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The role of the natural growth inhibitor (Abscisin II) in apple seed germination and the changes in the content of phenolic substances during stratification

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Apple seeds at the time of fruit harvest do not germinate unless they are after-ripened at low temperature (2°—7°C) under moist conditions for two to three months. The removal of the integuments, however, permits the germination of the naked embryos but the seedlings are dwarfed. The literature on the dormancy of the seeds is very large, but only recently new evidence was brought forward concerning this subject. The review of the problem is limited to the most pertinent papers.

Côme, 1965 a, b, made a detailed study of the germination of apple seeds taken out of the fruit stored at various temperatures. After three months of storage at 0° to 4°C (the normal length of stratification) the seeds taken out of the fruit germinated very poorly unless the seed coats were removed. Côme concluded that the distinction should be made between the true dormancy of the seed and the inhibition imposed on the embryo by the integuments. According to the author the phenolic substances, mainly chlorogenic acid, present in the integuments, play an important role in this inhibition. They absorb some of the oxygen passing through the integuments and impair its penetration to the embryo. The embryo is thus deprived of the oxygen necessary for the germination.

Recently, abscisin II, a new plant hormone was isolated and identified in young cotton fruit (Ohkuma 1963; Addicott 1966). Eagles and Wareing (1963) on the other hand, have found an inhibitory substance in the leaves and buds of *Acer* and *Betula* which they named "dormin". This substance when applied to growing seedlings arrested their growth and induced dormancy. Cornforth et al. established in 1965 that dormin is chemically identical with abscisin II, and they accomplished its synthesis. Evidence is accumulating that abscisin II can account for most of the inhibitory activity found in the acid fraction of plant extracts, (Cornforth 1965). In 1967 Pieniążek and Rudnicki isolated this plant hormone from apple leaves and apple fruit juice.

Kamiński (1967) obtained interesting results soaking for 5 days stratified apple seeds in apple juice. The germination of naked embryos was greatly inhibited after this treatment, and the growth of seedlings impaired. Since the presence of abscisin II in apple fruit was confirmed (Cornforth, personal communication) it is probable that natural abscisin II among others, was responsible for the poor germination of the embryos.

Lipe and Crane (1966) were able to show the presence of the inhibitor, probably abscisin II, in dormant peach seeds on the basis of chromatographic analysis, bioassays and absorption of ultraviolet light. Termination of rest was correlated with the disappearance or the inactivation of the inhibitor during stratification of the seeds. The inhibitor was located mainly in the outer and inner integuments of the seed and was distributed equally throughout them.

Sondheimer and Galson (1966) demonstrated the inhibitory effect of low concentrations of abscisin II (1μg/ml — 10 μg/ml) on the germination of excised nondormant ash embryos. No attempt, however, was made to isolate the natural inhibitor from the seed coats in order to try its effect on the germination of nondormant embryos.

It should be pointed out that 10 years ago Luckwill (1957) postulated the presence of *Malus* inhibitor 2, and suggested that the inhibition caused by this substance was of different nature than that induced by coumarin or chelidonic acid. Since abscisin II was isolated and identified in apple leaves and apple fruit juice, *Malus* inhibitor 2 might be identical with this plant hormone.

The purpose of this work was to investigate the role of the natural inhibitors from seed integuments, apple fruit juice and synthetic abscisin II in the germination of nondormant apple embryos.

In view of the numerous conflicting reports concerning the inhibitory action of the phenolic substances present in the seed coats, a special study of the changes in the content of those compounds during stratification was made.

MATERIAL AND METHODS

The seeds from 'Idared', 'Bancroft' and 'Antonovka' apples treated in several ways were analyzed for the content of phenolic substances and their germination studied in detail using 100 seeds per treatment.

