LOCATION OF ERGOT ALKALOID AND FUNGI IN THE SEED OF RIVEA CORYMBOSA (L.) HALL. f., "OLOLIUQUI"[1]

W. A. TABER AND R. A. HEACOCK[2]

Abstract

Ergot-type alkaloids were found to be present in the embryo but not in the seed coat, seed membranes, or resinous layer under the seed coat of the hallucinogenic seed "ololiuqui" (*Rivea corymbosa* (L.) Hall. f.). The alkaloids were typically present in the seeds. Fungi were found to be present in the seed coat but not embryo of surface-sterilized seeds. They also were typically present in the seed and were concentrated about the hilum. The only fungi present in the seed were *Chaetomium globosum*, *C. reflexum*, *C. spinosum*, *C. funicolum*, and *Fusarium moniliforme*. The absence of fungi in the region of the seed which contained the alkaloid suggests that fungi are not the source of alkaloid.

Introduction

Rivea corymbosa (L.) Hall. f. (*Ipomaea burmanni* Choisy) is a rare tropical member of the Convolvulaceae whose seed contains substances which, upon ingestion, cause mental disturbances (3, 4, 5). Aztecs and others have used this seed in religious rites, and the seed is still eaten by certain Indians in Mexico (5). The hallucinogenic and psychotomimetic properties of this seed have been verified recently (2, 3). Further, Hofmann and Tscherter (2) have isolated ergine (isolysergic acid amide), isoergine (lysergic acid amide), and chanoclavine from this seed and they concluded that these substances are at least part of the psychotomimetic principles of the seed. This is the first report of the occurrence of ergot-type alkaloids in the tissues of higher plants. They were previously known to be present in nature only in the sclerotic mycelium of *Claviceps* species when these had parasitized the ovaries of grasses. In view of the uniqueness of this find, it is important to establish whether the alkaloid is actually produced by plant tissue or whether it is produced by fungi or bacteria present in the seed. Seed-coat-borne fungi are well known (6, 7) and fungi can infect the ovary (i.e., *Claviceps* spp. in grasses) and therefore it is not inconceivable that a microorganism is the source of alkaloid. Requisites to determining the actual source of ergot alkaloid are knowledge of the location of alkaloid in seed, and, if fungi are present, the location and identity of the seed fungi. Alkaloid, fungi, or both could be located in the seed coat, embryo, or the characteristic internal seed membranes and resinous layer situated just beneath the seed coat. If the alkaloid is produced by the mature plant it might be located in the seed coat, or perhaps the resinous layer, but if it is produced by the new generation it would be in the embryo. Fungi could be in the seed coat or in the embryo if the seed were infected. This paper reports the location of

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[2]Psychiatric Research Unit, University Hospital, Saskatoon, Saskatchewan.

ergot-type alkaloids and the fungi which were found in the seed. Reference is also made to the general structure of the seed since, except for a brief report by Santesson (4), no description of this unusual seed has been published.

Materials and Methods

Seeds

The seeds were purchased from the Atkins Garden and Research Laboratory, Cienfuegos, Cuba.

Ergot Alkaloid Assay

The assay procedure has been described previously (11). Briefly, crushed seeds, seed fragments, or freeze-dried microbial cultures were wetted with 10% ammonium hydroxide, and the alkaloid extracted into ether and then back-extracted into 0.2 N H_2SO_4. The cell material was shaken 30 minutes in the alkaline ether. A single such extraction removed approximately 60% of the seed alkaloid. The acid extract was mixed with Van Urk reagent, and the resulting blue color was read at 550 mμ within 2 minutes.

The assay was checked against the fluorometric assay (11). Assay of lots of 10 seeds gave values of 20.2 and 25.0 μg alkaloid per seed by the colorimetric and fluorometric methods, respectively. Further, the peak of maximum emission (Fig. 1) is characteristic of ergot alkaloids (11).

