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# Gibberellin-like substances and growth inhibitors in relation to the dormancy and after-ripening of ash seeds (*Fraxinus excelsior* L.).*

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The problem of seed dormancy from the biochemical point of view has already been discussed in many papers (Pollock and Olney 1959; Olney and Po llock 1960; Bradbeer and Colman 1963; Duczmal 1963; Vegis 1964).

Much attention has been given to the role of endogenous growth regulators in this process. The existing data of this problem gave often discrepant results. Nevertheless they led to certain essential ideas concerning the phenomenon of seed dormancy as imposed by the growth regulating system.

According Nikolaeva (1963 a), Nikolaeva and Daletskaya (1963), seed dormancy is caused by a high concentration of auxin, the level of which may be diminished during the process of stratification.

The results obtained by Kawase (1958) point to the fact that during the period of after-ripening of dormant apple seeds a change occurs in the balance between certain growth-promoting and gwroth-inhibiting substances. In recent years several papers were published according to which the naturally occurring growth inhibiting substances may play a considerable role in the control of seed dormancy Villiers and Wareing 1960, Yoshida 1960, Szalai 1963. For more references see Vegis 1964.

Some other experiments Villiers et al. 1963, Villiers and Wareing (1965a) concerning the dormancy of ash seeds have shown that the growth inhibitors appearing in large amounts in the endosperm do not have a direct connection with the dormancy state of the embryos. The level of these inhibitors remained undiminished after the period of chilling.

Frankland and Wareing (1962) have found essential differences in the content of gibberellin-like substances in stratified and non stratified seeds of *Corylus avellana* and *Fagus silvatica*. The possibility of breaking the dormancy of seeds by exogenously applied gibberellin has also been investigated (Richardson 1959; Rémy 1961; Frankland 1962; Nikolaeva 1963 b).

Villiers and Wareing (1960) having applied gibberellic acid to dormant embryos of *F. excelsior* obtained a stimulation of germination and growth, but gibberellin did not prevent the dwarfism of seedlings. A positive influence of gibberellin in dormant embryos of ash was also observed by Szalai (1963). The treatment of

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intact seeds did not give any effiect. According to the opinion of Nikolaeva (1963 b) the effect of exogenously applied gibberellin on the dormancy state of the embryo is not specific as, in fact, its activity causes only a stimulation in the growth of the embryo.

In experiments concerning the influence of low temperature on the break of dormancy of ash seeds it has been found (Villiers and Wareing 1965 a), that chilling caused the production of a substance which promoted germination of the embryos. According to the opinion of the authors it was, however, not a gibberellin because the extracts did not give the typical growth effect in relation to a specific test for gibberellins. Not to speak of the character of the substance discovered the conclusion from these experiments is that low-temperature treatment leads to some essential changes within the growth regulating system of after-ripening seeds.

As may be concluded from the appropriate literature, the possible role of growth regulators, among others also gibberellins in breaking seed dormancy is, as yet, obscure. It was therefore decided to investigate the metabolism of endogeonus gibberellins during the period of stratification and dormancy of ash seeds.

## MATERIALS AND METHODS

Fruits of *Fraxinus excelsior* obtained from the Forest Konstancjewo near Golub were used.

Gibberellin-like substances were determined in fully matured seeds harvested in October 1964, and stored at 8—10°C in dry state for 6 months. Analyses were carried out at 3-week intervals starting with Dec. 10th, 1964. Before analysis, the naked seeds were imbibed in moist sand for 48 hours.

In order to examine the changes in the content of gibberellin-like substances during the process of stratification, whole fruits of F. *excelsior* were stratified as follows: a. at 3°C (cold stratification), b at 20°C (warm stratification) c. for 12 weeks at 20°C and then transferred to 5°C (warm-followed-by-cold stratification). Before stratification the fruits were soaked in water for 72 hrs and then rubbed through a sieve together with gravel in order to remove partly the external coverning structures. Then the seeds were placed into a mixture of moist peat and sand and exposed to appropriate temperature. The experiments have been interrupted after the beginning of germination of seeds stratified at low-followed-by-cold temperature.

