A STUDY OF CHEMOTROPISM OF POLLEN TUBES *IN VITRO*

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(WITH THREE FIGURES)

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Introduction

Although much has been added to our knowledge concerning the requirements for pollen germination and pollen tube growth in recent years (**1, 2, 4, 10, 17, 18**), very little progress has been made in studying chemotropism of pollen tubes. Literature dealing with this subject prior to 1924 has been reviewed by BRINK (**3**). There is an obvious lack of agreement among the different workers as to: (a) whether chemotropism of pollen tubes to pistil parts exists at all; and (b) if so, the role that chemotropism plays in directing the pollen tubes toward the egg. The present investigation is an attempt to further our knowledge of this process.

Methods

The plant materials used during the course of this investigation came chiefly from the greenhouses of the Botany Department and the garden of the Horticulture Department of the University of Wisconsin. An attempt was made to use as many plant species as were available and had been reported in the literature to show positive pollen tube chemotropism, with a view to confirming and testing the earlier results. Of these plants, *Hippeastrum Johnsoni,* a hybrid, was found to be the most favorable material for the study of chemotropic response because of its large stigma and pollen grains. Its use, however, was limited by its restricted flowering season.

The medium found most suitable for culturing pollen tubes of most plants contained 1% agar, 10% sucrose and yeast extract at a concentration of 100 p.p.m. Yeast extract was used since, according to BRINK (**3**), it decidedly accelerated pollen germination and promoted pollen tube growth.

While still hot, the agar medium was poured into a Petri dish to form a layer about 4 mm. thick. Pistil parts including whole ovules and sliced stigma, style, placenta, and ovary wall were imbedded near the surface when the agar started to gel. Pollen grains were spread with a camel's hair brush around the pistil slices after the medium had hardened. For low power examination the Petri dishes were inverted; cover slips were placed directly on the agar plate for high power examination.

Pollen grains of most species germinate within one to two hours, and since observations were usually completed within two hours, no special

[1] This work was done at the University of Wisconsin, where the author was a University Fellow in the Department of Botany.

sterilization procedures were employed. All experiments were carried out at room temperature which did not vary more than two degrees from 25° C.

The preliminary investigation consisted of an attempt to determine whether chemotropism could be demonstrated in vitro. After this phenomenon was demonstrated, two methods of experimentation were employed. One involved determining the nature of the active factor which is present in the pistil parts; the other was an attempt to duplicate the effect of the natural tissue with known substances.

The technique found most suitable for the application of known substances involved preparation of agar containing known concentrations of the substance used. Two-millimeter cubes were cut from this solidified agar. These cubes were placed on the surface of the culture plates in the same fashion as the pistil parts. By this method, a number of substances in varying concentrations were tried. These included sucrose, White's medium containing inorganic salts (**21**), indole-3-acetic acid, organic acids, amino acids, proteins, and various other nitrogen-containing substances. Some of these substances had been reported to have pronounced effect on the stimulation of pollen tube growth. Some are believed to be effective in inducing chemotropism of pollen tubes, while others are essential in plant metabolism.

Various attempts were made to isolate the active substance or substances from the pistil parts. These included extraction with water, ether, and 95% alcohol. The tissues were also placed on agar blocks at different pH levels, with the idea that the active substance might diffuse out into the agar. The agar blocks were then tested for activity.

In order to determine some of the properties of the active factor, the active tissues were subjected to various treatments before being used. To study the thermo-stability of the active factor, the tissues were heated at 100° C. To get some idea of the molecular size of the substance, pieces of the tissues were wrapped in commercial cellophane membranes, and then imbedded in the agar of the test plates. Activity was also determined after other pieces of the tissues had been hydrolyzed with 5% HCl and then neutralized with NaOH.

