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GROWTH OF POLLEN TUBES IN VITRO AND THEIR REACTION ON POTENTIAL DIFFERENCES

BY

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I. CULTURE OF POLLEN IN VITRO

About a century ago the first attempts were made to germinate pollen grains in vitro. At present several methods have been developed which facilitate the germination of pollen grains and the growth of the pollen tubes emerging from them, in an artificial medium.

The moist chamber with the hanging drop, well known in microscopical technics, is now commonly used. The pollen grains and the tubes produced here can thus be continuously examined. Instead of a moist chamber for one drop of culture solution, a petri dish in which more drops hang down from the cover is used, so as to observe a series of cultures under the same circumstances.

Pollen tubes should grow many centimeters, for which they need more space than there is in one drop of culture solution, so we successfully used culture tubes containing 4 ml sterilized medium plugged with non-absorbing cotton, well known in microbiological technics. In this way not only sterile or semi-sterile cultures can be obtained, and, besides, the large volume of culture medium is a more stable medium than a little drop.

Pollen grains of many species of plants germinate in pure water but those of other species are even damaged in it. Moreover, when pollen grains are surrounded by a liquid medium they lack oxygen, which is necessary for the intensive respiration during the germination period (VAN TIEGHEM 1869, OKUMUKI 1932, LINSKENS 1955). Therefore, agar is usually added to the culture medium; so the pollen grains are sown on a surface. The addition of gelatine has been abandoned, and silica gel (JOHNSON 1943) and calciumpectate gel, as we established in preliminary investigations, did not give satisfactory results. In most cases 1 to 2 % of agar added to the medium is sufficient. It is necessary to wash out the agar thoroughly with distilled water before mixing it with the culture solution, otherwise the germination of the pollen grains will be severely influenced.

Many investigations have shown a favourable effect of carbohydrates on the germination of pollen grains as well as on the growth of the pollen tube. These substances partly function as nutrient and partly they stabilize the osmotic properties of the medium.

The sucrose commonly used may be replaced by one of a few other carbohydrates as glucose (MIYOSHI 1894 b), sometimes fructose (BURCK 1900, BAIR 1941) and even lactose (BISHOP 1949). The optimum concentration of the sugar in the medium must be determined for each species of pollen, but it has for every species a wide range. Often there is an optimum at about 10 % sucrose.

The germination of pollen is very sensible for the pH of the medium. There is an optimum at about pH 5, but individual differences occur. The maximum percentage of germination is not always obtained at the same pH as that at which the pollen tubes reach their greatest length (VOM BERG 1930). The addition to the culture medium of organic acids such as 0.01 % malic acid or citric acid had some favourable effect especially with the pollen of *Ericaceae* (MOLISCH 1893, LIDFORSS 1896, JOST 1907, KÜHLWEIN 1937).

A favourable effect was shown by addition of 0.01 % to 0.001 % of boric acid as well on the germination as on the growth of the pollen tubes of *Nymphaeaceae*, *Liliaceae* and *Amaryllidaceae* (SCHMUCKER 1933, 1935, EHLERS 1951). It is probable, however, that the function of boric acid is not only to bring about the acid reaction, but there may also be an influence as a trace element, for the effect of boron on the pollen tube growth seems to be dependent on the boron contents of the plant (VISSER 1955).

Other trace elements Mn and Zn are reported to have some beneficial effects (HUANG 1948). Most salts, however, proved to be injurious to the pollen tube growth except perhaps calcium salts at 5 to 25 milliaequivalents (OKUMUKI 1932, BUNGENBERG 1934).

Pollen tubes are grown at temperatures which range from 20° C to 30° C with an optimum for most plants at about 25° C. Usually temperatures under 15° C and over 35° C have growth-retarding effects. There is some ecological adaptation, so pollen grains of tropical plants need more heat than plants from temporate regions, just as waterplants possess more water-resistant pollen than desert plants.

II. THE INFLUENCES ON THE DIRECTION OF POLLEN TUBE GROWTH

One of the problems in the pollen tube growth was, how did the pollen tube find its way into the stylar canal and to the ovules in the ovary.