Germination assay of the stratified seeds was carried out in Petri-dishes lined with filter paper moistened with distilled water and kept in laboratory conditions.

The method of extraction and paper-chromatography of gibberellin-like substances has been described in a previous paper (Kentzer 1966). For analysis 5 g samples of embryos and 30 g of endosperm were taken from each variant at 3 weeks intervals. As basic test in determining the activity of the investigated substances, the test of the first leaf of oat was applied (Michniewicz 1961).

When some active zones were found in the chromatograms in an atypical position for gibberellin within the given solvent system the dwarf pea test (Kelvedom Wonder) was used additionally, after the method described by Köhler and Lang (1963).

The inhibitory substances present in the dormant seeds were examined in relation to the growth of the embryos of the same species and in the dwarf pea test. Ten full — sized embryos of seeds imbibed for 3 months were excised and placed directly on the chromatogram strips moistened with distilled water. Before the assay the embryos were measured individually and after four days their growth increase was determined. The joint action of the eluates and gibberellic acid ($GA_3$ conc. 0.1 μg/ml) on the growth of embryos and dwarf peas was also investigated.

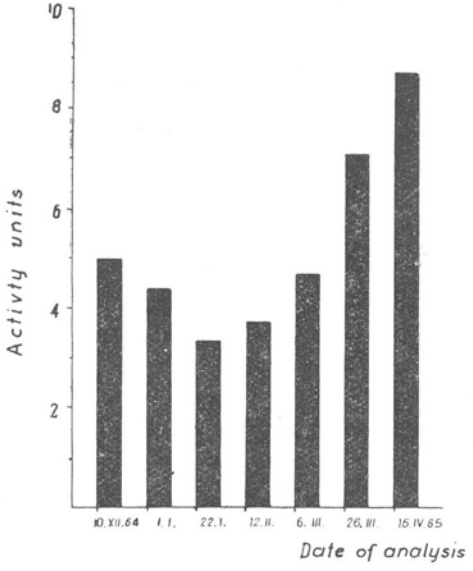


Fig. 1. Activity of growth-inhibiting substances from the endosperm of ash seeds after various periods of storage, expressed in activity units (AU).
(1 AU = 10% inhibition of growth of the test plants)

The extracts from the germinating embryos (after warm-followed-by-cold stratification) and from embryos stratified only at warm temperature were also examined by thin-layer chromatography (TLC), using layers of activated silica gel G. The plates were developed in chloroform, ethylacetate, acetic acid (90:10:5) (Sembdner et al. 1962). Gibberellin spots were detected in ultra-violet light after spraying the plate with 5% sulphuric acid and heating at 60°C. The lettuce hypocotyl assay (Frankland and Wareing 1960), was adopted as biotest for the detection of gibberellin-like substances.

The content of the gibberellin-like substances was calculated per 100 g fresh and dry weight of the examined plant material.

## RESULTS

### 1. Gibberellin-like and growth-inhibiting substances during storage of ash seeds

Fully matured seed did not show the presence of gibberellin-like substances either in the embryos or in the endosperm through the whole time of storage.

In the extracts of endosperm, however, there always was present a certain inhibitor which delayed the growth of the oat first leaf. It was localized in the chromatogram at $R_f$ 0.3—0.6. The level of this inhibitor remained, in general, unchanged during a long period of time. A considerable increase of its activity was observed in the seeds after several months of storage. Particulars regarding the changes in the content of this inhibitor during the time of storage are presented in Fig. 1.

