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# A PRELIMINARY STUDY OF ORCHID POLLEN GERMINATION AND THE CHROMATOGRAPHIC ISOLATION OF A STIMULANT FROM COLUMNS

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**Abstract**—A relatively low percentage of pollen germination of 29 orchid species was found to occur on an inorganic-salt agar medium containing sucrose. Additions of sorbitol, inositol, indole-3-acetic acid, coconut milk, pineapple juice did not increase the percentage of germination. A material from *Oncidium* alliance columns did, however, appreciably increase germination and tube growth. This stimulating material was found to be neutral, more soluble in water than in ether and stable to autoclaving. It separated at $R_f$ 0·3–0·5 under the conditions of paper partition chromatography followed. The stimulating material was active in low concentrations and did not appear to be highly species or genera specific.

WITHNER[1] has recently summarized the scant information available on orchid pollen germination. The most extensive work was done by Curtis and Duncan[2] who germinated several species of orchid pollen and reported that traces of boron were stimulating to germination, and that stigmatic fluid was considerably more so. Both boron and stigmatic fluid have long been recognized as general stimulants of pollen growth. No information has been brought forward to suggest in what way, if any, the initial germination of orchid pollen differs in its requirements from that of most other plants, although difficulty of germination has often been noted. As early as 1922 Von Kirchner[3] cited the inability of orchid pollen to germinate on the stigmas as one of three factors involved in sterility. The senior author of the present paper has found that orchid pollen does not readily germinate *in vitro*. It seemed worthwhile, therefore, to investigate conditions promoting orchid pollen germination.

Brewbaker and Kwack,[4] working with a variety of pollen, but not including that of any of the *Orchidaceae*, have found no pollen growth promotion given by indole-3-acetic acid and several other growth substances, but have found stimulation to result from the addition of either coconut milk or yeast extract to a basal medium of 10% sucrose and 100 ppm boric acid. They also have found stimulation to result from the addition of extracts of various plant parts: anther, leaf, stem, root, pistil and petal. They reported that stimulation in all these cases was due to the presence of calcium ion and could be fully compensated for by addition of any of a number of soluble inorganic calcium salts (e.g. White's medium with $Ca(NO_3)_2 \cdot 4H_2O$ at 280 ppm gave satisfactory results.) They also found that variation in sugar concentration over a wide range (2–40 per cent) had little effect on pollen germination. The effect of lower concentrations of sucrose was expressed by pollen cell bursting.

The basal medium used throughout the present experiments was that of Tulecke,[5] slightly modified, which contains calcium nitrate and boric acid as well as other inorganic materials and thus eliminates the type of ion deficiency discussed by Brewbaker and Kwack.[4] On this

[1] C. L. WITHNER, *The Orchids, A Scientific Survey*, p. 350. Ronald Press, N.Y. (1959).
[2] Cited by C. L. WITHNER, *The Orchids, A Scientific Survey*, p. 350. Ronald Press, N.Y. (1959).
[3] Cited by C. L. WITHNER, *The Orchids, A Scientific Survey*, p. 353. Ronald Press, N.Y. (1959).
[4] J. L. BREWBAKER and B. H. KWACK, *Am. J. Botany* **50**, 859 (1963).
[5] W. TULECKE, *Am. J. Botany* **35**, 29 (1960).

medium most of the 29 species of orchid pollen tested showed low germination of from 0 to less than 8 per cent after 7 days. None, except *Phalaenopsis* hybrids and *Vanda* hybrids showed more than 20 per cent germination. In general, lowest germination was found in the *Oncidium* alliance, highest in the *Sarcantheae* and moderate germination in the *Laelieae* (see Table 1.) Further work was largely restricted to the *Oncidium*. In general, there was no or little increase in germination after 7 days. Germination was quite variable from test to test and usually began at the periphery of the pollinia.