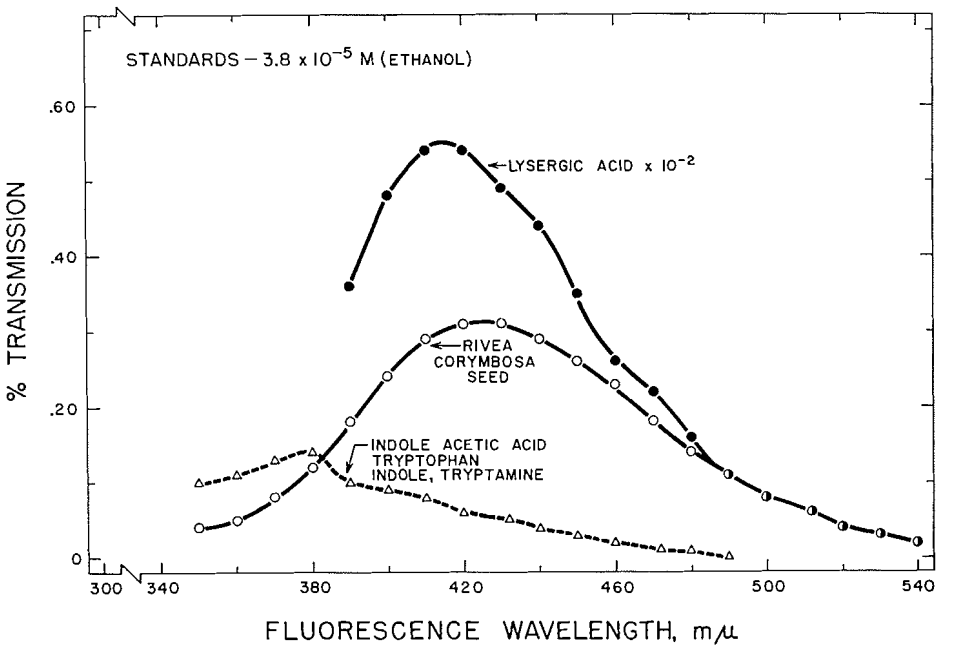

FIG. 1. Fluorescence spectrum of 50% aqueous ethanol – tartaric acid extract of seed. Activating wavelength was set at 330 mμ. The emission maximum at 420 mμ is characteristic of ergot alkaloids (11).

Since the alkaloids which are found in these seeds (2) were not available as standards, the values are expressed as ergometrine equivalents.

Surface Sterilization of Seeds

The surface of seeds was sterilized by soaking them 1½ minutes in commercial Javex, diluted 1:10. They were rinsed in six changes of 100 ml of sterile distilled water. Since the hilum region is surrounded with hairs (Fig. 2) and tended to trap air which prevented contact of the seed and germicide, the seeds were first moistened with 90% ethanol.

Fragmentation of Seeds

The surface-sterilized seeds were cracked singly under aseptic conditions with a sterile steel rod and were then teased into seed coat fragments, hilum button, and seed meat (hypocotyl, cotyledon, internal membranes, and resinous layer). The various fragments of each seed were plated onto WGA agar (8) containing neomycin. The seed coat fragments were drawn through sterile agar to remove adhering meat fragments before plating. The fragments of seed were approximately 0.3 to 1 mm square. Approximately one-third of the meat and three-quarters of the seed coat of each seed was plated out. The hypocotyl usually remained intact and was plated as one piece. The complete seed could not be plated because the brittle nature of the thick coat (Figs. 3 and 4) caused some pieces to spring out of the sterile working area while the seeds were being teased apart. Teasing was carried out at 6× magnification.

The alkaloid assay of seed regions was carried out in three separate tests. The seeds were then divided into regions as follows: test *a*—hypocotyl, cotyledon fragments, and remainder of seed; test *b*—membrane, embryo, and seed coat; and test *c*—resinous layer under coat and remainder of seed. In each test an equal number of crushed but unseparated seeds were assayed as controls. The "remainder of the seed" fraction in test *a* contained appreciable cotyledon which could not be separated. The cotyledon fraction, however, contained only cotyledon.

Identification of Fungi

Isolates were transferred to potato dextrose agar slants and to sterile straw partially immersed in water and contained in test tubes. Isolates of *Chaetomium* were identified from the keys of Skolko and Groves (6, 7) and of Udagawa (10). One species, which resembled both *C. cochloides* and *C. globosum*, was found to be the latter after comparison with an authentic *C. cochloides*. Isolates of *Fusarium* were identified from Gilman (1). Transfers of *Fusarium* were sent to Dr. W. L. Gordon, Canada Department of Agriculture, Winnipeg, for confirmation of identification.

Test for In Vitro Alkaloid Production

Eleven isolates of fungi and five isolates of bacteria were tested for alkaloid production on three and five media, respectively. The media were similar to those described previously (9) but since production was not detected, the composition of the media will not be given.