The seeds were removed from the fruits stored at 7°C for 3 months (Bancroft) and 6 months (Idared). They were kept for a month or 6 weeks under following conditions: a) in dry conditions at room temperature, b) on wet filter paper at 25°C, c) on wet filter paper at 4°C, d) embryos on wet filter paper at 25°C, e) intact seeds stratified in moist sand at 4°C for 12 weeks.

Two extraction methods for phenolic substances were used.

A — the seeds separated into outer integuments, endosperm and embryos were extracted after grinding with a few ml of 0.1 N HCl, and after centrifuging the supernatant was decanted and an aliquot spotted on Whatman No. 1 chromatographic paper.

B — exactly the same sample of seeds (by weight) was extracted with boiling methanol for 20 minutes, left for 18 hours, decanted and evaporated to dryness. The residue was dissolved in a few drops of ethanol and an aliquot spotted on Whatman No. 1 paper.

Two solvents were used for developing the chromatograms: a) 2% acetic acid, and b) n-butanol-acetic acid-water (4:1:1 v/v/v). For the identification of phenolic substances the chromatograms were examined under UV light, and after spraying with diazotized sulfanilic acid and diazotized benzidine.

'Antonovka' seeds were stratified in moist sand for 12 weeks, and every week 100 seeds separated into integuments and embryos were extracted with boiling methanol, and an aliquot of the extract spotted on Whatman No. 1 paper. Two solvents were used: a) 2% acetic acid and b) n-butanol-ammonia-water (8 : 1 : 1 v/v). Diazotized benzidine was used as color reagent.

The non-phenolic inhibitor from the nonstratified and stratified seeds of Wealthy apples was extracted from 45 g of seed coats using the method described by Lipe and Crane (1966). 250 g of nonstratified 'Antonovka' seeds were extracted intact. After extraction, evaporation and developing the chromatograms in isopropanol-ammonia-water (10 : 1 : 1 v/v/v) the wheat coleoptile straight growth test, Nitsch (1956), served to locate the inhibitory zone.

The effect of the non-phenolic inhibitor from 'Whealthy' and 'Antonovka' seeds was also tested on the germination of stratified embryos of those seeds. The inhibitory effect was compared with that of synthetic abscisin II and natural apple juice inhibitor previously isolated by Pieniążek and Rudnicki (1967) and identified at the Milstead Laboratory of Chemical Enzymology, Sittingbourne, England, by drs. J. W. Cornforth and B. V. Milborrow. Synthetic abscisin II was a gift of dr J. W. Cornforth which is gratefully acknowledged.

The embryos were germinated on petri dishes (15 embryos per dish in 2 replicates with 2 ml of water (controls), and known concentrations of abscisin II for comparison (0.3 μg/ml to 10 μg/ml). The non-phenolic inhibitor from the seeds was eluated from the inhibitory zone on Whatman No. 1 paper, and its concentration was unknown. 30 embryos were used per treatment.

RESULTS

In the seeds of analyzed apple varieties — phloridzin, phloretin and chlorogenic acid were the main phenolic substances (Table 1, 2). The other phenolic substances like p-coumaric acid, ferulic acid, and others not identified on the chromatograms — were present in negligible amounts. Phloridzin was very abundant in the integuments of nonstratified seeds of 'Antonovka' (Table 1). During stratification it has disappeared from them completely. On the other hand, the embryos of the nonstratified seeds were free of phloridzin but after seven weeks at the temperature of 2°—4°C there appeared a very large spot of phloridzin on the chromatograms.

The data presented in Table 2 show also the presence of phloridzin in the integuments of dry 'Bancroft' seeds that were removed from the fruit stored for three months at 7°C, and just a trace of it in the embryos. On the contrary, the germinating embryos after they turned green were rich in ploridzin like the fully stratified 'Antonovka' embryos.

Phloretin was always present in the integuments — before and after the stratification, its spot was always about the same size (Table 1). The embryos were free of phloretin, and only at the end of 11 weeks of cold treatement a trace of it was noticed.