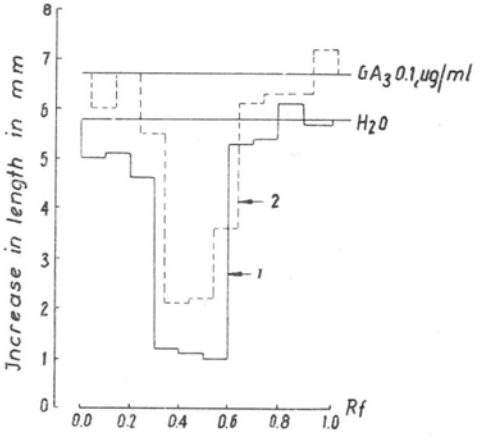


Fig. 2

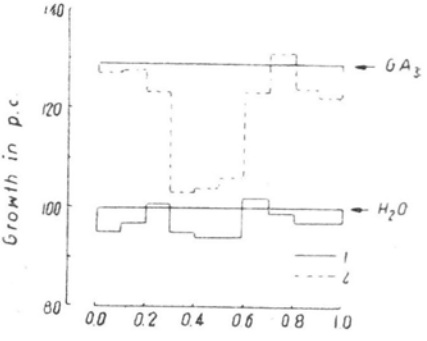


Fig. 3

Fig. 2. Influence of the inhibitor, isolated by paper chromatography from the dormant ash seeds, on the growth of the embryos.

1 — eluates only; 2 — eluates + $GA_3$.

Fig. 3. Effect of inhibitor from the dormant ash seeds in relation to gibberellin as bio-assayed by the dwarf pea test.

1 — eluates only; 2 — eluates + $GA_3$ 0.002 µg/plant.

The inhibitor extracted from the dormant seeds showed also a strong growth-retarding effect on the embryos themselfes (Fig. 2.). The effectiveness of the eluates together with gibberellic acid ($GA_3$) was also investigated. As seen in the diagram, $GA_3$ alone, (conc. 0.1 µg/ml) had only a slight effect on the growth of the embryos but it was able to diminish the inhibitory action of the eluate. Comparison with Fig. 3. shows that the same eluate also interfered with the growth response of dwarf peas to gibberellin.

## 2. Gibberellin-like substances in the embryos and in the endosperm of ash seeds stratified at various temperatures

The content of gibberellin-like substances is presented only in relation to the fresh weight of the plant material analysed. The results did not show any essential changes when recalculated on dry matter.

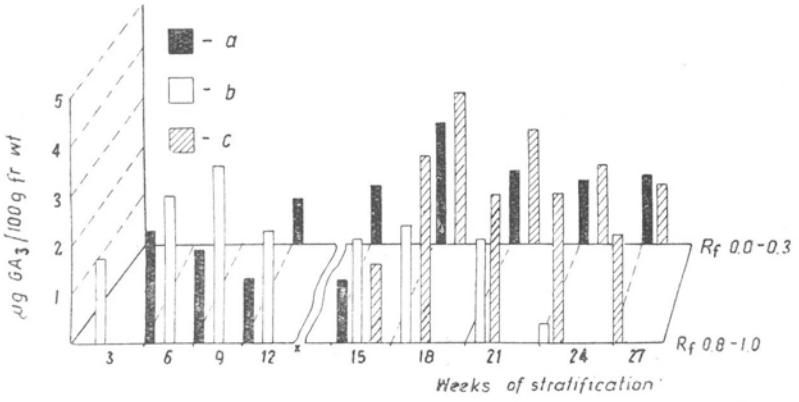


Fig. 4. Dynamics of giberrellin-like substances isolated by paper chromatography from the embryos of ash seeds, stratified at various temperatures.

*a* — stratification at 3°C; *b* — stratification at 20°C; *c* — stratification at 20°C and subsequently at 5°C; *x* — transfe from warm to cold temperature (in variant c).

Results of analysis of the embryos are presented in Fig. 4. Paper chromatography and bioassay on the first leaf of oat revealed that extracts from embryos stratified continuously at 20°C gave only one active zone at $R_f$ 0.8—1.0 which corresponded to that of gibberellic acid in the control chromatogram. The active substance was present already after three weeks duration of the experiment. In the initial period of stratification the amount of this substance increased and then gradually diminished, disappearing completely towards the end of the experiment. At that time the embryos were already full-grown within the seeds, but they were not germinable.

The chromatograms of extracts from chilled embryos (continually at 3°C) have shown in the first period of stratification the presence of two active zones: $R_f$ 0.8—1,0 and $R_f$ 0.0—0.3 which promoted the growth of the oat first leaf. The gibberellin-like substance present at $R_f$ 0.8—1.0 appeared here later than in the embryos from seeds stratified at 20°C. It also disappeared completely in the further periods of chilling. The decreased level of the substance with $R_f$ 0.8—1.0 after 15 weeks of stratification was accompanied by the appearance of the second active compound which showed a considerable activity up to the final period of the experiment, but the seeds were here still not germinable.