Results

Uniformity of Alkaloid in Seeds

Single seeds contained too little alkaloid for individual estimation. Since only 16% of scarified seeds germinated, 18 lots of three seeds each were assayed on three different days to determine whether or not alkaloid was present only in occasional, and perhaps infected, seeds. Statistical analysis of the data obtained (Table I) showed no significant difference in the groups or among replicas and that the standard error was small. It was concluded therefore that the alkaloids were uniformly distributed among the seeds.

TABLE I

Evidence that alkaloid is typically present in the seed

	Assay, days		
	1	2	3
Replica	μg alkaloid per seed		
1	8.3	10.0	9.6
2	8.0	11.1	5.2
3	9.0	7.0	6.4
4	12.6	6.5	7.4
5	10.0	8.0	9.6
6	8.3	9.0	8.0
Mean	9.3	8.6	7.7
Standard error	± .6	± .7	± .7
Grand mean and standard error is 8.5 ± 0.8 μg (calculated from interaction error)			

NOTE: *Analysis of variance*—variance ratio (F) for days is 1.16 and F for replicas is 2.42. There was no significant difference ($P > .20$) either among the three assays or among the replicas within the assays.

The total amount of alkaloid, expressed as ergometrine equivalents, was determined from three serial extractions of seed with alkaline ether. The average alkaloid content of seed having fresh weight of 25.9 ± 0.6 mg was 20.2 μg. A lot of seed averaging 17.2 mg fresh weight contained an average of 12.2 μg alkaloid.

Location of Alkaloid in Seed

When halved seeds were moistened with 65% H_2SO_4 and then immersed in Van Urk reagent (Fig. 4), the inner seed coat layer and internal seed membrane colored a pink-brown. Within 2 hours, the hypocotyl and cotyledon, which were previously colorless, became true blue. These observations suggested that the ergot alkaloids were localized in the seed but did not reveal which of the two color reactions reflected the presence of alkaloid.

Preliminary tests on groups of 10 seeds which were separated into seed coat and meat revealed that the alkaloid was present only in the meat. This meat fraction contains hypocotyl, cotyledon, internal membrane, and a distinctive resinous layer lying beneath the seed coat and apparently in contact with the internal membrane. When seeds were separated into hypocotyl, clean cotyledon, and the remainder of the seed, which still contained appreci-

