THE USE OF RADIOACTIVE PHOSPHORUS IN DETERMINING POLLEN-TUBE PENETRATION IN TOBACCO

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(With Three Text-figures)

(*Received* 4 *January* 1955)

In a study of the mechanism causing sterility in amphidiploid *Nicotiana* hybrids it was necessary to obtain a measure of the extent of pollen-tube elongation within styles of plants having different genotypes. A gene transferred to *N. tabacum* from *N. setchellii* was observed to retard pollen-tube growth in the style, regardless of the type of pollen applied (Ar-Rushdi, 1953). Comparisons with normal material were effected in preliminary trials by microscopic examination of pollen-tube ends in the stylar tissues, by a modified version of the dissection technique described by Buchholz & Blakeslee (1927). The procedure was readily adaptable to the present material but was found to be very time-consuming, and its usefulness was restricted to a period of about 6 days following pollination. Accordingly, the author set out to develop a technique which would be as accurate and would be applicable to a large-scale study of styles over a longer period of time. This was accomplished by labelling pollen grains with radioactive phosphorus and observing pollen-tube development as revealed in autoradiographs. This paper records the results which indicate that the autoradiograph is a valuable tool in such investigations.

Radioactive phosphorus (^{32}P) was chosen in preference to other agents because it is inexpensive, readily available, and is quite suitable for autoradiography. In addition, it is favourable for this type of work from a safety standpoint owing to its relatively short half-life. The first attempts to incorporate it in pollen grains involved soaking and vacuum infiltration of the grains in solutions containing the radioisotope as described by Colwell (1951). This treatment failed to induce a sufficiently high level of radioactivity in the pollen. Colwell's method of placing excised inflorescences in nutrient solution containing ^{32}P was also found unsatisfactory for tobacco. Young flower buds abscissed prematurely and older buds, although remaining on the inflorescence, did not become tagged under the condition of the trials.

Procedure

Satisfactory labelling of pollen with radioactive phosphorus was accomplished by transferring mature plants of Red Russian tobacco from soil to culture solution after washing the root system continuously for 24–48 hr. The 4 l. crocks used contained 3 l. of Hoagland's solution in which the phosphorus was replaced by ^{32}P in the form of KH_2PO_4 or H_3PO_4. A pH of 5·5–6·0 was maintained by periodic adjustment. Flower buds in which the pollen mother cells were undergoing meiotic prophase were found to attain maximum labelling. Those which were at about the stage of first microspore mitosis did not produce pollen with adequate radioactivity. Buds at earlier developmental stages than either of the above abscissed a few days after treatment was initiated.

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Radioactivity could be detected in the floral parts about 52 hr. after radioactive phosphorus was added to the culture solution. After anther dehiscence radioactivity of the pollen was measured by means of a Geiger-Müller counter to determine whether a suitable level had been reached. Before and after each exposure of pollen the background count of a cover-slip was determined. The radioactive pollen (25–50 counted grains) was placed on the cover-slip using a small drop of lactophenol to keep it in place. Radioactivity was expressed as a function of the time required to complete 1024 counts. Counts per pollen grain per minute were determined for each sample by subtracting the average background value from the mean count of the sample and dividing by the number of pollen grains. To obtain autoradiographs of sufficient intensity after short exposure a level of 7 counts per pollen grain per minute was desirable. This was achieved by using a plant about 3 ft. tall with no side branches and with about six buds of the appropriate size. Such a plant required 10 mc. of ^{32}P to give the desired radioactivity in the pollen. The treated pollen was demonstrated to be functional by applying it to stigmas of normal flowers. Capsules containing viable seeds were produced.

Styles were pollinated using radioactive pollen and collected at daily intervals over a period of 2–8 days. They were washed, the stigmas removed and then squashed between microscope slides. The slides were separated and the style again washed, allowed to dry, then exposed to no-screen X-ray film. For pollen with a level of radioactivity of 7 counts per grain per minute the exposure time was 30 min. Where the sample count dropped to 3 or 4 a 2 hr. exposure was required.

Results

The objective of this investigation was to obtain a comparison of the results depicted by autoradiographs with those of direct microscopic observation of pollen-tube penetration within stylar tissues. Accordingly, styles containing labelled pollen tubes were excised at various intervals from 48 to 192 hr. after pollination. The extent of pollen-tube penetration was determined by microscopic examination. The cover-slips were removed and the same styles were dried and exposed to X-ray film. Measurements of pollen-tube elongation as revealed by the resulting autoradiographs were compared with those obtained by dissection and observation of the stylar core. These comparisons are graphically represented in Fig. 1, wherein each pair of replications gives the results for two flowers of a plant, both of which were pollinated and collected at the same time. Thus, discrepancies in column height within pairs represent the variation to be expected from flower to flower under uniform conditions of treatment. Measurements of tube penetration obtained by the autoradiograph and dissection methods were in close agreement in all instances. Of the fourteen styles compared in this way twelve were from plants having the tube-retarder gene as, in these, pollen-tube elongation could be followed for a longer period than the 72 hr. required for tubes to reach the ovules in normal plants. If radioactive fluid diffused or was otherwise transported down the style, such material should magnify the effect.