In the warm-followed-by-cold stratification method, the transfer of seeds to low temperature caused a temporary increase in the level of the gibberellin-like substances with $R_f$ 0.8—1.0. After several weeks, there also appeared a second active substance at $R_f$ 0.0—0.3. The active substance near the start line showed a blue

spot when sprayed with ethanolic solution of sulphuric acid and viewed under ultraviolet light.

The eluates showing gibberellin-like activity in the oat's test also promoted the growth of the dwarf peas (Fig. 5).

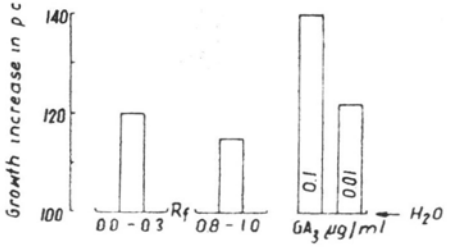


Fig. 5. Growth-promoting effect of gibberellin-like substances from chilled embryos on dwarf peas.

A comparison of the extracts of chilled (warm-followed-by-cold stratification) and unchilled embryos was also made by means of thin-layer chromatography (TLC, Fig. 6). By this method of separation, three different substances showing gibberellin-like activity in the chilled, and two in the unchilled embryos could be

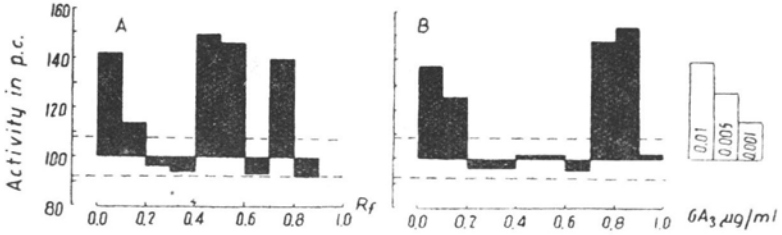


Fig. 6. TLC of gibberellin-like substances from chilled and unchilled embryos of ash seeds. A — chilled embryos; B — unchilled embryos.

revealed. These results point also to some qualitative changes in the content of these substances which occurred in the embryos owing to by the treatment with low temperature.

The dynamics of gibberellin-like substances in the endosperm of seeds stratified in different temperature conditions is illustrated in Fig. 7. The extracts from the endosperm of seeds stratified at warm temperature showed in the chromatograms the presence of only one active zone at $R_f$ 0.8—1.0. The substance localized there was detectable only in the early stages of seed imbibition.

In the endosperm of stratified seeds kept continually in a low temperature, the gibberellin-like substance did not appear until after a longer period of seed imbibition, at the time when the same substance in the embryos was no more detectable.

Extracts from the endosperm of seeds after a stratification by warm-cold temperature contained, like the embryos, two gibberellin-like substances between 0.0—0.3 and 0.8—1.0 *Rf*.

The level of the substances showing gibberellin-like properties present in the endosperm was in all variants always much lower than in the embryos.

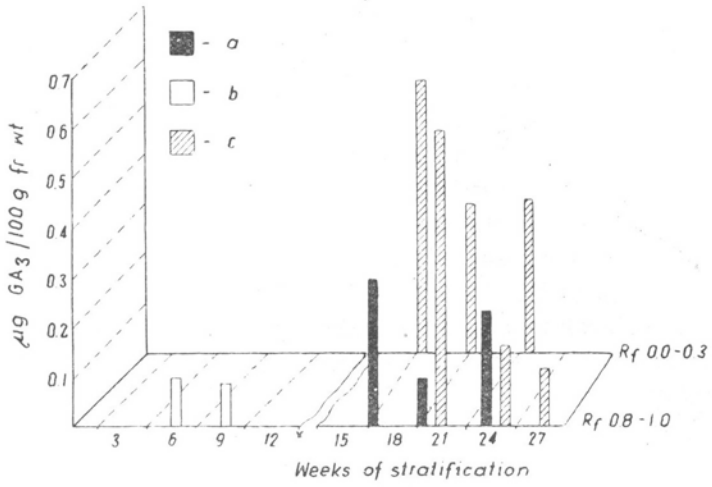


Fig. 7. Dynamics of gibberellin-like substances isolated by paper chromatography from endosperm of ash seeds, stratified at various temperatures.