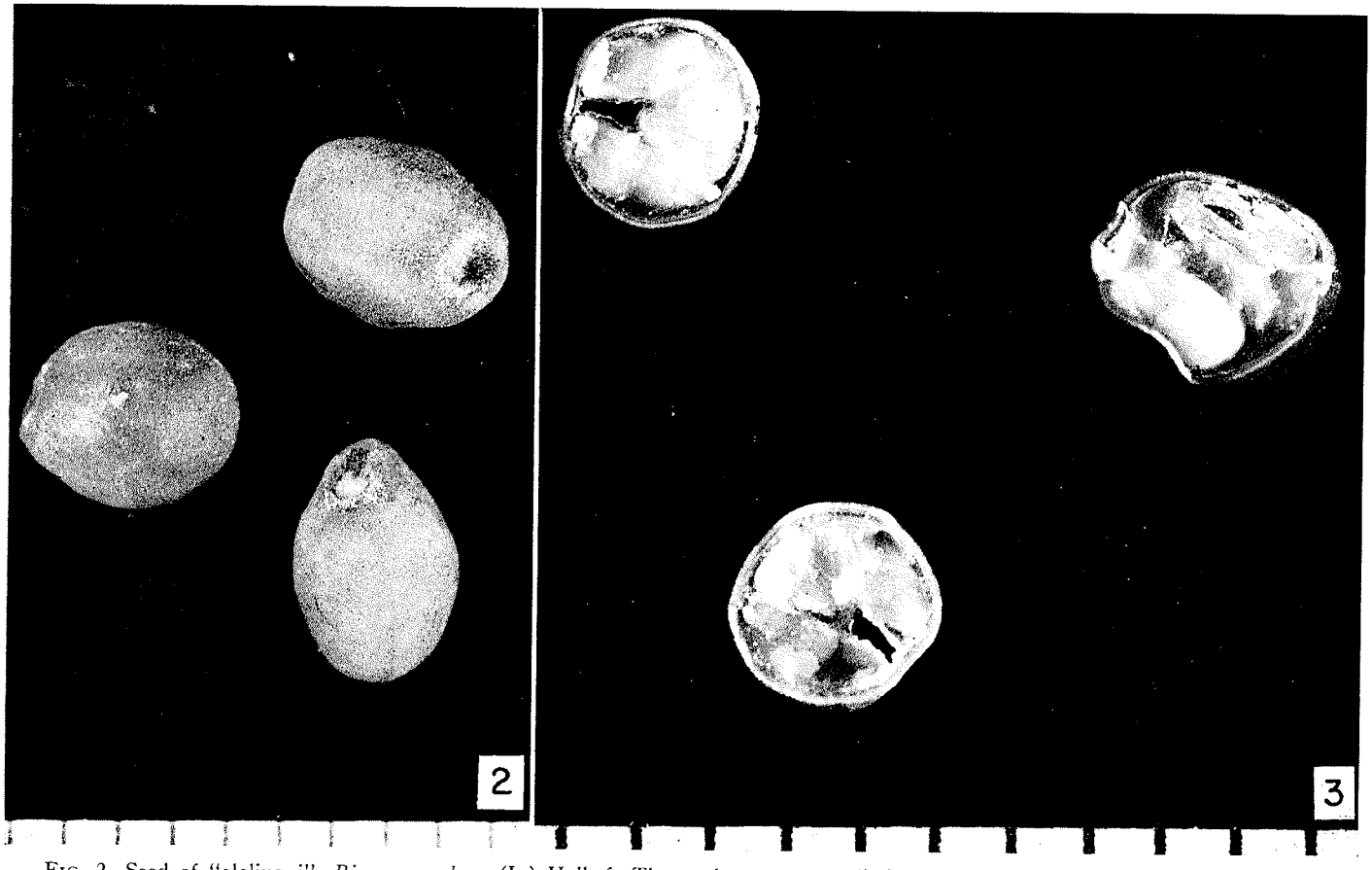

FIG. 2. Seed of "ololiuqui", *Rivea corymbosa* (L.) Hall. f. The seed possesses a distinct hilum which is ringed with minute hairs. The scale is in millimeters. FIG. 3. Seed opened parallel to (right) and perpendicular to the long axis. The embryo is large and occupies most of the seed. Note the double-layered coat and layer of clear resinous material just beneath the coat. The thick, clear membrane outlines the cavity shown in cross section. The membrane is at least partly cellular.

PLATE II

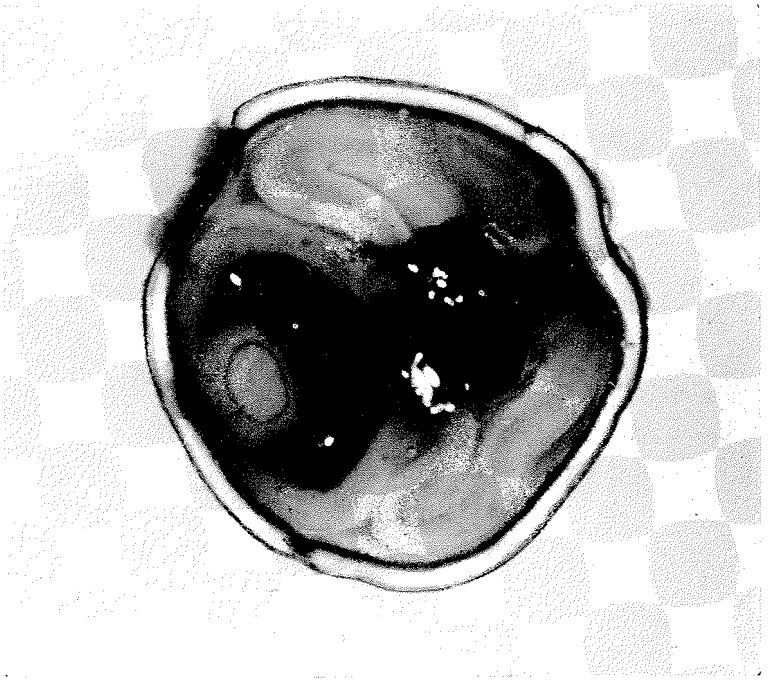

FIG. 4. Seed tissue stained with Van Urk reagent. The surface was wetted with 65% H_2SO_4 and then submerged in Van Urk reagent for 30 minutes. The central membrane, seed layer adjacent to the seed coat, region adjacent to hypocotyl (seen in cross section on left side of seed), and thin layers within the hypocotyl and cotyledon are stained pink-brown (dark areas in photograph). This color is probably not due to reaction with ergot alkaloid. Two hours of incubation led to formation of true blue color only in the hypocotyl and cotyledons proper. This color is probably due to reaction of ergot alkaloid with the reagent. The resinous layer, which is not visible in the photograph, does not react with the color reagent. See Table II and Text for results of chemical assay of these various regions of seed.

able cotyledon, alkaloid was found to be present in all three (Table II). The embryo tissue, then, contains alkaloid and that present in the remainder of the seed could be due to the presence of residual cotyledon. Further separation of the seed into membrane, embryo, seed coat, and resinous layer (Table II) revealed that only the embryo tissue contained alkaloid.

