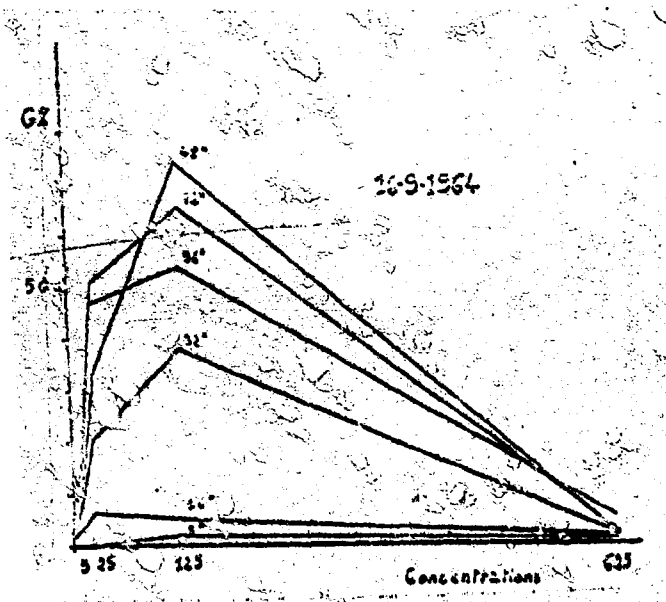

Fig. 7. Variation of the rate of germination (% G) of seeds of O. minor Sutt. as a function of the concentration of biologically active compounds in the test solutions (Exp. September 16, 1964).

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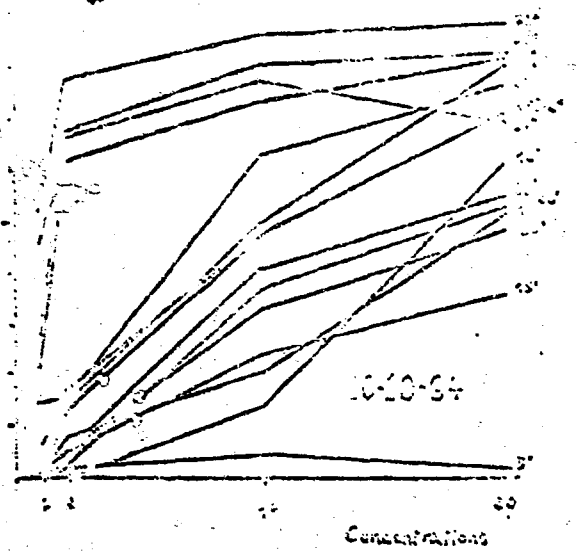

Fig. 6. Variation of the rate of germination (% G) of seeds of O. minor Sutt. as a function of the concentration of biologically active compounds in the test solutions (Exp. October 10, 1964).

c) Conclusions

a) The value of G=61% for the concentration 200 and an exposure time of 10 minutes (Table II, Fig. 6) is probably an error in technique which occurred when the seeds were washed. The curves derived from the same concentration were sufficiently alike, except in this case. We believe that a bad placement of the filter caused incomplete washing.

b) Examining curves of equal concentrations in Fig. 5, we are led to conclude that the culture liquids of Centaurea scabiosa L. contained two compounds A and B which stimulated the germination of the seeds of O. minor Sutt. These compounds had a miximum efficiency after exposure times of 8 and 72 hrs, respectively, for the seeds of the parasite.

The dormancy of the seeds of O. picridis Vauch. was only rapidly broken by Compound A.

c) Comparing curves of equal concentration in Figures 4 and 5 indicated that compound B was present in the culture liquid of C. scabiosa L. at a time when the plants were in the two-leaf stage, but that compound A appeared much later.

d) Examining Fig. 6 indicated the probable existence of a third compound (C), capable of breaking the dormancy of the seeds of O. minor Sutt. after a very short exposure time, i.e., after 25 minutes.

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e) Curves of Fig. 4, 5 and 6 show that on this side of a certain exposure time, some compounds broke the dormancy of the seeds, while beyond that time they blocked, at least partially, the germinating mechanism.

f) With respect to exposure time, the concentration of the active compounds passed through an optimum value beyond which they produced an inhibition of germination (curves in Fig. 7 and the 96 hr curve in Fig. 8).

g) The group of curves of Fig. 4 was divided into two groups which were quite different. This confirmed the existence of at least two active compounds A and B, while the third one (C) was not apparent.