An attracting influence of the stigma on the growing pollen tube in vitro has been indicated in the case of several plants such as *Primula* (CORRENS 1889), *Amaryllis*, *Clivia* (MOLISCH 1893) and *Narcissus* (BRINK 1924). Detailed research on 36 species of plants showed attractivity of the stigma on the pollen tubes of 10 species, among which *Hippeastrum*, *Lilium*, *Narcissus* and *Antirrhinum*. The style and the stigma attracted the pollen tubes only in young and mature condition whereas the placenta and the ovules did so in every case, but the distance was never more than 1 to 1.5 mm (TSUNG 1949).

Capillaries filled with sucrose are said to attract pollen tubes of *Digitalis* and *Oenothera* at a distance of about 1 mm (MIYOSHI 1894 b), but this chemotropism is strongly influenced by the culture medium

(LIDFORSS 1909). Other chemical substances failed to attract the pollen tubes (TSUNG 1949).

Effects of gravitation and influence of light on the direction of the pollen tube growth could not be established (KNY 1881, MIYOSHI 1894 a).

The influence of potential differences upon the growth of the pollen tube is not at all clear yet.

The pollen tubes of *Impatiens* grew to the anode when sown on agar between two platinum electrodes with a direct current of about 0.03 mA (WULFF 1935). We calculated that there must be a potential difference of about 1.3 volts per cm. The length of the pollen tubes was less than 1 mm, whereas about 4 mm is reached in vitro (EHLERS 1951).

Contrary to this the pollen tubes of *Vinca rosea* grow for 80 % in the direction of the cathode at a direct current of 0.55 mA per mm^2 (MARSH AND BEAMS 1945). The potential difference was perhaps more than 10 volts per cm and in this case the pollen tubes reached only a length of 0.1 to 0.5 mm, whereas in vitro they usually grow up to 10 mm (BOBILIOFF-PREISER 1917).

If pollen tubes react on potential differences one would expect that potential differences should be shown in the style of the flowers.

Between several parts of a plant potential differences even up to 200 millivolts have been established. They are attributed to diffusion processes in the cells or to oxido-reduction systems acting in the tissues.

The potential differences measured depend on the electrodes used, hence the difficulty of definitively establishing the value of such potential differences (UMRATH 1928). These measurements are further complicated by electrical effects occurring at the surface of wounds by which the injured place becomes negative with respect to the uninjured part. The maximum difference is reached a few minutes after the injury took place, and then it gradually fades away (KÜMMEL 1930).

The style of *Narcissus* and *Primula* has a preferential conductivity. When a current of about 0.0003 mA was run form the style to the ovary the conductivity was greater than when the current was run in the opposite direction (GUHA 1927). So the resistance for a current running in the direction from the ovary to the stigma is greater than when conducted in the opposite direction and from this we expect that there may exist a potential difference in which the stigma is positive and the ovary negative. If this is so, the pollen tubes under natural circumstances grow to the negative part of the carpel.

Thus we were interested to know what potential differences could be shown in the styles of the flower and how the pollen tubes reacted upon potential differences in vitro.

III. PLANT MATERIAL AND METHODS USED IN OUR INVESTIGATIONS

The plant material we used, *Narcissus Pseudonarcissus* L. var. *Golden Harvest*,[1]) was available throughout the year. There was little difference

[1]) Obtained from the Laboratorium voor Bloembollenonderzoek at Lisse, by kind permission of Prof. Dr. E. van Slogteren.

between pollen from forced plants and normal pollen (viz. NOHARA 1924). The pollen was gathered as soon as possible after the opening of the flowers and stored over calciumchlorid in the dark at room temperature. We used the pollen for about a month, during which time no decrease of vitality of the pollen tube growth was observed. The length of the style was about 60 mm.

The culture medium consisted of 100 ml bidistilled water, 2 g washed agar, 10 g sucrose and 10 mg boric acid puriss. The agar was obtained from agar strips which were placed in distilled water for weeks and finally in bidistilled water. We refreshed the water almost daily. The washed agar was melted on a waterbath and filtered through glasswool before use. The culture medium had a pH 5.

Each culture tube contained 4 ml medium sterilized on a waterbath for 10 minutes and then placed in a sloping position.