Results

The occurrence of chemotropism

Among the 36 species of plants tested (see table I and text below), only a small number showed pollen tube chemotropism under the specified experimental conditions. Those which showed positive results are listed in table I. In no case was chemotropism shown to pistil parts of flowers of other species. For example, pollen tubes of Hippeastrum were indifferent to the stigma of *Vinca major,* and pollen tubes of *Vinca major* were indifferent to the stigma of Hippeastrum. Pollen tubes of *Primula obconica* showed no re-

sponse to the stigma of Antirrhinum, nor did the tubes of Antirrhinum respond to the stigma of Primula. Pieces of young embryo, seed coat, sepal, petal and leaf of *Antirrhinum majus* (2N) did not attract pollen tubes of that species.

There is a maximum effective distance beyond which the influence of the active tissue does not extend. Beyond this distance, the pollen tubes grow at random. This is shown in figures 1 and 2. The effective distance in *Antirrhinum majus* and Hippeastrum was measured and was found to be 1 – 1.5 mm. This is in agreement with observations by MOLISCH (**13**) in *Narcissus Tazetta*. The agreement of the measurements of the effective distances presumably indicates similar diffusion patterns and therefore may mean the effective substances in the two cases have approximately the same molecular weight.

If, while still growing, the tubes were turned gently with a camel's

TABLE I

PLANTS IN WHICH POLLEN TUBE CHEMOTROPISM OCCURRED

PLANTS	TISSUES THAT ATTRACTED POLLEN TUBES
Hippeastrum Johnsoni	Stigma only
Hippeastrum puniceum	Stigma only
Narcissus Tazetta	Stigma, ovule and placenta
Narcissus poeticus	Stigma, ovule and placenta
Antirrhinum majus (2N)	Ovule and placenta always; stigma and cut end of style under certain conditions
Antirrhinum majus (4N)	Ovule and placenta, other parts uncertain
Lilium superbum	Stigma, cut end of style, placenta and ovule
Paeonia sp.	Ovule and stigma
Hemerocallis fulva	Stigma, placenta and ovule; not shown late in the season
Hemerocallis sp.	Stigma, placenta and ovule; not shown late in the season

hair brush, they would again grow toward the source of the chemotropic stimulus.

Early in the investigation, it was found that the pollen tubes of *Antirrhinum majus* showed chemotropic response only to ovules and placentae. However, later in the season chemotropic response both to the stigma and to the cut end of the style was occasionally observed. Since the medium used was the same and the laboratory conditions did not change appreciably, some change in the plant itself is suggested. Since these plants were growing out of doors, possible causal factors are the age of the plants, day length, and temperature, singly or in combination.

A study of the effect of age of Antirrhinum flowers was made. Pollen grains obtained in July from freshly dehisced anthers were sown around the imbedded pistil parts of flowers and flower buds of different ages. The presence or absence of attraction is shown in table II. The placenta and the ovule attracted the pollen tubes at all stages of their development, while the stigma and style affected the pollen tubes only if they came from very young buds or from mature flowers after anthesis. The age of the

flower, therefore, seems to influence the induction of chemotropic response in Antirrhinum, but it does not seem to be the only factor involved. That other factors are also involved is indicated in Hemerocallis (table I) where the stigma, placenta and ovule all strongly attracted the pollen tubes early

