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PSORALEN, AN INHIBITOR IN THE SEEDS OF *PSORALEA SUBACAULIS* (LEGUMINOSAE)

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Abstract—In order for germination to occur in a maximum percentage and minimum time, the seeds of *Psoralea subacaulis* T. and G. require both scarification and leaching. The leachate was shown to be inhibitory to seed germination and root growth, not only to *P. subacaulis* itself, but also to four unrelated species. The inhibitory substance was shown to be present in the seed coats, but not in the embryo. Crystalline material isolated from the leachate, both from the non-glycosidic fraction and from the hydrolyzed glycosidic fraction, was identified as the furocoumarin, psoralen. This compound, hitherto known to occur in two other species of *Psoralea*, is believed to be responsible for the inhibition of seed germination and root growth observed in this species.

INTRODUCTION

Psoralea subacaulis T. and G. is a herbaceous perennial whose distribution is centred in the cedar glades of Middle Tennessee, with a few disjunct populations in glades in northern Alabama and northern Georgia. The plants flower in April and the fruit matures in May. The seeds are shed in late May and lie dormant until the following spring when germination occurs. In this study, dormancy has been found to be due to (1) the presence of a "hard" seed coat and (2) one or more chemical inhibitor(s) present in the seeds. Thus, unlike most plants whose seeds have hard coats, the seeds of this species have a second block to germination; namely the presence of a water-soluble inhibitor(s).

Bonner[1] and Grümmer[2] have discussed the role of toxic substances produced by certain plants in relation to their interactions with other higher plant species. The topic of germination inhibitors has been reviewed by Evenari[3,4] and Billings.[5] These inhibitors may function to the advantage of the species: (1) by prevention of premature germination, (2) by extension of the period during which germination can occur, (3) by insuring that an adequate supply of water for seedling establishment is present, and (4) by prevention of establishment of other species in the immediate vicinity.[3,4]

RESULTS

Germination of Seeds

In an experiment to determine the effect of scarification on subsequent germination, none of the control seeds had germinated after one week, while 54 per cent of the scarified seed had germinated. The roots of the resulting seedlings, however, appeared to be stunted. Since none of the non-scarified seeds germinated, the seeds were routinely scarified in all subsequent experiments.

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[1] J. BONNER, *Botan. Rev.* **16**, 51 (1950).
[2] G. GRÜMMER, *Soc. Exp. Biol. Symp.* **15**, 219 (1961).
[3] M. EVENARI, *Botan. Rev.* **15**, 153 (1949).
[4] M. EVENARI, *Soc. Exp. Biol. Symp.* **11**, 21 (1957).
[5] W. D. BILLINGS, *Ann. Rev. Plant Physiol.* **8**, 375 (1957).

In an experiment to determine the effect of leaching, the leached seeds gave 91 per cent germination after one week, while only 46 per cent of the non-leached seeds had germinated. Measurement of the length of primary roots showed that those of leached seedlings averaged 1·5 cm while those of the non-leached averaged only 0·5 cm in length.

Inhibitory Effects upon Other Species

Three replicates of fifty seeds each of lettuce, tomato, cucumber, and radish were sown in petri dishes containing fifty seeds of *P. subacaulis* which had been allowed to imbibe water and leach for 24 hr. Another three replicates of fifty seeds each were sown in petri dishes not containing seeds of *P. subacaulis*. After 4 days the number of seeds which had germinated was determined and the length of the primary root of each germinated seedling was measured. The results are summarized in Table 1. The seeds of lettuce and tomato showed a pronounced decrease in percentage of germination in the presence of the seeds of *P. subacaulis*, and the primary roots exhibited greatly reduced growth in all of the species tested.

TABLE 1. PERCENTAGE GERMINATION AND ROOT GROWTH OF FOUR SPECIES AFTER 4 DAYS IN PRESENCE OF SEEDS OF *P. subacaulis* AND IN WATER

Species	% Germination		Primary root length (cm)	
	Leachate	Water	Leachate	Water
Lettuce (*Lactuca sativa* L.)	60	90·3	0·7	3·2
Tomato (*Lycopersicum esculentum* Mill.)	25·3	92·6	0·3	1·3
Radish (*Raphanus sativus* L.)	74·0	98·0	0·4	4·7
Cucumber (*Cucumis sativus* L.)	73·3	80·7	1·3	3·5

Location of the Inhibitor

The seeds of *P. subacaulis* do not contain an endosperm. In order to determine whether the inhibitor was located in the seed coat or in the embryo, embryos were excised, and their subsequent root growth was compared with that of other excised embryos germinated in dishes containing the removed seed coats.