Table 1

Changes in phloridzin and phloretin content during stratification of 'Antonovka' seeds

Treatment	Phloridzin	Phloretin
Nonstratified seeds		
integuments	+++	+++
embryos	—	—
Stratified seeds (6 weeks)		
integuments	—	+++
embryos	—	—
Stratified seeds (7 to 11 weeks)		
integuments	—	+++
embryos	+++	trace

—, +++ — as in Table 2

Table 2

The main phenolic substances in the seeds of 'Bancroft' and 'Idared' varieties

Treatment and variety	Phloridzin	Phloretin	Chlorogenic acid
IDARED			
endosperm (6 weeks at 25 C)*	—	++	trace
endosperm (6 weeks at 4 C)**	—	++	trace
BANCROFT			
dried seeds:			
integuments (2)	+++	+	++
embryos	trace	—	trace
non-germinating seeds after 1 month at 25 C			
integuments	+	+	—
endosperm	trace	++	—
embryos	—	—	—
germinating seeds (removed from the fruit stored for 3 months at 7 C)			
embryos (white)	trace	trace	—
embryos (green)	+++	—	—

*) all seeds dormant

**) all seeds germinated

+, ++, +++ — refers to the size of the spot after spraying the chromatograms with diazotized sulfanilic acid and diazotized benzidine.

— no spot on the chromatogram

Table 3

Percentage of germination of Idared seeds* from different treatments

	1	2	3
	after 2 weeks at 20°C	non-germinating seeds from (1) after 2 months at 20°C	the seeds from (2) after 3 weeks at 2—4°C
Intact seeds	22	0	76
Outer integuments removed	24	0	84
Naked embryos (endosperm removed)	100	83	

* The fruit was stored for 6 months at 7°C, then the seeds were removed and kept dry at room temperature for one month before they were put to germinate on wet filter paper.

The third phenolic compound — chlorogenic acid (Table 2) that was abundant in dry seed coats started to disappear when the seeds were placed on wet filter paper. Chlorogenic acid disappearance from the endosperm was not correlated with treating the seeds with different temperature, and their increasing ability to germinate.

Table 4

The effect of abscisin II (synthetic and natural apple juice abscisin) and the inhibitor from seed integuments on the germination of stratified apple embryos

Treatment	Percentage of embryos germinated after three days	
	Wealthy	Antonovka
Control (water)	95.3	92.0
Inhibitor from integuments of Wealthy:		
nonstratified seeds	46.6	
stratified seeds	86.6	
Inhibitor from nonstratified intact Antonovka seeds		21.0
Apple juice abscisin II equivalent to μg/ml of synthetic abscisin II:		
5.0 μg/ml		0
2.5 ,, ,,		0
1.25 ,,		0
0.6 ,,		6.7
0.3 ,,		26.6
Abscisin II (racemic) conc.:		
10.0 μg/ml	0	
5.0 ,,	0	0
2.5 ,,	6.7	0
1.25 ,,		20.0
0.6 ,,		46.7
0.3 ,,		53.3