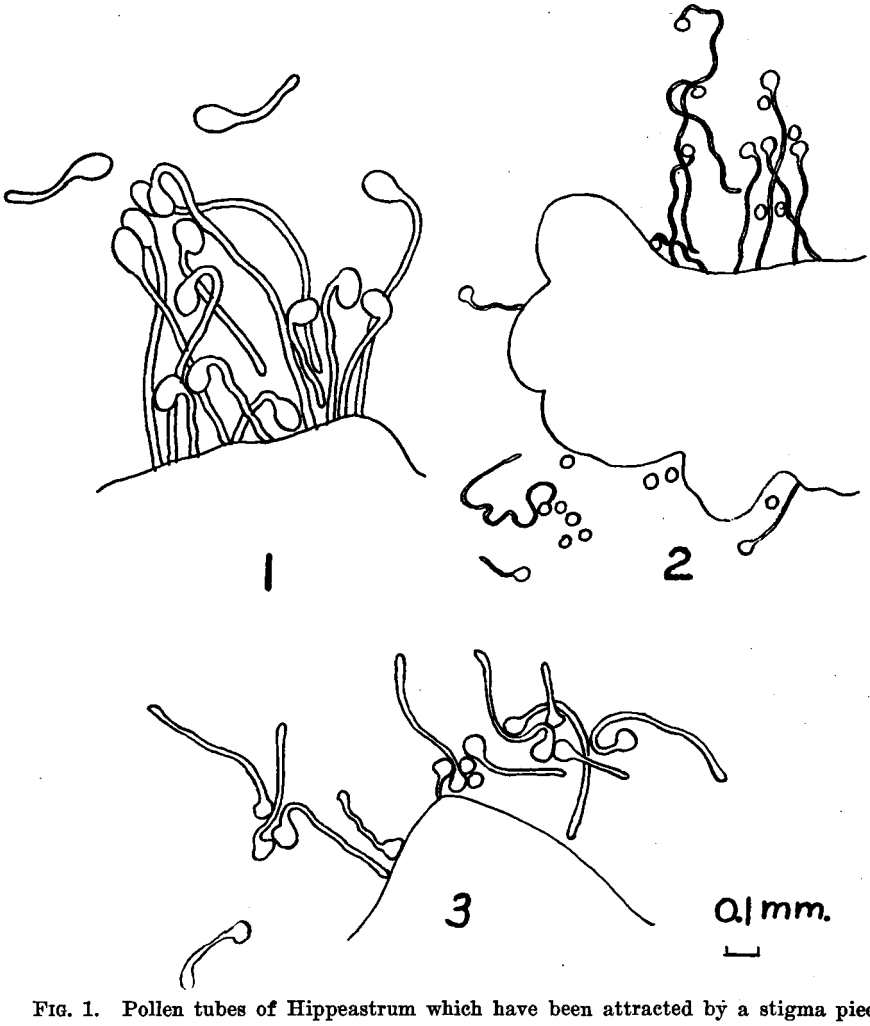

FIG. 1. Pollen tubes of Hippeastrum which have been attracted by a stigma piece of the same species. The maximum effective distance in this case is approximately 1.3 mm.

FIG. 2. Pollen tubes of *Antirrhinum majus* showing chemotropic response to a piece of placental tissue of the same species. The maximum distance is at least 0.7 mm.

FIG. 3. Pollen tubes of Petunia hybrida growing in all directions regardless of the imbedded stigmatic tissue.

All drawn with camera lucida to the same scale.

in the season, but not after the beginning of July. The season for this seasonal change remains to be discovered.

In most of the plants investigated, pollen grains grown on the artificial

medium in the vicinity of imbedded pistil parts sent out their tubes in all directions quite regardless of the presence of the imbedded tissue (figure 3). This was true with the following species: *Primula malacoides, Primula kewensis, Petunia hybrida, Vinca major, Muscari botryoides, Scilla sp., Tradescantia paludosa, Pisum sativum, Tulipa Gesneriana var. Darwinia, Eucharis grandiflora, Lilium eximium* (only stigma was tried), *Papaver orientale, Oenothera biennis, Rudbeckia tricolor, Impatiens balsamina, Allamanda sp.*, and *Lupinus sp.* In all but one case, all of the various pistil parts were used. The pollen of the following plants did not germinate at all in the medium used: *Fuchsia sp., Pelargonium sp., Taraxacum officinale, Abutilon hybiscus, Senecio cruentus, Browallia speciosa major,* and *Dendrobium thyrsiflorum.*

TABLE II

THE EFFECT OF AGE OF FLOWERS ON THE INDUCTION OF CHEMOTROPISM IN *Antirrhinum majus.* + = PRESENCE 0 = ABSENCE + ? = DOUBTFUL

AGE (DAYS FROM ANTHESIS)	CHEMOTROPISM TO: STIGMA	STYLE	PLACENTA	OVULE
8 before	+	+	+	+
5 "	+	+	+	+
4.5 "	0	0	+	+
4 "	0	0	+	+
3 "	+?	0	+	+
2.5 "	0	+?	+	+
2 "	0	0	+	+
1.5 "	0	+	+	+
1 "	0	+?	+	+
1 after	+	+	+	+
2 after	+	+	+	+