TABLE 1. SPECIES OF ORCHIDS TESTED FOR POLLEN GERMINATION UNDER CONTROL CONDITIONS

Oncidium Alliance
*Oncidium ansiferum* Reichb. f.
*O. baueri* Lindl.
*O. cebolleta* (Jacq.) Swartz
*O. cornigerum* Lindl.
*O. flexuosum* Sims
*O. guttatum* (L.) Reichb. f. (*O. luridum* Lindl.)
*O. lietzei* Regel
*O. nanum* Lindl.
*O. obryzatum* Reichb. f.
*O. parviflorum* L. O. Will.
*O. pubes* Lindl.
*O. pulchellum* Hook.
*O. robustissimum* Reichb. f.
*O. sarcodes* Lindl.
*O. sprucei* Lindl.
*O. stenotis* (var. from Ecuador) Reichb. f.
*Rodriguezia secunda* H.B.K.
*R. venusta* (*fragrans*)
Laelieae
*Brassavola subulifolia* Lindl. (*B. cordata* Lindl.)
*Cattleya harrisoniana* Bate. ex Lindl. (*C. loddigesii* Lindl.)
*Epidendrum cochleatum* L.
*Laelia gouldiana* Reichb. f.
Sarcantheae
*Phalaenopsis mannii* Reichb. f.
*Phalaenopsis* Blume Grace Palm × P. Doris
*Renanthera imschootiana* Rolfe
*Vanda* Reichb. f. rothchildiana × *V. suavis*
Dendrobium
*Dendrobium farmeri* Paxt.
*D. nobile* Lindl.
*D. pierardii* Roxb.

Of special interest were the pollen grains of *Rodricidium* Tahiti (*Rodriguezia secunda* × *Oncidium flexuosum*). No germinating pollen cells were found in any examination. In most cases extensive cell decomposition was seen and no, or very few, distinct nuclei were observable after formation of the microspore mother cells. The senior author[6] has previously reported the probable sterility of this hybrid and that he has not found ovary stimulation to result from use of its pollen on a number of related species.

Additions, to the basal medium, of up to 20 per cent coconut milk had no determinable effect on the germination of the species tested (*Oncidium obryzatum, O. sarcodes*). The same species did not respond to either sorbitol or inositol at 25 mg/l. nor to the two sugars in combinations (12·5, 25·0 and 50·0 mg each/l.). Addition of indole-3-acetic acid at 84 and 167 ppm had no stimulating effect on pollen germination of the species: *Oncidium flexuosum, O. guttatum, Rodriguezia venusta* (*fragrans*). Addition of 20% canned pineapple juice did not stimulate, but possibly slightly inhibited, germination in 6 species (*Oncidium baueri, O. flexuosum, O. guttatum, O. stenotis* (var. from Ecuador), slightly different from the type, *Phalaenopsis mannii, Rodriguezia venusta* (*fragrans*)).

To test for the presence of possible stimulating compounds in stigmatic material, chromatographic methods were used. Pollinia were planted in 1–2 mm films of basal medium covering filter paper squares obtained from paper chromatographs of *gymnostemia* (see Experimental). In such tests, *Oncidium flexuosum* control pollen germination was from 0–4 per cent, (beginning and very short tubes), while germination over compounds from *O.*

[6] W. W. SANFORD, *Sexual Compatibility Relationships of the Oncidium and Related Genera, Am. Orchid Soc. Bull.* In press, (1964).

*guttatum* column material with $R_f$ 0·2–0·6 was greater than 10 per cent with beginning, medium and long tubes, and germination over other $R_f$ areas was the same as that of the control; when control germination was zero, germination over *O. flexuosum* column material at $R_f$ 0·2–0·75 was from slight to more than 10 per cent, with many medium and long tubes, and germination over column material at other $R_f$ areas was zero; both control and experimental germination over column material of *Brassavola subulifolia* were from zero to slight and no conclusion could be drawn from the data. *Oncidium guttatum* pollen germination of the control was zero, while over column material of *O. guttatum* at $R_f$ 0·4–0·55 germination was greater than 10 per cent with mainly medium tubes, and over $R_f$ 0·55–0·7 was slight, with short and mostly abortive tubes. Pollen of *Rodriguezia venusta* (*fragrans*) control germination was from zero to very few short tubes, while above *O. flexuosum* column material at $R_f$ 0·5–0·75 germination was greater than 10 per cent, with many medium and long tubes and less than 10 per cent germination, with short tubes at $R_f$'s 0·2–0·5 and 0·75–1·0. Pollen of *O. ansiferum* control germination was less than 1 per cent while it was about 4 per cent above *O. ansiferum* column material at $R_f$ 0·4–1·0.