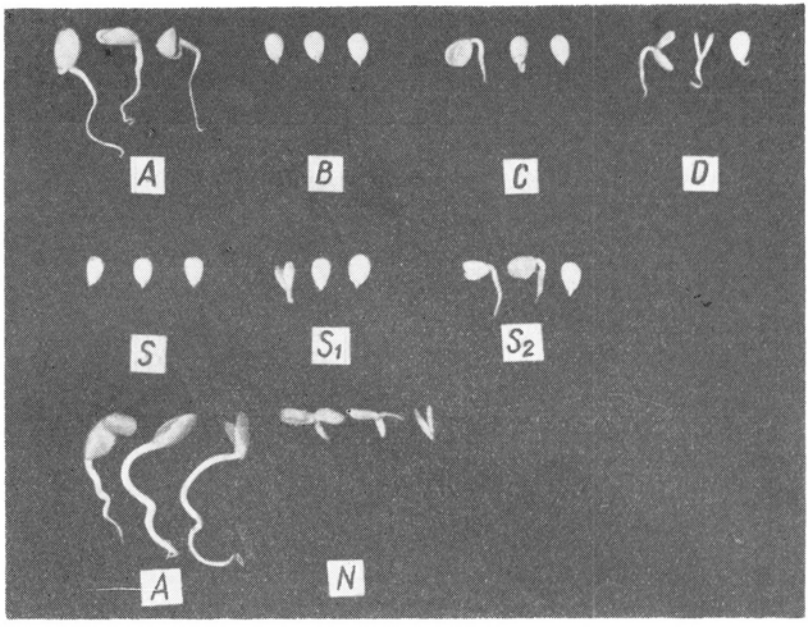

Fig. 1. The germination of stratified 'Antonovka' embryos (1 st row)
A — controls; *B* — 5 μg/ml of abscisin II; *C* — 2.5 μg/ml; *D* — 1.2 μg/ml
S, S_1, S_2 — natural abscisin II from apple juice equivalent to 1.25 μg/ml, 0.6 μg/ml, 0.3 μg/ml synthetic abscisin II ('Antonovka' embryos) (2 nd row)
The germination of stratified 'Wealthy' embryos (3rd row). *A* — controls; *N* — inhibitor from nonstratified 'Wealthy' seeds.

Trace amount of chlorogenic acid was found in the endosperm of dormant seeds as well as in the endosperm of readily germinating which were exposed to low temperature treatment for six weeks (Table 2).

The data presented in Table 3 pertain to the germination of 'Idared' seeds, and they show that the endosperm plays the most important role in the germination of apple seeds. 'Idared' seeds deprived of outer integument germinated similarly as intact ones, whereas the removal of the endosperm resulted in 100 per cent germination.

The non-phenolic inhibitor isolated from the seed coats of nonstratified 'Wealthy' and 'Antonovka' seeds inhibited strongly the growth of wheat coleoptiles (37 per cent), and its R_f value was 0.65—0.74. On the contrary the extracts from stratified seeds produced but slight inhibition of the wheat coleoptiles growth (12 per cent).

In the apple embryo bioassay, two petri dishes (15 apple embryos in a dish) with 2 ml of uknown amount of the inhibitor from seed coats dissolved in water were used. Abscisin II both synthetic and natural from the apple juice at known concentrations was used for comparison (Table 4 and Fig. 1).

The inhibitor from the integuments of nonstratified Wealthy seeds inhibited the germination of stratified embryos by 53.4 per cent. The extract from intact 'Antonovka' seeds produced stronger inhibition of embryo germination — 79 per cent.

The extract from the similar sample of the integuments from the stratified seeds, on the contrary, inhibited only slightly the germination of stratified embryos.

The inhibitory effect of abscisin II and the natural apple inhibitor from the fruit juice on the germination of the embryos ended after the embryos were rinsed for about 10 minutes in water and transferred to other petri dishes. Despite the rinsing the germination of seeds was somewhat inhibited at the highest concentrations of abscisin II (5 μg/ml, 10 μg/ml). The seedlings, however, obtained from the seeds treated with natural abscisin II and the inhibitor isolated from the integuments had very short hypocotyls as compared with control seedlings and they showed other morphological abnormalities (Fig. 2). The effects of abscisin II and the natural inhibitor on the growth of seedlings will be described separately.

DISCUSSION

The results of germination experiments and the changes in phenolic substances — phloretin and chlorogenic acid in different parts of apple seeds during stratification showed that none of them seemed to play an important role in dormancy. It was shown previously that phlorodzin does not inhibit the growth of *Malus* (Pieniążek, 1965).

No evidence was found in support of Côme's hypothesis that chlorogenic acid naturally present in the integuments inhibits the germination of non-fully after-ripened seeds. Chlorogenic acid as stated by Côme, (1965) and as found in our experiments is present in fairly large amounts (UV light and color reagents) in the integuments. No difference, however, was noticed in our experiments in its content in the dormant and germinating seeds.

