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Growth regulators in the pollen of pine (*Pinus silvestris* L.)*

L. MICHALSKI

INTRODUCTION

There is much evidence for the presence of growth regulators in flower pollen. Laibach (1932) defined them as "pollen hormones" owing to their similarity to substances stimulating growth. Now it is considered that Laibach's "pollen hormone" is identical with growth hormone. This is based on results of experiments reported by Thimann (1934), Yasuda (1934) and Gustafson (1937), who have found that there is a similarity between the action of "pollen hormones" and typical growth substances like indole-3-acetic acid and α-naphthalene acetic acid.

Later — qualitative examinations, e.g. of extracts from *Zea mays* pollen pointed to the possibility that, besides some substances of the auxin type, there may occur in them also other growth-promoting substances. Mitchell and Witehead (1941) using these extracts, obtained a more intensive growth of the internodes in bean seedlings, whereas the application of indole-3-acetic acid and other growth promoting substances of the auxin type, showed only a low stimulation. Jakuszkina (1947), experimenting with extracts from the pollen of hazel and pine, suggested also that in the pollen, besides auxins, also some other growth-promoting substances are present.

The experiments of Jakuszkina gave the start for investigating growth regulators in the pollen of tree plants continued by Larsen and Tung (1950), Anhaeusser (1953), Tanaka (1958), Michalski (1958, 1959, 1967). It was found that the pollen of trees contained, besides growth-stimulators also growth-inhibiting substances (Larsen and Tung 1950; Tanaka 1958; Michalski 1958, 1967). After Jakuszkina only Tanaka experimented with the pollen of pine and confirmed her findings with regard to the presence of inhibitors.

Some of the stimulating substances in pollen may have the character of gibberellin-like substances. They have been found in the pollen of oak and in some *Betulaceae* (Michalski 1967).

The aim of the present work is the analysis of growth substances of the auxin type and also gibberellin-like substances in extracts of pine pollen (*Pinus silvestris* L.).

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METHODS

Auxins

Auxins have been extracted from 25 g samples of air-dry pine pollen collected in the seasons of 1964—1966, by the method of double extraction in n-hexane and methanol. First the neutral substances and then acidic substances have been separated. The methanol extracts were fractioned by the method of Larsen (1955)

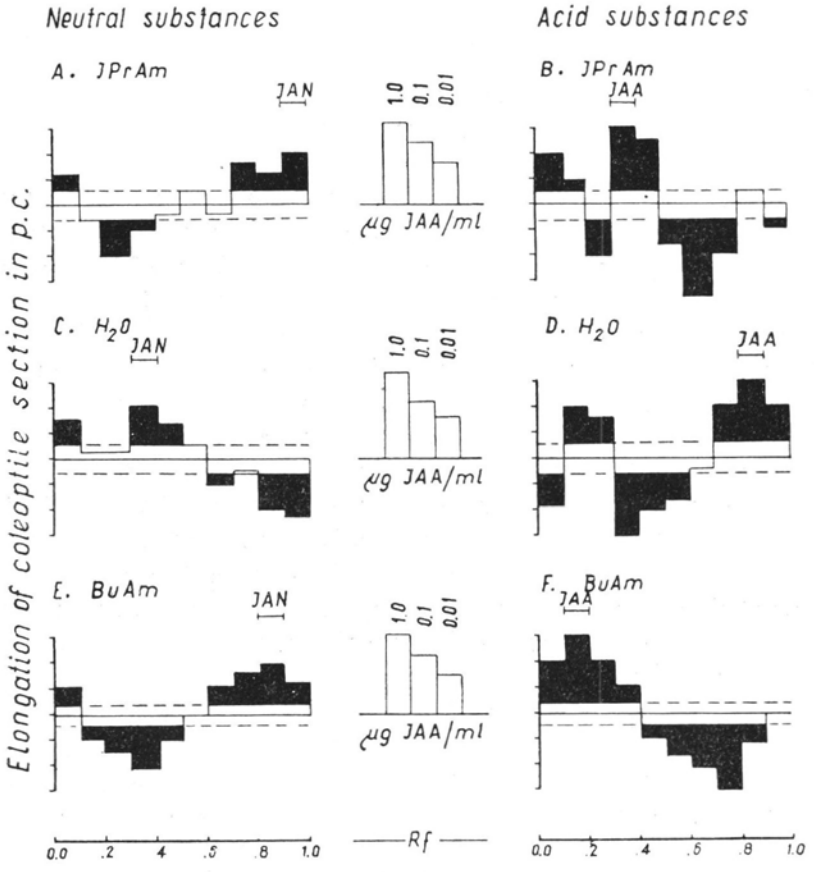

Fig. 1. Bioautography of auxins from the pollen of *Pinus silvestris* L., partitioned by paper chromatography in the solvent systems:

IPrAm — isopropanol-ammonia-water (10 : 1 : 1 v/v.); H_2O — water; BuAm — n-butanol-ammonia-water (100 : 3 : 18 v/v.)

and subsequently concentrated under reduced pressure to 1 ml. The concentrated extract was partitioned by paper chromatography on Whatman's No. 3. The TLC method — silica gel G — proved unsuitable for auxin assays. The chromatograms have been developed in three solvent systems:

isopropanol-ammonia-water (10 : 1 : 1 v/v.),
water,
n-butanol-ammonia-water (100 : 3 : 18 v/v.).

The physiological activity of the separated auxins was examined by the Avena coleoptile section test (Victory oats — Svalöf). For the chemical detection, Salkowski's and Prochazka's reagents have been used. The analytical procedure concerning auxins estimation has been described in previous papers (Michalski 1958, 1966).

The results of investigations were analyzed statistically and presented in the form of histograms (Fig. 1).

Gibberellin-like substances

The gibberellin-like substances were extracted from 25 g pollen samples with 70% aqueous acetone. After acetone evaporation the water residue was acidified to pH 2 and fractionated with ethyl acetate. The concentrated acetate fraction was first partitioned by two-dimensional paper chromatography with a water — water solvent, to remove the growth-inhibitors (Mowat 1963). The gibberellin-like substances were then separated by TLC method on silica gel G (Merck's prod.) with following solvent systems:

water,
benzene-acetic acid (10 : 3 v/v.),
0.1 M phosphate buffer pH 6.3 (gel impregnated with caprylic alcohol),
benzene-n-butanol-acetic acid (70 : 25 : 5 v/v.).

The physiological activity of gibberellin-like substances was examined by: the oat's first leaf test (Michniewicz 1961), the lettuce hypocotyl test (cult. Böttner) after Frankland's and Wareins's modified method (1960), the dwarf pea stem growth test (McComb and Carr 1958) and the barley endosperm test (Paleg 1961).

The results of the analysis are given in fig. 2.

RESULTS AND DISCUSSION

Auxins

In the pollen of pine some neutral substances have been found, extractable with hexane and some acidic ones which could be extracted with methanol.

Neutral substances. In chromatograms, developed with the solvent system isopropanol-ammonia-water (Fig. 1 Hist. A) the growth-stimulating substances appeared at R_f 0.7—1.0. The physiological activity of the „neutral" stimulators was unsignificant.

As shown by comparative examinations with standardized substances, and judging by data from the literature there have been detected: at R_f 0.86 indole-3-aldehyde (IH), at R_f 0.97 ethyl indole -3-acetate (IAE) and at R_f 0.99 indole-3-acetonitrile (IAN). The chromatographic characteristic of these substances in other solvent systems used would indicate the presence of one of these substances. In the case of development of chromatograms with water, the R_f values for IH, IAE and IAN

were limited to R_f 0.3—0.6 (Hist. C), whereas in the alkaline butanol solvent's system (Hist. E) they appeared at R_f 0.8—1.0.

A more exact identification of these substances was rather difficult because the detection of spots with Prochazka's as well as Salkowski's reagents did not result in colour reactions which are characteristic for these substances. It may be assumed that this is the consequence of a too small concentration of the partitioned substances. The amounts of extracts applied for chromatographic examination corresponded to an equivalent of only 5 g air-dry pollen, because the application of larger amounts, proved difficult for an exact chromatographic separation.