TRIALS WITH KNOWN SUBSTANCES

A series of substances in a wide range of concentrations (0.01, 0.1, 1.0, 10.0, 100.0, 1000.0 p.p.m.) were tested for possible effects on the direction of pollen tube growth. Studies were made on *Lilium superbum* and *Hippeastrum Johnsoni* with water soluble vitamins and a number of nitrogen-containing substances. The results were negative. The experiment was repeated on *Antirrhinum majus* using 17 different substances (table III). The number of pollen tubes growing in various directions was counted and the results analyzed statistically.

In table III a plus sign indicates growth towards the substance, a minus sign growth away from it. Growth was called positive if the pollen tube pathway between pollen grain and test block was within the quadrant subtending the grain, negative if within the opposite quadrant, and neutral if within the lateral quadrants. Neutral figures are not presented in the table. All values are derived from five replicated samplings for each dilution of each substance.

It appears that in every case the numbers of pollen tubes growing in

the positive and negative directions are about the same. Application of the chi-square test showed that even in the extreme case (thiamin at 0.1 p.p.m.), the deviation from random is hardly significant.

It can be seen readily in figures 1 and 2 that, in every case of positive chemotropism induced by tissues, all the pollen tubes within the maximum effective distance grew in the positive direction. This further supports the statement that none of the substances, at any concentration tried, had a comparable effect.

To determine whether sugar might be involved in the chemotropism, pieces of Hippeastrum stigma were imbedded in plain agar, and adjacent

TABLE III

EFFECT OF SOME WATER SOLUBLE VITAMINS AND SOME PHYSIOLOGICALLY IMPORTANT NITROGENOUS SUBSTANCES ON THE DIRECTION OF POLLEN TUBE GROWTH OF *Antirrhinum majus*. FIGURES REPRESENT THE NUMBER OF TUBES GROWING IN EACH DIRECTION (SEE TEXT).

SUBSTANCES	CONCENTRATION (P.P.M.)											
	.01		.1		1		10		100		1000	
	+	−	+	−	+	−	+	−	+	−	+	−
Asparagine	19	18	21	26	22	16	18	14	18	22	15	12
Urea nitrate	21	19	15	19	18	14	15	13	16	12	20	20
Urea	19	22	20	17	21	16	14	18	17	19	19	15
Na ureate	18	16	17	18	17	12	20	15	15	17	18	23
Choline	14	17	19	15	12	18	15	15	25	20	22	18
Adenine	15	14	19	14	13	12	17	17	29	22	14	15
Guanine	14	17	18	16	16	15	19	15	17	13	18	11
Uracil	20	24	16	20	14	17	19	13	20	18	26	23
Barbituric acid	26	17	16	14	17	14	14	18	18	15	21	23
Na nucleate	14	15	25	20	15	8	18	13	21	14	23	12
Thiamin	21	28	24	12	17	17	16	11	13	18	.....	.....
Riboflavin	24	15	22	13	25	24	16	17	18	11	.....	.....
Pyridoxine	26	18	17	12	23	21	15	17	26	19	.....	.....
Niacin	17	18	27	21	30	24	23	11	12	13	.....	.....
Nicotinamide	16	20	14	18	22	12	14	19	16	11	.....	.....
Inositol	14	20	18	20	20	16	17	16	20	13	.....	.....
P-Aminobenzoic acid	22	13	23	19	18	18	17	16	13	21	.....	.....

to these pieces, blocks of agar were removed and replaced with equal size blocks containing 10% sucrose without stigma pieces. Hippeastrum pollen grains were sown along the line separating the sucrose-containing block from the agar containing the stigma. All the tubes grew to the stigma side, none to the sugar side. Of course this does not exclude the possibility that the stigma contains a higher concentration of sugar than 10%, but this is very unlikely. In the same way, the major and minor mineral elements for plant growth were found to be ineffective in inducing chemotropism, as were also yeast extract and casein. (The casein, which is only sparingly soluble in water, was tested by imbedding crystals directly in the agar.)