As it has been shown endosperm plays an important role in germination of seeds. The composition of phenolic compounds in the endosperm of germinating and dormant seeds was, however, the same in spite of their quite different ability to germinate.

Great difference was observed in the content of phloridzin in dormant and stratified seeds. The nonstratified seeds had phloridzin mainly in the integuments whereas the embryos were free of it, and only after some weeks of stratification phloridzin started accumulating. The germinating embryos of Bancroft after they turned green showed a remarkable change in the amount of phloridzin.

Not much is known about the synthesis of phloridzin in *Malus* although Hutchinson (1959) postulated that young leaves are mainly responsible for its synthesis. Our results suggest that the synthesis of phloridzin, or its release from other compounds, takes place in cotyledons during final period of stratification, and that this compound starts to accumulate rapidly at the time when they begin to turn green.

Phloretin, the aglucon of phloridzin, found in the integuments seems to be the product of bacterial degradation of phloridzin during the stratification (Börner 1959).

The evidence presented herein suggests the identity of the inhibitor from the seed coats with abscisin II found previously in apple leaves and apple fruit juice (Pieniążek, Rudnicki 1967). R_f value of the synthetic abscisin II and the inhibitor from the integuments was about the same (0.65—0.74). The inhibitory zone after chromatographic separation was almost free of the phenolic substances. After stratification of the seeds the inhibitor almost disappeared from the integuments.

It seems probable that like in case of peach seeds, Lipe and Crane, (1966) a substance similar to abscisin II, or identical with it, disappears to a large extent from seeds or is inactivated during their stratification.

Future experiments will show whether physiological dwarfing as suggested by Lipe and Crane (1966) and Kamiński (1967) is caused by a certain concentration of this inhibitor nevertheless not high enough to prevent the germination of seeds.

The inhibitory action of abscisin II on the germination of stratified apple embryos was established beyond doubt. The study will follow on the changes in the level of this compound in the seeds during stratification.

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Rola naturalnego inhibitora wzrostu (abscysyny II) w kiełkowaniu nasion jabłoni i zawartość związków fenolowych w czasie stratyfikacji

Streszczenie

Nasiona jabłoni nie kiełkują bez tzw. stratyfikacji w temperaturze 2—7°C przez dwa do trzech miesięcy. Po zdjęciu okryw nie stratyfikowane nasiona kiełkują, ale otrzymane siewki nie rosną normalnie. Côme (1965), postawił hipotezę, że związki fenolowe, a przede wszystkim kwas chlorogenowy znajdujący się w okrywach nasiennych odgrywa ważną rolę w hamowaniu kiełkowania nasion.

Autorki przebadały ekstrakty z okryw nasiennych stratyfikowanych i nie stratyfikowanych nasion i stwierdziły występowanie w nich kwasu chlorogenowego obok innych fenoli, jak florydzyna i floretyna. Nie znaleziono jednak różnic w ilości kwasu chlorogenowego w okrywach nasion przed i po stratyfikacji.

Znaleziono natomiast naturalny inhibitor wzrostu nie wykazujący charakteru fenolowego, który hamował nie tylko wzrost na długość koleoptili pszenicy, ale również hamował w znacznym stopniu kiełkowanie stratyfikowanych zarodków jabłoni. Po zakończeniu stratyfikacji zawartość inhibitora w łuskach nasion znacznie zmalała.

Na podstawie testu pszenicznego i jabłoniowego, jak również wartości R_f związek ten wydaje się identyczny z syntetyczną abscysyną II i abscysyną naturalną poprzednio znalezioną w soku jabłek.

Doświadczenia wykazują, że mechanizm ustępowania stanu spoczynku nasion jest związany z zanikiem hormonu (prawdopodobnie) abscysyny II, a nie związków fenolowych.