Inocculations of the pollen on the agar medium, as well as manipulations with electrodes were performed in as sterile a way as possible by the usual microbiological methods. The cultures were placed in the dark at 21° C and examined after about 24 hours.

For the cultivation of pollen tubes under influence of a potential difference platinum electrodes were used, 5 mm wide and 5 or 10 mm long, connected with a platinum wire of 20 cm length. The electrodes were placed in the culture tubes at a distance of 30 mm before the agar was cooled (Fig. 1).

Preliminary observations on the pollen tubes were made by means of a magnifying glass but afterwards microscopic preparations were made in the following way. The whole agar medium was pulled out

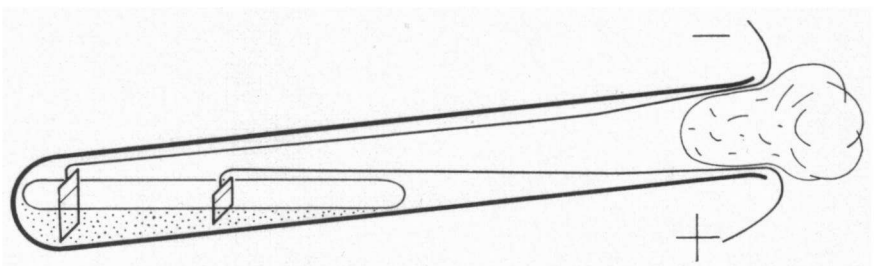

Fig. 1. Culture tube with agar medium and platinum electrodes.

of the culture tube by means of a hook with a flat end. The pollen tubes were then fixed and stained with a few drops of iodine tincture. When necessary the agar medium was cut into pieces and by means of a few drops of glycerine a cover glass was attached.

Potential differences in the flowers were measured by means of an Electrofact potentiometer, from which millivolts can be read directly within a few seconds. The electrodes used in this case were platinum needles of 0.25 mm diameter.

IV. Experiments on some factors which direct pollen tubes in their growth

a) The influence of sugar

In the sugar containing medium the pollen tubes of *Narcissus Pseudonarcissus* grow radially from the place where the pollen grains were inoculated. The length of such tubes normally ranged from 5 to 7 mm. Pollen grains sown on an agar medium containing no sugar produce pollen tubes that do not even reach half those lengths. When some grains of sugar are placed on a sugar-free medium at a distance of about 5 mm from the pollen grains, then the pollen tubes grow in all directions in the agar medium but in the direction of the sugar they are about twice as long as in the other directions.

So we could not show the existence of a real chemotropism, only an acceleration of the growth of the pollen tubes caused by sugar. This explanation is not quite the same as that suggested by Miyoshi (1894 b) and Lidforss (1909).

b) The influence of gravitation

It was observed in our cultures that the pollen tubes grew in all directions from a group of grains; they did not only grow along the surface but sought their way into the agar, too. So it was obvious that gravitation had little influence on the direction of pollen tube growth. Further, we could not establish any difference in the growth of pollen tubes, whether the culture tubes with the agar surface were placed horizontally or vertically, in both cases the pollen tubes grew radially on and in the agar medium.

Just like Kny (1881), Miyoshi (1894 a) and some others, we therefore conclude that gravitation exerts no influence on the growth of the pollen tubes.

c) Potential differences in the style

We supposed that potential differences in the style could be a guiding factor in pollen tube growth. Therefore we first investigated whether potential differences were present in the flowers of *Narcissus Pseudonarcissus*. Two platinum needles connected with the Electrofact potentiometer were introduced into parts of the flower under investigation and the potential difference read within a few seconds.

TABLE I

Potential differences in the flowers of *Narcissus Pseudonarcissus* L. var. *Golden Harvest*. Electrofact potentiometer. Electrodes: platinum needles. Three parallel measurements.

Potential difference between the top of the ovary and the corona	Potential difference between the top of the ovary and the stigma
20 millivolts	120 millivolts
70 „	120 „
10 „	120 „
Average 33 millivolts	Average 120 millivolts

The ovary proved to be negative both with regard to the pedicel and as regards the stigma and the style. There was, further, a small potential difference between the top of the ovary and the corona while a greater one existed between the top of the ovary and the stigma (Table I). Thus it is obvious that potential differences may influence pollen tube growth in the style.