Legend as in Fig. 4.

## DISCUSSION

It was established in some previous experiments (Kentzer 1966) that in fully matured, air-dried seeds of *F. excelsior* a certain inhibitor was present showing a considerable biological activity.

The present studies have shown that this inhibitor is localized in the endosperm and maintains its full activity over a long period of time after the harvest of seeds. The inhibiting effect of extracts from the endosperm of seeds stored during 5 and 6 months was higher than just after the harvest. This fact stands in disaccord with the data of Villier and Wareing (1965 b), who did not establish the presence of inhibiting substances in the dry seeds of F. *excelsior*. According to the opinion of those authors, a water-soluble inhibitor is formed metabolically only during seed imbibition. Accepting this assumption it should be expected that the level of these substances would be always the same, whether the seeds were stored long or shortly, providing the same time for swelling had been given.

The results of the present experiments have shown that seeds stored during several month, produced, if imbibed, more inhibitors as the seeds just after the harvest. So, it can be supposed that even at such low life-activity as that of air-dried seeds there occur certain biochemical processes which change the potential ability of the seeds to produce some inhibitors during the time of imbibition.

The differences between the results of the above mentioned investigators and those previously described might have been also caused by the different methods

used for extraction and estimation of this kind of substances. The investigations of Villiers and Wareing were directed to estimate the active substances in relation to the *Avena* coleoptile test. Whilst in the present experiments the growth-inhibiting substances showed an inhibitory action on the growth of the first leaf of oats, a test typical rather for gibberellin.

It is interesting to note that the inhibitor extracted from the endosperm of the dormant ash seeds had also the ability to lower the promoting effect of gibberellic acid on the growth of dwarf peas (Fig. 3). Villiers and Wareing (1965 a) established that the effect of certain inhibiting substances from the embryos of ash seeds could be reversed by the application of gibberellic acid. It seems possible that this inhibitor, or inhibitors, interfere also with the naturally appearing gibberellin-like substances within the seeds.

The results of analysis of gibberellin-like substances during the stratification of seeds in various temperature conditions, indicate that embryos treated with low temperature, contained a certain active substance *Rf* 0.0—0.3 having the character of gibberellin, which did not appear in embryos stratified continuously at 20°C. The presence of this substance was established by paper chromatography as well as by the method of thin-layer chromatography. The same substance has already been found in maturing ash seeds (Kentzer 1966). It seems probable that during the after-ripening of these seeds a cycle of metabolic changes occurred which also characterized some stages of seed development.

The gibberellin-like substance occurring in the embryos from warm-stratified seeds disappeared completely during several weeks after the embryos had grown to full size. Utilization of some substances of hormonal nature during the growth of the embryo within the imbibed seed was also stated by Villiers and Wareing (1965 b). Thus it seems possible that among other also some gibberellin-like substances are fully utilized during the period of an intensive growth of the embryo.

The production of an additional gibberellin-like substance has been observed in the embryos from seeds kept continually at low temperature as well as in the germinating embryos after the warm-followed-by-cold stratification. This fact seems therefore to indicate that the substance of gibberellin-like properties produced during the time of chilling is not the only factor determining the interuption of dormancy of the embryo. Its level must have been probably balanced by the presence of some other substances of this type which were not present at the given time within the embryos which were stratified continuously at low temperature (Fig. 4).

Of essential importance might have also been the differences in the content of gibberellin-like substances in the endosperm of the examined seeds. The highest activity of these substances was found in the extracts of the the endosperm of seeds after the stratification at the variable temperatures. The extracts of endosperm of the unchilled seeds showed a slight gibberellin-like activity only in the first weeks of stratification.