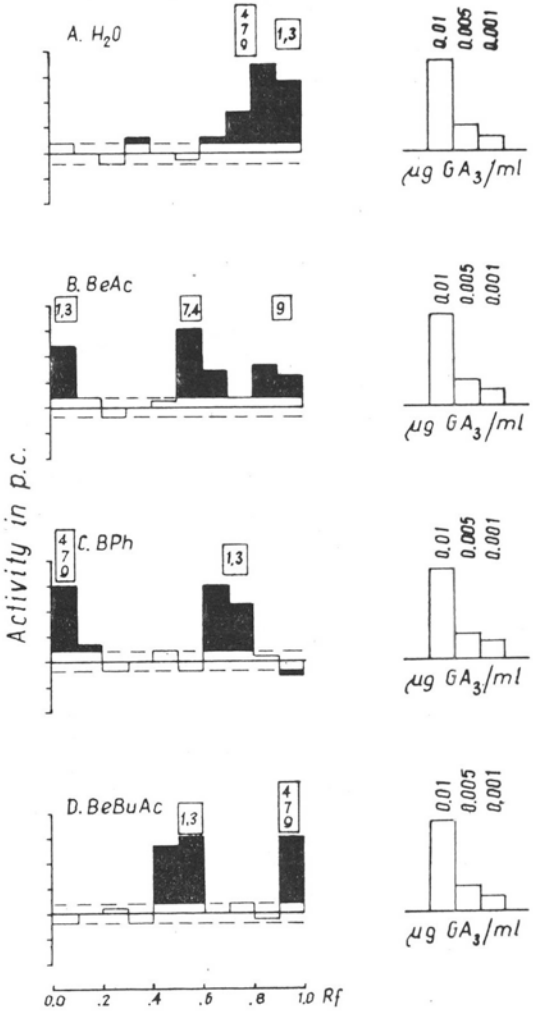

Fig. 2. Bioautography of gibberellin-like substances from the pollen of *Pinus silvestris* L., partitioned by the TLC method on silica gel G in the solvent systems:

H_2O — water; *BeAc* — benzene-acetic acid (10 : 3 v/v.); *BPh* — 0,1 M phosphate buffer pH 6.3 (gel impregnated with caprylic alcohol); *BeBuAc* — benzene-n-butanol-acetic acid (70 : 25 : 6 v/v.)

Among neutral substances in the pollen of pine appeared besides stimulators, also an inhibitor (R_f 0.2—0.3) which markedly decreased the growth of the test material. The eluate from the spot of chromatogram containing this inhibitor, applied together with indole-3-acetic acid of about 0.05 μg IAA/ml, decreased its stimulating activity in relation to the test material applied. The presence of an inhibitor together with neutral substances was observed independently of the solvent's systems used (Hist. C and E). It is supposed that the inhibitor, acting antagonistically in relation to IAA, may be the cause of difficulties in the analysis of the auxin-like compounds in the extracts of pine pollen. This refers especially to crude extracts. The chromatographic development of such extracts does not supply, in many cases, the possibility for an exact separation of the "neutral" inhibitor's from IAA. In the solvent systems used, the R_f values of the inhibitor are equal to those of the IAA R_f.

Acid substances. In the acidic fraction of methanol extract (Fig. 1 Hist. B, D and F), two growth stimulators have been found. The more active one has the chromatographic properties of indole-3-acetic acid (R_f 0.38). In the alakaline solvent system, consisting of isopropanol and ammonia (Hist. B) or butanol and ammonia (Hist. F), it showed the value of $R_f < 0.5$ whereas, when chromatographed in water (Hist. D) it was localized at $R_f > 0.5$. This feature is typical for growth substances of the character of acids (Guern 1959). It is probably IAA because it stimulates clearly the growth of the test plants, and the maximum of this stimulation in the various systems corresponds to the position of this compound. This stimulator loses its growth activity when tested together with a "neutral" inhibitor. The chemical detection of the spot of this stimulator with Prochazka's and Salkowski's reagent was unsuccessful until extracts of quite large amounts of pollen, up to 35 g per start-point were applied. The colour reactions however were not typical of IAA, and the quality of the partition in such cases is unsatisfactory because of the large amounts of accompanying substances which are very difficult to remove. In the fractioned methanol extracts, besides stimulators, there appeared also two growth--inhibiting substances showing a quite strong physiological activity. They, however, do not posses the specific properties of the inhibitor found among the neutral substances.