Organic acids and some of their salts were tried on pollen of Hippeas-

trum by PFEFFER'S (**15**) capillary method. Concentrations of 100 p.p.m. of succinic acid and its sodium salt, dl- and l-malic acids, fumaric, citric, lactic, pyruvic, glutaric, and glutamic acids were used. None of these showed any effect. Similar results were also obtained with the same plant material using an adaptation of the penicillin assaying cylinder method (**5**) and acids at a concentration of 200 p.p.m. Peptone, over a concentration range from 2% to 0.002% and indole-3-acetic acid at 10 p.p.m. did not have any effect on the direction of pollen tube growth of *Hippeastrum*.

ATTEMPTS TO ISOLATE THE ACTIVE FACTOR

Various methods were used in an attempt to isolate the active factor from the pistil tissue. Agar was made up at different acidities (pH 4.0, 6.9, 9.1) and allowed to solidify. Lobes of fresh stigma tissue of *Hippeastrum Johnsoni* (the stigma is tri-lobed) and masses of ovules of Antirrhinum were placed directly on the agar surface. Cubes of agar just large enough to hold the tissue were then cut out. These small cubes were put on a pad of moistened filter paper in a covered Petri dish. Diffusion into the agar was allowed to take place at room temperature for three and one half hours. The tissues were then removed and the agar blocks inverted on another culture plate containing solidified agar medium, so that the surface with which the tissues had been in direct contact touched the surface of the culture medium. Pollen grains were spread around the cubes in the usual manner. The results indicated no transfer of an active factor to the agar.

Positive results were finally obtained with *Lilium superbum* using 3% agar at pH 6.9 instead of the 2% as above and allowing diffusion to continue for five hours. The pollen tubes were markedly attracted by these cubes. Apparently part of an active factor existing in the tissue had diffused out into the agar blocks.

Water extractions were prepared in the following way. Fresh stigma tissue from five flowers of Hippeastrum was ground in a mortar with 2 ml. of distilled water and then filtered through filter paper. The filtrate was subjected to activity tests by several methods. In each test the filtrate failed to induce positive chemotropism. Similarly, the filtrate from ground ovules of eight flowers of Antirrhinum did not show any activity. The extraction was repeated by grinding the tissue with a little $CaCO_3$ to neutralize any acid which might have been produced from the tissue breakdown. The filtrate was still not active.

The active tissue was also ground with ethyl ether. Filter paper strips were soaked in the filtrate, the ether was allowed to evaporate completely, and the filter paper strips were used for the activity test. The pollen tubes were found to be indifferent to them. A trial with 95% ethyl alcohol extracts gave the same result. Although the filtrates were not active in any of the above cases, the mashed residues of the parts used remained active.

Stigmas of Hippeastrum still retained their activity in attracting the pollen tubes after being boiled in 50 ml. of distilled water for 10 minutes. Hydrolysis with 5% HCl for 10 minutes followed by neutralization with NaOH also failed to destroy their effectiveness. However, stigmas wrapped in a commercial cellophane membrane and imbedded in agar, completely failed to attract the tubes growing around them. This indicates that the active substance could not pass through the pores of the membranes. The exact porosity of the membrane was not determined, but these facts suggest that the molecular weight of the substances must be relatively high.

Discussion of results

The above observations substantiate earlier reports on chemotropism of pollen tubes. The failure to demonstrate this phenomenon under certain experimental conditions does not necessarily mean that chemotropism does not exist in nature, nor that it is without significance in directing the growth of pollen tubes to the embryo sac.

It is true that this phenomenon can be more consistently demonstrated in some species than in others. Pollen tube chemotropism of *Narcissus Tazetta,* previously reported by Molisch (**13**), Lidforss (**7**), and Tokugawa (**20**), was verified in the present investigation. Similarly, the present findings agree with those of previous authors in the case of Antirrhinum. On the other hand, the present work did not confirm Miyoshi's results on Scilla and Primula (**12**), nor Brink's work on Hippeastrum (**3**). These discrepancies are understandable when one considers, first, that the situation varies even within a species handled under identical laboratory conditions and with the same kind of medium as was demonstrated in this study; and second, that the occurrence of pollen tube chemotropism is affected by the concentration of the culture medium, as has been shown by Lidforss (**8**). It seems likely that there are some undetermined internal and external factors which regulate production of some unidentified substance which is active in the induction of chemotropism.