An increase of the content of gibberellin-like substances, as caused by low-temperature treatment was, observed by Smith and Rappaport (1961) in potato

tubers. Frankland and Wareing (1962) working with seeds of some forest trees, have pointed out certain changes of a qualitative nature in the system of gibberellin-like substances under the influence of chilling. After Eagles and Wareing (1963) the break of dormancy of seeds of *Betula pubescens* seemes to be closely related to the increased level of endogenous gibberellin caused by low-temperature treatment.

Thus it may be stated that, within the biochemical changes which take place in the process of seed stratification, also gibberellin-like substances are involved. The direction of these changes is controlled by the conditions of temperature and depends also on an appropriately advanced growth of the embryo.

It seems possible that the effect of low temperatures induces certain quantitative as well as qualitative changes in the metabolism of gibberellin-like substances which are responsible for the germination of seeds.

## CONCLUSIONS

1. The endosperm of fully matured seeds of F. *excelsior* contained some inhibitors which showed a high biological activity during a long period of time after the harvest. The inhibitor had the ability to diminish the growth-promoting effect of gibberellic acid in relation to the dwarf pea test.

2. In the embryos and endosperm of chilled seeds a gibberellin-like substance was present, which did not appear in seeds stratified continuously at 20°C.

3. The experiments also showed that a low-temperature treatment of ash seeds leads to some quantitative as well as qualitative changes in the metabolism of gibberellin-like substances. It can be assumed that these changes may be to a certain degree responsible for breaking of dormancy in the ash seeds.

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## *Substancje giberelinopodobne i inhibitory wzrostu w okresie spoczynku i wtórnego dojrzewania nasion jesionu (Fraxinus excelsior* L.)

Streszczenie

Oznaczano zawartość substancji giberelinopodobnych w nasionach spoczynkowych jesionu oraz w okresie stratyfikacji w różnych warunkach temperatury.

Aktywność tych związków badano oddzielnie w embrionach i endospermie nasion w odstępach trzy tygodniowych.

Stratyfikację nasion prowadzono w trzech różnych kombinacjach temperaturowych: a) Stratyfikację chłodną w temp. 3°C, b) Stratyfikację ciepłą w temp. 20° C, c) Stratyfikację ciepło-chłodną (12 tygodni w temp. 20° C a następnie w 5° C). W trakcie trwania zabiegu przeprowadzano próby kiełkowania nasion. Doświadczenie prowadzono do momentu skiełkowania nasion w wariancie stratyfikowanym w zmiennej temperaturze (stratyfikacja ciepło-chłodna).

Ekstrakty acetonowe z badanego materiału roślinnego, uprzednio odpowiednio oczyszczone, rozdzielono stosując chromatografię bibułową i cienkowarstwową. Aktywność biologiczną ekstraktów oznaczano testem pierwszego liścia owsa i karłowatego grochu.

Wpływ substancji hamujących badano również na wzrost izolowanych embrionów nasion jesionu. Oznaczano jednocześnie łączny wpływ eluatów z hamujących stref chromatogramu i giberelliny na wzrost embrionów i karłowatego grochu.

Rezultaty badań pozwoliły na wysunięcie następujących wniosków.

1. W pełni dojrzałe nasiona jesionu nie zawierają substancji giberelinopodobnych ani w embrionach, ani w endospermie. W endospermie tych nasion występuje natomiast inhibitor, który zachowuje pełną aktywność biologiczną przez długi okres czasu po zbiorze. Inhibitor ten hamuje wzrost izolowanych embrionów jesionu i ma zdolność niwelowania stymulującego wpływu giberelliny na wzrost karłowatego grochu.

2. Embriony i endosperm z nasion poddanych chłodnej stratyfikacji zawierają pewną substancję aktywną o własnościach gibereliny, która nie występuje w nasionach stratyfikowanych w temperaturze 20° C.

3. Wyniki badań wskazują, że działanie niskiej temperatury w okresie stratyfikacji prowadzi do zasadniczych zmian ilościowych i jakościowych w metabolizmie substancji giberelinopodobnych. Wydaje się prawdopodobnie, że zmiany te stanowią jeden z warunków decydujących o przerwaniu stanu spoczynku nasion jesionu.

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