The presence in the pollen of pine of a substance of the auxin type, having clearly differentiated physiological properties, is confirmed by the literature (Jakuszkina Tanaka 1958).

Gibberellin-like substances

In the preliminary experiments the presence of gibberellin-like substances in the pollen of pine in chromatographically unpartitioned, purified acetone extracts, was established, by the lettuce hypocotyl test. A significant increase of hypocotylgrowth, accompanied by an elongation of cotyledons, an enlargement of their surface and a clear characteristic chlorosis, indicates the gibberellic character of

the examined substances. This was also confirmed by the barley endosperm test and the growth response of the oat's first leaf and the dwarf pea test. The partition of the examined substances by the TLC-silica gel method gave, to a certain degree, the possibility to their closer identification. The chromatographic properties of these substances, in different solvent systems, indicates that they are quite different. (Fig. 2).

In the solvent system: benzene:acetic acid (Fig. 2 Hist. B), they were partitioned into three groups of a various physiological activity. This differentiation is obviously the result of an ununiform reaction of the test plants on the particular gibberellins (Brian, Hemming and Lowe 1962).

The first group of stimulators (R_f 0.0—0.1) included some active substances giving, besides growth effects, also a chlorosis of the test plants. Taking into consideration data from the literature (Sembdner, Gross and Schreiber 1962) and the results of experiments with some standard substances, it can be assumed that R_f 0.0—0.1 corresponded to the position of gibberellins A_1 and A_3. Their presence has been proved in higher plants. The separation of a similar group with the help of other solvent systems (Hist. C and D), as well as the similarity of the fluorescence of spot after spraying with H_2SO_4, indicate that in the extracts of pine pollen a substance may be present similar to gibberellic acid.

In the second zone, at R_f 0.5—0.7, some substances were present which also caused a chlorosis of the test plants. This zone corresponds, in the developing system, used to the position of gibberellins A_4 and A_7 (R_f 0.60 and 0.57). According to the literature, they have not been found, as yet, in higher plants.

In the third zone (R_f 0.8—1.0), gibberellin-like substances of chromatographic properties corresponding to gibberellin A_9 (R_f 0.90) which is not typical for higher plants were found.

So far standards of all the gibberellins known up to date are not available. This was an obstacle for making a proper comparison between the gibberellin-like substances examined.

It seems that the presence of gibberellin-like substances in pollen has been confirmed by experiments made by Mitchell and Whitehead, who have found in pollen extracts an active, non-auxin, growth substance. The similarity of this substance to gibberellin was indicated by the growth reaction of the internodes of beans observed in the experiments of the authors. This test has certain common features with the bean test on gibberellin of Mitchell and Angel (1950).

Jakuszkina's suggestions regarding some growth stimulators of the non-auxin type noted in the pollen of pine and hazel have been confirmed by the results of theses studies, as well as in the previous studies on growth substances in the *Betulaceae* (Michalski 1967). The gibberellic character of these substances was shown by their physiological action in relation to plants used for the specific biological tests.

SUMMARY

Chromatographic analysis of purified extracts from pine pollen showed the presence of an acidic stimulator having the properties of IAA.

Also a neutral inhibitor has been found which applied simultaneously with IAA or the native growth promoter isolated from pine pollen decreased markedly the growth response of the Avena coleoptile section to both the substances.

By the TLC — method three active groups of gibberellin-like substances have been found. One of them included an active substance showing similar physiological and chemical properties as gibberellic acid.

Department of Plant Physiology
Nicolaus Copernicus University
Toruń — Poland.

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Regulatory wzrostu w pyłku sosny zwyczajnej
(Pinus silvestris L.).

Streszczenie

Z ekstraktów z pyłku sosny wyodrębniono metodą chromatograficzną "kwaśny" stymulator, posiadający właściwości kwasu 3-indolilooctowego.

Stwierdzono również obecność "neutralnego" inhibitora, który stosowany w teście razem z IAA lub ze stymulatorem wyodrębnionym z pyłku, hamował ich stymulujące działanie na koleoptile owsa.

Metodą TLC wyizolowano trzy aktywne grupy substancji giberelinopodobnych. Jedna z tych grup posiadała fizjologiczne i chemiczne własności kwasu giberelowego.