Preceding the experiment, all seed were allowed to imbibe for 12 hr, in order to facilitate removal of the seed coats, which is practically impossible otherwise. The experiment involved three treatments. In one treatment, the seed coats removed from the seed were placed in the petri dishes along with the embryos. In a second treatment the seed coats were discarded, and filter paper and water in the dishes with the embryos were not changed. The leachate from the embryos was thus allowed to accumulate during the entire course of the experiment. In the third treatment, the seed coats were again discarded, but the filter paper and liquid contents of the petri dishes in which the embryos were placed was changed at 12-hr intervals throughout the germination period. All the embryos were allowed to grow for 4 days, after which the length of the primary root was measured. The results are summarized in Table 2.

From these results it can be seen that the growth of the embryos in the presence of the seed coats was greatly reduced in comparison with that of the embryos, either leached or non-leached, grown alone. No apparent difference was observed in the growth of leached and non-leached embryos in the absence of the seed coats. It was therefore concluded that the inhibitor is located in the seed coat.

TABLE 2. GROWTH OF EXCISED EMBRYOS AFTER 4 DAYS IN PRESENCE OF SEED COATS OF *P. subacaulis* AND IN WATER

Treatment	Length of primary root (cm)
Embryo + seed coat	0·6
Embryo leached	2·2
Embryo non-leached	2·0

Chemical Nature of the Inhibitor

Rather early in the course of the experiments just described, it was noticed that the leachate from the seeds was dark brown in color, and had a pronounced coumarin-like odor. Significantly, Indian workers[6, 7, 8] have isolated from the seed of the related *Psoralea corylifolia* L. two furocoumarins psoralen and isopsoralen (angelicin).

By sublimation of the leachate obtained from 50 g of seed of *P. subacaulis*, a white crystalline material was obtained which in water and ethanol (3 : 1 by volume) had absorption maxima at 295 nm, 245 nm, and 205 nm. By comparison with the spectra of furocoumarins in this solvent as given by Fowlks,[9] it is clear that the material obtained from seeds of *P. subacaulis* is a furocoumarin.

Both the crystalline material from *P. subacaulis* and an authentic sample of psoralen were fluorescent, and, upon excitation at 300 nm, both showed a broad fluorescence peak in the range of 440–480 nm. When the material from the non-glycosidic portion was recrystallized twice from benzene it yielded approximately 100 mg of a crystalline substance which had a melting point of 160–162° undepressed when mixed with an authentic sample of psoralen. The i.r. of the crystalline material from *P. subacaulis* also had a superimposable i.r. spectrum in comparison with an authentic sample of psoralen. Upon GLC (in a temperature programed instrument) both the material from *P. subacaulis* and an authentic sample of psoralen had the same elution time, and addition of psoralen to the non-glycosidic material from *P. subacaulis* resulted in increasing the height of the effluent peak.

The glycosidic portion of the extract, recrystallized twice from benzene, yielded approximately 50 mg of a crystalline product with a wide melting range. Both GLC and TLC showed the presence of two components. On gas-liquid chromatography there was the large peak corresponding to psoralen was preceded by a much smaller one. On TLC two spots were seen, one of which had an R_f identical to that of psoralen.

DISCUSSION

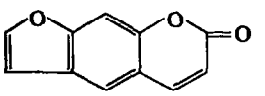
Other members of this genus from the seeds of which psoralen has been isolated include *Psoralea corylifolia* L., a native of india,[6, 7, 8] and *Psoralea drupacea* Bunge, a native of Turkestan and Persia.[10] Psoralen has also been isolated from the seeds of several species of *Coronilla*.[11]

[6] H. S. JOIS, B. L. MANJUNATH and S. VENKATA RAO, *J. Indian Chem. Soc.* **10**, 41 (1933).
[7] K. K. CHAKRAVARTI, A. K. BOSE and S. SIDDIQUI, *J. Sci. Ind. Res. (India)* **7B**, 24 (1948).
[8] H. N. KHASTGIR, P. C. DATTAGUPTA and P. SENGUPTA, *Indian J. Appl. Chem.* **22**, 82 (1959).
[9] W. L. FOWLKS, *J. Invest. Dermatol.* **32**, 249 (1959).
[10] I. N. KARTASHKINA, M. R. I. SHAMSUTDINOV and T. T. SHAKIROV, *Khim. Prirodn. Soedin. Akad. Nauk. Uz. SSR.* **2**, 65 (1966); *Chem. Abstr.* **65**, 1038 (1966).
[11] A. STOLL, A. PEREIRA and J. RENZ, *Helv. Chem. Acta* **33**, 1637 (1950).