It is well known that pollen tubes can grow through pieces of the style when these are cut carefully from the flower and placed in a moist chamber (PFEFFER 1886, MIYOSHI 1894 a, JOST 1907, SCHOCH-BODMER 1932, STRAUB 1946, HAECKEL 1951). We were also able to establish this for the styles of *Narcissus* and *Gladiolus*.

We compared the potential differences in the style of some flowers with those of the same styles after isolating them. We found that a potential difference was left after the style had been cut (Table II).

TABLE II

Potential differences in the flowers of *Narcissus Pseudonarcissus* L. var. *Golden Harvest* compared with those in cut styles. Electrofact potentiometer. Electrodes: platinum needles. Five parallel observations.

Ovary - Corona	Ovary - Stigma	Cut Styles
20 millivolts	70 millivolts	10 millivolts
20 ,,	60 ,,	30 ,,
30 ,,	60 ,,	20 ,,
50 ,,	80 ,,	30 ,,
60 ,,	80 ,,	40 ,,
Average 36 millivolts	70 millivolts	26 millivolts

This is in agreement with the possibility that the pollen tubes in the style are influenced by potential differences. The pollen tubes of *Narcissus Pseudonarcissus* then grow to the cathodal side of the style with a small potential difference.

d) The influence of potential differences on pollen tube growth

We were interested to know whether pollen tubes are influenced in their growth in vitro by potential differences and in what way they would show their reaction. So we grew pollen tubes of *Narcissus* between two platinum electrodes placed in the agar medium culture tubes (Fig. 1). The potential differences applied ranged from 0.2 to more than 2 volts per cm. During the whole growing period the potential difference was maintained between the electrodes and examinations were made after about 20 hours.

From table III it may be seen that at a low potential difference pollen tubes do not react at all. They reach a length of about 5 to 7 mm, just like the blank tests and they grow in the same way radially in and on the agar medium.

In those cases in which a high potential difference was applied pollen tube growth was totally inhibited. Nevertheless some germination

occurs but the pollen tubes then produced are only a few times longer than the pollen grains.

At a potential difference of about 0.5 volts per cm the pollen grains were prevented to grow their tubes in the direction of the anode but they did produce their tubes rather straight in the direction of the cathode, the latter reached a length of 5 to 7 mm.

TABLE III

The influence of potential differences on the pollen tube growth. Pollen of *Narcissus Pseudonarcissus* L. var. *Golden Harvest*. Resistance of the culture medium 30 to 45 kΩ. Platinum electrodes at a distance of about 30 mm. Direct current from batteries. Avometer.

Potential differences applied in V per cm.	Current calculated in mA.	Results
0.2	0.015	Pollen tubes growing in all directions
0.4	0.03	„ „ „ „ „ „
0.4	0.05	Tubes only in the direction of the cathode
0.5	0.04	„ „ „ „ „ „ „ „
0.5	0.04	„ „ „ „ „ „ „ „
0.6	0.05	Short tubes in the direction of the cathode
0.6	0.09	„ „ „ „ „ „ „ „
0.7	0.09	Tubes totally inhibited; agar liquefies near cathode
1.76	0.15	„ „ „ „ „ „ „

The potential difference applied to obtain these results with *Narcissus* is about half that applied to *Impatiens* (WULFF 1935) while the direction of the pollen tubes is exactly the opposite one. Further, whereas the direction of the pollen tube growth of *Narcissus* was the same as in *Vinca* (MARSH AND BEAMS 1945), the required potential difference is much lower.

If all these results are trustworthy they suggest that there may be in various genera a specific difference in pollen tube growth with regard to potential differences.

SUMMARY

1. Pollen tube growth in vitro is accelerated by addition of sugar.
2. Gravitation has no influence whatever on the direction of growth of the pollen tubes.
3. In the flowers of *Narcissus Pseudonarcissus* L. var. *Golden Harvest* potential differences could be shown, in such a way that the ovary is negative both with respect to the pedicel and the stigma.
4. There is a small potential difference between the stigma and the top of the ovary.
5. Pollen tubes of *Narcissus* grow in vitro in the direction of the cathode and are inhibited in the direction of the anode at a potential difference of about 0.5 volt per cm.

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