Observed differences in results obtained by the two methods may be influenced by various factors. Removal of the cover-slip and subsequent drying may lead to some expansion or shrinkage of the stylar core following the microscopic study. There is probably a difference also in the accuracy with which the longest tubes may be detected. In autoradiographs there may be too few of these to be resolved on the film. This would be particularly true in squashed styles where the cortex probably has the effect of scattering

the radiation. There may be a small amount of diffusion, although the use of plants with the tube-retarder locus indicates that it is not extensive. A disadvantage of the method is the lack of resolution of individual tubes in autoradiographs, presumably resulting from the excessive numbers of tubes within the style and the relatively high dispersion of β-particles emitted.

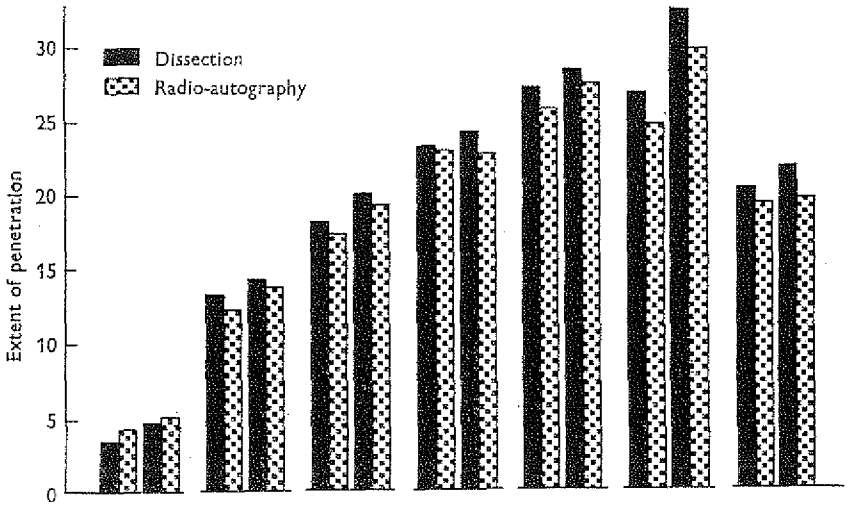

Fig. 1. Comparison of the results obtained by dissection and autoradiography. Diagrams depict extent of pollen-tube penetration in millimetres at various intervals after pollination. Each pair of replications presents the results for two flowers of the same plant pollinated and collected at the same times.

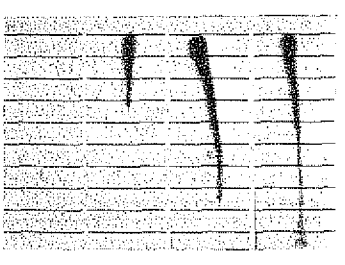

Fig. 2

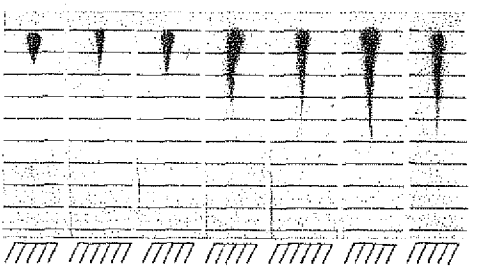

Fig. 3

Fig. 2. Autoradiographs of styles from fertile plants collected 24, 48 and 72 hr. after application of radioactive pollen in the greenhouse. Complete pollen-tube penetration after 72 hr. is indicated by radioactivity in the attached tip of the ovary. The control represents exposure of a style containing untreated pollen tubes.

Fig. 3. Autoradiographs of styles from *N. tabacum* plants having the introduced tube-retarder gene (greenhouse conditions). Mean lengths of styles are indicated by cross-hatched lines.

Fig. 2 presents autoradiographs of four styles of normal *N. tabacum* to show the extent of pollen-tube penetration at 24-hr. intervals following pollinations in the greenhouse. The control represents an exposure of a normal style containing non-radioactive pollen tubes. The fourth column shows that 72 hr. after pollination pollen tubes had entered the ovary. In Fig. 3 is depicted the extent of pollen-tube penetration in material in which the pollen tubes were prevented from reaching the ovules by the genic constitution of the maternal plant. Maximum penetration was achieved after a period of about 7 days.

Determination of the method of incorporation and localization of the radioactivity in the male gametophyte was beyond the scope of this investigation. Recent work of Taylor & Taylor (1953) suggests that a significant part of the radioisotope is present in the nuclear DNA, being incorporated during the synthesis of the nucleic acid. In their material this occurred during meiotic prophase and again prior to prophase of the microspore division. The earlier stage of pollen development was found to be appropriate for maximum labelling in the present study. Labelling at the time of microspore division resulted in a lower level of activity in the pollen which might have given comparable autoradiographs only by lengthening the time of exposure to X-ray film.

SUMMARY

A procedure is outlined for the use of autoradiographs in determining the extent of pollen-tube penetration in the styles of tobacco flowers. The results were in close agreement with those obtained by direct observation. Radioactive pollen was obtained by transferring mature plants to nutrient culture containing radioactive phosphorus. At intervals after pollination styles were collected and exposed to X-ray film, the resulting autoradiographs showing the location of pollen-tube ends in relation to the length of the style. Normal flowers, in which fertilization occurs about 72 hr. after pollination, were compared with those in which genetically controlled sterility is effected by inhibition of pollen-tube development.

The author wishes to thank Dr A. H. Gold for his supervision during the work. He also acknowledges the helpful suggestions of Prof. R. E. Clausen and the assistance of Dr D. R. Cameron in preparing the manuscript.

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