TABLE II

Distribution of alkaloid in seed*

Test	Region of seed	μg/seed
A	Hypocotyl	1.2
	Cotyledon	1.8
	Remainder (coat, membrane, adhering cotyledon, resinous layer)	3.7
B	Membrane	0.0
	Embryo	4.6
	Coat	0.0
C	Resinous layer under coat	0.4†
	Remainder	4.1

*Assay of extract of from five to eight seeds.
†No visible color reaction.

Location of Fungi in Seed

Thirty surface-sterilized seeds were separated into 476 meat fragments and 113 seed coat fragments. Four of the former and 10 of the latter yielded fungi on plating, giving an incidence of 0.8% and 10%, respectively. Except for two cultures of *Fusarium*, all of the isolates were species of *Chaetomium*. The low incidence in meat suggested that this might be due to contamination of the meat fragments with seed coat fragments during teasing. The test was repeated on 12 seeds and, in this test, care was taken to assure that the meat fragments did not come into contact with seed coat fragments during the teasing procedure. None of the meat fragments contained fungi (Table III). The fungi were concentrated about the hilum. Fungi were not present, then, in the region of the seed which contained ergot-type alkaloids.

Species of Fungi and Bacteria

Of the 52 fungi which were isolated from fragments, 50 were species of *Chaetomium* and the other 2 were isolates of *F. moniliforme* Sheldon. The *Chaetomium* species were *C. globosum* Kunze, *C. funicolum* Cooke, *C. reflexum* Skolko, and *C. spinosum* Chivers. Of the 100 intact, surface-sterilized seeds which were plated out in groups and of which 73 to 100% yielded fungi, only *Chaetomium* species were present. *C. reflexum* and *C. globosum* were the most frequent species found. On the basis of these samplings from the small quantities of seeds available, it would appear that the seeds possess a somewhat characteristic seed coat population.

A yellow bacterium which grew out in high frequency from surface-sterilized seeds plated on the medium containing neomycin was isolated. It was not identified.

TABLE III

Incidence of fungi (*Chaetomium* species) in various parts of surface-sterilized seeds

Seed	No. fragments		No. fungi	Identification
1	Hilum	1	0	
	Meat	12	0	
	Coat	8	2	*C. reflexum*
2	Hilum	1	0	
	Meat	11	0	
	Coat	6	1	*C. spinosum*
3	Hilum	1	1	*C. reflexum*
	Meat	15	0	
	Coat	8	1	*C. reflexum*
4	Hilum	1	0	
	Meat	14	0	
	Coat	9	1	*C. reflexum*
5	Hilum	1	1	*C. globosum*
	Meat	11	0	
	Coat	5	0	
6	Hilum	1	0	
	Meat	10	0	
	Coat	9	1	*C. reflexum*
7	Hilum	1	1	*C. reflexum*
	Meat	11	0	
	Coat	9	0	
8,9,10	Hilum	1,1,1	0	
	Meat	9,11,12	0	
	Coat	7,7,5	0	
11	Hilum	1	1	*C. globosum*
	Meat	9	0	
	Coat	5	1	*C. reflexum*
12	Hilum	1	1	*C. spinosum*
	Meat	11	0	
	Coat	6	0	

NOTE: Seventy-five per cent of the seeds bore fungi. Of 34 seeds which were surface sterilized and washed with the above but not broken up, 73% contained fungi. Forty-one per cent of the hilum fragments, 8% of the seed coat fragments, and none of the meat fragments yielded fungi.

Conclusion

The ergot alkaloids are typically present in the lot of seeds examined and were located in the embryo but not in the seed coat, in the resinous layer adjacent to the seed coat, or in the membrane located centrally in the seed. Both the hypocotyl and cotyledon contained ergot-type alkaloids. The fungi and bacterium were present in the seed coat but not in the embryo. Fungi were most frequent about the hilum. *Claviceps* species, the previously known source of ergot alkaloid, were not detected in the seeds. These data are consistent with the hypothesis that the seed rather than a fungus or bacterium in the seed is the source of alkaloid.

Acknowledgments

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