These results pointed quite definitely to the presence of material in the columns of *Oncidium* species capable of stimulating pollen germination in related species. The $R_f$ position of the active material(s) appeared to be roughly in the area 0·2–0·7. To test this possibility further, large numbers of columns, without wings, pollinia or anthers, were ground and extracted with ether, the filtrates were then chromatographed, the chromatograms eluted sectionally, and the eluants incorporated in basal agar medium for pollen planting. Results of such a test are shown in Table 2.

TABLE 2. RELATIONSHIP BETWEEN POLLEN GERMINATION AND $R_f$ POSITION OF ELUANTS FROM CHROMATOGRAPHED *Oncidium ansiferum* COLUMNS

| Pollen type | $R_f$ range of eluant and incubation period (days) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0·0–0·1* | | 0·1–0·3 | | 0·3–0·5 | | 0·5–0·6 | | 0·6–0·8 | | 0·8–1·0 | |
| | 7 | 10 | 7 | 10 | 7 | 10 | 7 | 10 | 7 | 10 | 7 | 10 |
| | Percentage germination† | | | | | | | | | | | |
| *Oncidium flexuosum* (24 samples) | 17 | — | 15 | 14 | 23 | 84 | 8 | 34 | 3 | 3 | 0·5 | 4 |
| *Oncidium parviflorum* (7 samples) | 3 | — | 0 | — | 42 | — | 0 | — | 0 | — | 0 | 0 |
| *Laelia gouldiana* (20 samples) | 11 | — | 11 | 24 | 40 | — | 33 | 25 | 9 | 20 | 11 | 26 |

* 0·0–0·1 $R_f$ position, extending from the base of the paper just through the sample position, may be considered a valid control.

† Percentage germination was obtained by dividing the number of germinating pollen cells by the number of tetrads observed in at least five scattered fields per sample.

Results indicated clear pollen germination stimulation from *Oncidium ansiferum* column material at the $R_f$ position 0·3–0·5. Two attempts were made to chemically characterize the stimulating material of the *Oncidium* alliance. In one test, ether extracts from the columns of *O. sarcodes* were partitioned to separate acid and basic materials. Results of this test are shown in Fig. 1. In the other test, water extracts of *Rodriguezia secunda* were adjusted to

different pH's and partitioned into ether in order to accumulate water and ether-soluble, neutral, acid and basic materials. These results are shown in Fig. 2.

From the results of ether–water partitioning, it is concluded that the pollen germination stimulating material is more water- than ether-soluble and is neutral. Treatment of the culture medium precludes the possibility that the stimulator is very heat labile. As the amounts of material separated by chromatography were extremely small and subsequent dilution by the