The multiplicity of substances able to break the dormancy of the seed of O. minor Sutt. was in good agreement with the spectrum of plants which stimulated the germination of that parasite.

4. Chromatography

a) Experimental Protocol

Ascending chromatography was used. Crude concentrated solutions were spotted with a micropipet on a line on Whatman No 1 paper. A mixture of butanol:acetic acid:water 4:1:5, v/v, was used in a bell-jar (cloche) for 14 hours. The lighter phase of the mixture served as solvent, the heavier phase served to saturate the atmosphere of the bell-jar.

After the migration, the solvent front was marked, the chromatogram was dried in a stream of air at ambient temperature. Equidistant lines, 1 cm apart, parallel to the starting line, were drawn. Strips perpendicular to that line, approximately parallel to the direction of the migration, were then cut out. Amino acids were made visible by ninhydrin, sugars - on other sheets - by aniline phthalate (5) and p-anisidine phosphate (4). Biological tests were also tried. Strips 0.5 cm wide were cut at each spot detected by the above sprays. A total of 27 spots was noted. Three samples were used: the first spot clearly shown by the solvents, the 27th spot which was only revealed by saturated vapors and a blank piece of filter paper.

Twenty-eight capsules filled with agar were prepared on one filter paper on which were also placed seeds of Orobanche, previously washed with distilled water (Fig. 2). Strips of the chromatogram were placed over each unit prepared in this manner.

One Petri dish was used per strip per species.

Germinated seeds were counted after 15 days, just as was done before.

b) Results

Culture liquids of the experiment of September 16, 1964.

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minor:

R_fx100	% G
78.3	1.1
82.6	4.1
86.9	26.8
91.2	77
95.5	75.6
99.8	0

The R_f value was calculated for the center portion of each compound visible on the chromatogram.
% G is the percentage of germinated seeds
O. picridis: no germination at all

Unfortunately, we were unable to chromatograph the culture liquids of the experiment of October 10, 1964.

Starting with compounds that moved farthest, % G for O. minor was a maximum for compounds having R_fx 100 values of 90.1, 93.2, 94.0 and 91.1.

The % G which reached about 20% was increased by compounds having R_fx 100 values between 67 and 72. They were followed by compounds which decreased % G to almost zero and were, in turn, followed by compounds which improved germination, starting with R_fx100=75. The improvement continued to increase until the maximum, mentioned above, was reached.

With O. picridis seeds, maximum % G was obtained with those portions of the chromatogram which were also most active for the germination of O. minor seeds, except in one case, in which slower moving compounds were best. We also observed that the active region having lower R_f values was much more limited in activity.

Each spot which produced zones of germination was not always revealed by the reagents used.

No germination occurred in the controls.

c) Conclusions

Two compounds broke the dormancy of the seeds of Orobanche minor Sutt. and appeared on the chromatograms of the culture liquids of Centaurea scabiosa L. They had an R_fx100 of about 70 and 92, respectively. The R_fx100=92 substance might have been compound B, made evident by testing the concentrations and length of exposure time. Compound A induced germination in seeds of O. picridis and might have had a similar R_f value or, perhaps, a slightly higher one. On that account, its activity on O. minor may have been confounded with that of compound B. The germinating activity which appeared at R_fx100=70 might have been due to compound C.

These results resemble those obtained by Brown et al. (2) in 1951, who studied the role played by root excretions in the germination of parasitic plants. The root excretions contained an active compound at R_fx100=90 (approx.), produced by [illegible] and capable of germinating seeds of O. minor.

The work of Sunderland (6) with root extracts of seedlings, culture liquids and suspensions of root fragments of Zea mais, grown in glucose solutions, may have a bearing as shown by his results: a water-soluble compound with R_fx100=60 and another factor, called "Mb", ether-soluble, R_fx100=92, were considered by Sunderland to be able to break the dormancy of seeds of O. minor.

CONCLUSIONS

We observed the dormancy of seeds of Orobanche minor Sutt. could be broken by several compounds. Nevertheless, when these substances are very concentrated or when they remain in contact with the seeds for a long time, they may become inhibitory for germination.

Bio-chromatographic studies must be interpreted with caution. Sometimes, the maximum percentage of germination does not correspond to the most abundant compound which breaks the dormancy. Also, at the other extreme, a very small portion of compounds can exert some inhibition because their concentration is too strong.

Very fine chromatography of active zones and a study of each portion of these zones actually aided tests involving concentrations and exposure times and they seem to be the better methods for characterizing promising compounds.

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