Despite the failure to identify the active factor, the above studies give some idea of its properties. It must be a slowly diffusing, heat stable, water soluble substance of considerable molecular size. It is rather resistant to acid hydrolysis. Since the stigmas of Hippeastrum still retained activity after being boiled 10 minutes in 50 ml. of water, the active substance must be effective in very small concentrations.

The comparable effect of sodium malate both on fern spermatozoids (**13**) and on the pollen tubes of Antirrhinum (**3**) suggests that the active substance may not be specific. This suggestion is supported by the observation of some investigators that pollen tubes show chemotropism to the stigmas and ovules of quite distantly related species and is further strengthened by the fact that the maximum distance observed by Molisch in *Narcissus Tazetta* (**13**) and by the present author in Hippeastrum and in Antirrhinum was approximately the same.

The mechanism of pollen tube chemotropism does not seem comparable to the chemotactic movement of lower organisms, such as bacteria and some unicellular algae, and of the spermatozoids of fern. In those cases, the whole cell moves, usually by means of cilia or flagella, toward the chemical stimulus. It is, however, more or less like the chemotropism of hyphae from germinating fungus spores (**11**). Knowledge of this phenomenon is also scant. Positive chemotropism of seedling stems and roots of higher plants has been reported (**6, 9, 14, 16**), but the mechanism involved may be even more complicated. Since, in the present case, we are dealing with a single cell rather than a multicellular organ, like stem and root, it would appear that the bending response of the pollen tube might occur as a result of unequal growth of the wall on two sides of the tube. In pollen tubes the wall is composed mainly of pectic substances. If the growth of this wall varied inversely with the concentration of the active substance, in other words, if the substance inhibited the extensibility of the wall material, directly or indirectely, the observed response would result.

The ineffectiveness of the water extract of the tissue described above may be explained by assuming that some interfering wound substance is released. It would be difficult to explain otherwise, since the effective substance is water soluble. The explanation for the inactivity of the residue from ground tissue is possibly due to the fact that the active factor is continuously formed from the tissue, as in the case of auxin.

That the substance involved in chemotropism is probably not nutritive in nature is seen from the experimental results which show that substances known to be essential for tube growth failed to induce chemotropism. Therefore, "chemotropism" is to be distinguished from "trophotropism," which, as defined by STRASBURGER (**19**), is the movement of an organism or an organ toward a food source.

Summary

1. Of 36 species of plants studied, nine showed positive pollen tube chemotropism to their respective pistil part or parts, 20 did not, and the pollen of the other seven species did not germinate at all. The medium contained 10% sucrose, 1% agar and 100 p.p.m. of yeast extract in distilled water.

2. The chemotropism of pollen tubes was shown to be related to the developmental stages of the flower in Antirrhinum. It also seemed to vary with some undetermined internal or external factors.

3. Of all the pure substances tried, including sucrose, the major and minor essential minerals, water soluble vitamins, indole-3-acetic acid, organic acids, peptone, casein and other nitrogen-containing substances, none showed any effect in attracting or repelling the pollen tubes of any species. Compressed yeast and yeast extract also failed to show any effect. Tissues other than pistil parts, such as young embryos, seed coats, sepals, petals

and leaves of *Antirrhinum majus* did not attract pollen tubes of the same species.

4. The active factor in the stigma of *Lilium superbum* was shown to diffuse out from the tissue into an agar block, and subsequently from the block into the culture medium to exert its influence.

5. The active factor in all cases studied was shown to be a slowly diffusing, heat stable, water soluble substance of considerable molecular size. It must be effective in very low concentrations.

6. As an explanation of the mechanism of pollen tube chemotropism, it is postulated that the active substance may directly or indirectly inhibit the extensibility of the wall material of the tube, thus controlling the direction of growth.

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