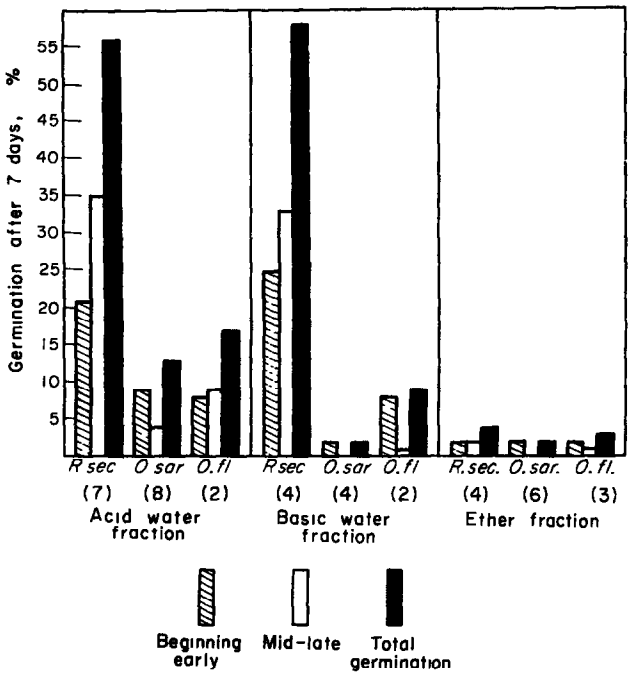


FIG. 1. POLLEN GERMINATION ON ETHER EXTRACTS OF COLUMNS OF *Oncidium sarcodes* CHROMATOGRAPHED TO $R_f$ 0·35–0·55.

Acid water fraction—partitioned from ether into pH 8·05 solution. Basic water fraction—into pH 5·05 solution. Ether fraction—the remaining ether extract.

Percentage germination was obtained by dividing the number of germinating cells by the number of tetrads observed in at least 5 scattered fields per sample. (Sample numbers are indicated by the figures below the species abbreviations.)

*R. sec.—Rodriguezia secunda; O. sar.—Oncidium sarcodes; O. fl.—Oncidium flexuosum.* Pollen incubated 7 days.

agar medium was considerable, it is improbable that the action of the material is due to osmotic effect or to nutritionary supplement. It appears, rather, that the stimulator has growth-substance-like activity. The water solubility and neutrality of the material remind one of the calcium ion stimulating effects reported by Brewbaker and Kwack.[4] This possibility seems, however, unlikely because the basal medium used contained approximately the optimum concentration of calcium nitrate reported by these workers. (Reported optimum, 288 ppm; present concentration, 280 ppm.) A more recent paper of Mascarenhas and Machlis,[7] however, reports a much higher optimum (about 400 ppm) of calcium for pollen of *Antir-*

[7] J. P. MASCARENHAS and L. MACHLIS, *Plant Physiol.* **39**, 70 (1964).

*rhinum majus*. The possibility of great sensitivity of orchid pollen to calcium and the identity of the presently reported stimulator with calcium are thus not precluded.

Stimulation to *Laelia gouldiana* pollen by column material from *Oncidium ansiferum*, together with some individual tests not reported here, suggest that the stimulator is not specific, although it is possible that it is particularly active within the *Oncidium* alliance.

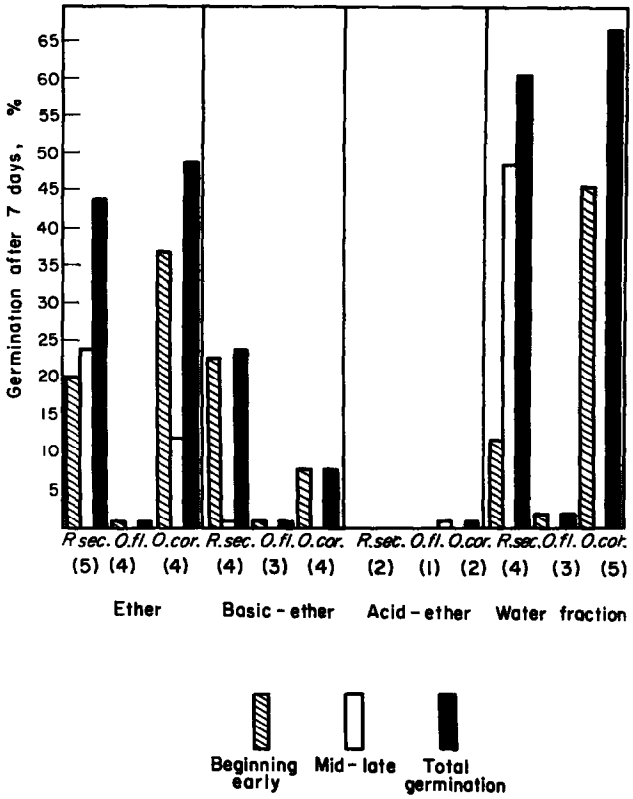


FIG. 2. POLLEN GERMINATION ON WATER EXTRACTS OF COLUMNS OF *Rodriguezia secunda* CHROMATOGRAPHED TO $R_f$ 0·35–0·55.