Rodighiero[12] tested the inhibitory properties of nine furocoumarins to lettuce seeds and found that three of these, psoralen, angelicin, and xanthotoxin, were effective inhibitors of seed germination and seedling and root growth. Bennett and Bonner[13] isolated three furocoumarins from whole plants of *Thamnosma montana* Torr. and Frem. and showed that these inhibited growth of tomato seedlings in nutrient solution. Fowlks[14] has shown that psoralen and 8-methoxypsoralen inhibit growth of *Tetrahymena*, rat tumors, and mammalian tissue cultures.

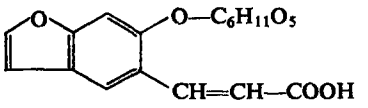
Fowlks[9] describes the results of other workers concerning the mutagenic properties of a number of furocoumarins. Psoralen and 5-methoxypsoralen were found to be very effective mutagens. Four hours of exposure of onion root tips to psoralen at $5{\cdot}6 \times 10^{-5}$ M or 5-methoxypsoralen at $5{\cdot}0 \times 10^{-5}$ M resulted in 40 per cent of chromosome mutations. The chromosome aberrations noted were agglutination of chromosomes and stickiness of the chromosomal surfaces. At higher concentrations there was a total inhibition of mitosis.

Chandra and Wacker,[15] using cell free extracts of *E. coli*, found that under the influence of light the furocoumarins, psoralen, xanthotoxol, and xanthotoxin, become bound to nucleic acids, preferentially to thymine and uracil, and inhibit the incorporation of AMP into RNA. Maximal inhibition was shown by psoralen. The template efficiency of DNA in the RNA polymerase reaction was also found to be decreased after treatment of DNA with these compounds.



Psoralen

(I)



Glycoside of Psoralen

(II)

The isolation of psoralen from both the ether-soluble and the hydrolyzed ether-insoluble fractions of the extract from *P. subacaulis* indicates that this compound exists in the seed both as a glycoside (II), and in the free state (I). It would seem possible that the minor component detected by both gas-liquid chromatography and thin-layer chromatography in *P. subacaulis* may have been isopsoralen (angelicin), since both furocoumarins have been shown to be present in *P. corylifolia*,[7,8] but since no authentic sample of this compound for comparison was available, no definite conclusion is possible.

EXPERIMENTAL

Seeds of *Psoralea subacaulis* were collected during May, 1966, from a glade in Maury County near Columbia, Tennessee, and were stored in plastic containers in the laboratory until required (fall 1966).

[12] G. RODIGHIERO, *Giorn. Biochim.* **3**, 138 (1954).
[13] E. L. BENNETT and J. BONNER, *Am. J. Botany* **40**, 29 (1953).
[14] W. L. FOWLKS, Personal communication (1966).
[15] P. CHANDRA and A. WACKER, *Z. Naturforsch.* **21b**, 663 (1966).

Germination tests were performed in petri dishes containing two sheets of Whatman No. 1 filter paper moistened with distilled water. Three replications of fifty seeds were used for each treatment. The tests were carried out in incubators at 25 ± 1°, a temperature which had been shown in other experiments to be near optimal for this species, and a 12/12 photoperiod. Leaching was accomplished by changing the seeds to clean petri dishes every 12 hr for 48 hr. The seeds were considered to be germinated when the radicle broke through the seed coat.

The active principle(s) in *P. subacaulis* seed coats was isolated using the method of Khastgir *et al.*[8], which is essentially as follows: 500 g of seed were ground and defatted with 2 l. of light petroleum. After the light petroleum was removed by filtration, the solid material was extracted several times with 95% ethanol over 48 hr using a total of 8 l. of ethanol. The ethanol was distilled under reduced pressure, until *ca.* 50–100 ml of a syrupy extract remained. This material was then extracted with several portions of ether, (2 l in all), to separate the non-glycosidic (ether soluble) and glycosidic (ether insoluble) fractions. Approximately 1 g of a crystalline material was subsequently isolated from the ether extract. From the ether-insoluble fraction, after hydrolysis with conc. HCl, approximately 200 mg of crystals were obtained.

GLC of the material from *P. subacaulis* was performed in a dual column, temperature programed instrument equipped with a hydrogen flame ionization detector (Aerograph Model 204, Wilkens Instrument and Research, Inc., Walnut Creek, California). The material was analyzed with a 300 cm × 0·3 cm column packed with 3% silicone gum rubber on 80–100 mesh Diatoport S.

TLC of the crystalline material from *P. subacaulis* was performed on a plate of silica gel using chloroform as the developing solvent. The spots were examined under u.v. light.

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