Ether fraction—partitioned from pH 7·0 water into ether. Basic ether—from pH 8·3 water into ether. Acid ether—from pH 5·5 water into ether. Water fraction—the remaining water adjusted to pH 7·0.

Percentage germination was obtained by dividing the number of germinating cells by the number of tetrads observed in at least 5 scattered fields per sample. (Sample numbers are indicated by the figures below the species abbreviations.)

*R. sec.—Rodriguezia secunda; O. fl.—Oncidium flexuosum; O. Cor.—Oncidium cornigerum.* Pollen incubated 7 days.

## EXPERIMENTAL

The basal medium used varied from that of Tulecke[5] in that 10 g sucrose and 18 g agar were added per litre and the ferric citrate was replaced by an equivalency of available iron provided by iron chelate (Linck, New Jersey, U.S.A.). The pH was adjusted to 5·4. Autoclaving was for 15 min at 15 lb/in$^2$. Although orchid pollinia were most often found to be aseptic on removal, occasional fungal contamination made sterilization necessary. Calcium hypochlorite (1 teaspoon per 55 ml water, filtered and used at once) was found effective and not greatly damaging providing the pollinia were not dipped into the solution for more than 1 sec

and were rinsed at once in sterile distilled water. Whole pairs of pollinia were placed on the agar surface in either petri dishes or small test-tubes. Planting was done in a sterile transfer box. Incubation was in bright, diffuse light in the greenhouse, where temperatures varied considerably with the season. Temperature variation probably accounts for some of the variation in germination from test to test.

The chromatographic solvent was freshly made, n-butanol: 36% ammonium hydroxide: water; 5:1:4 (top layer). Strips of Whatman No. 1 filter paper, 2 cm wide by 3 cm at the base for a length of 2 cm, followed by a constriction to 0·5 cm for 2 cm followed by the main strip of 2 cm width for a length of 15–25 cm were used. In cases where orchid columns rather than column extracts were chromatographed, the columns were crushed directly onto the sample area of the paper strips, following, in general, the method of Riley and Bryant.[8] Development was ascending for 12–15 hr.

Sections of chromatograms were eluted by extraction at 4–6° for 30–72 hr with ether. All evaporations were at room temperature (about 24°) under a moving current of air. Residues of eluants were taken up in hot agar medium and autoclaved. For direct pollen planting above chromatogram squares, 1 to 3 stigmas were used for each chromatogram. For the test of *Oncidium ansiferum* columns, 29 columns were ground in ether, the extract was then chromatographed, eluted and incorporated in 2 ml of basal medium per 2 cm of the chromatogram. Columns of 156 *O. sarcodes* flowers were used in the test reported in Fig. 1. The ether extract was partitioned by shaking three times with equal volumes of water. The residues were taken up in ether, chromatographed, eluted and each taken up in 4 ml of basal agar medium. Two hundred and forty-three columns of *Rodriguezia secunda* were used in the test reported in Fig. 2. The columns were ground in cold water, filtered under reduced pressure and partitioned by shaking three times with equal volumes of ether. Final residues eluted from the chromatograph strips were taken up in 6 ml basal agar medium. The pH adjustments were made with sodium bicarbonate and N HCl.

*Acknowledgement*—The authors wish to thank D. Dirmikis for technical assistance in this work and H. A. Dunn and Alvim Seidel for some of the plant materials used.

[8] H. P. RILEY and T. R. BRYANT, *Am. J. Botany* **48**, 133 (1961).