ACTA SACIETATIS
BOTANICORUM POLONIAE
vol. XXXIX — nr 1
1970

Changes in the leucoanthocyanidin content in the leaves of biennially bearing apple trees

ST. LEWAK, M. J. GROCHOWSKA, T. KURYŁ and D. WYZIŃSKA

INTRODUCTION

The phenomenon of biennial bearing of fruit trees, so important from the economic point of view, has attracted the attention of numerous investigators for many years (Hartley et al. 1959; Pieniążek et al. 1964). Attempts to elucidate the causes and mechanisms of this phenomenon led to the formulation of several hypotheses. According to some of them, growth regulators play here an important role (Grochowska 1963, 1968; Chan and Cain 1967). The competition for structural or energy supplying material between developing fruit and flowerbud is according to other hypotheses responsible for the behaviour of the plant (Bielińska 1957).

Our studies derive from Grochowska's observation (1963) that the phloridzin content in the leaves of biennially bearing apple-trees is correlated with the process of flower-bud formation. Phloridzin was not found to be present in biologically active concentrations in the leaves of the on-year trees whereas the leaves of off-year ones and those of annually bearing trees contained considerable amounts of phloridzin (as a strong growth inhibitor of test plants). Phloridzin is the main phenolic glycoside of apple tissues. According to many authors it is a physiologically important substance. It is known that phloridzin participates in the processes of growth (Hancock et al. 1961; Grochowska 1964, Sarapuu 1964, 1965) and germination (Kamiński, Pieniążek 1968), in control of high-energy bound formation (Marré and Servettaz 1955; Sarapuu 1964) and in the formation of defence mechanisms in apple trees against fungal infections (Holowczak et al. 1963).

Apple tissues contain also other phenolic compounds. Condensed plant tannins present a group of various compounds of little known structures and different abilities of binding enzymatic proteins. This feature may be considered as an indication that condensed tannins control some metabolic processes.

Leucoanthocyanidins occur in apple tree both in the form of low — molecular weight compounds and of polymers. Changes in the content of these two groups of substances in apple leaves were studied by Faust (1964) and by Lewak et al. (1967). In both cases annually bearing varieties of apple tree were studied. Assuming the possibility that leucoanthocyanidins participate in the control of generative processes of the apple tree we undertook studies on the changes in the leucoanthocyanidin content in the leaves of on- and off-year apple trees. These studies were carried out with the biennially bearing c.v. Landsberska, both on on-year and off-year leaves.

MATERIAL AND METHODS

Material. The experiments were carried out on the leaves of the apple tree, cv. Landsberska collected from 18 year-old trees in the garden of the Institute of Pomology in Skierniewice. Fourth and fifth leaves from the shoot top were collected simultaneously from on-year and off-year trees from May to September 1968. Analogous series of samples were collected in 1966 and 1967.

Ca 10 g samples (30 leaves) were used for experiments immediately after collection or were frozen in solid Co_2, freeze-dried and stored in paraffin wax sealed vessels at room temperature.

Samples 500 mg of freeze-dried leaves were extracted in the manner described earlier (Lewak et al. 1967). The extraction of fresh leaves was carried out in a similar manner using 1700 mg samples and 67% instead of 60% ethanol for the first extraction.

Methods. Determination of total leucoanthocyanidins as well as low- and high-molecular weight fraction were carried out as described in the previous paper. For transformation of leucoanthocyanidins in to anthocyanidins the method of Swain and Hillis (1959) or its modification developed by Govindarajan and Mathew (1965) were used. The original Swain and Hillis technique was applied only for comparative studies on the utility of both methods.

Respiration of fresh leaves was measured using 1 cm dia. discs cut from the central parts of the leaves with the use of the Warburg respirometer at 25°. Twelve such discs were placed in one manometric flask together with 0,5 ml of distilled water. In the well of the flask 0.2 ml of 10% KOH was placed, together with a piece of filter paper (1.5×2 cm).

RESULTS AND DISCUSSION

Series of determinations of the total content of leucoanthocyanidins and of content of low- and high-molecular weight leucoanthocyanidins in biennially bearing apple-trees, cv. Landsberska, were carried out in the vegetation periods of 1966, 1967 and 1968. The analyses were carried out simultaneously on the freeze-dried material, and on fresh leaves by the previously described technique of leucoanthocyanidin fractionation (Lewak et al. 1967) and the modification of the technique of leucoanthocyanidin determination described by Govindarajan and Mathew (1965).

Comparison of the results of direct determination of leucoanthocyanidins by the Swain and Hillis colorimetric method (1959) and by its modification indicated a better sensitivity of the latter (Table 1).

Table 1

Comparison of the values of absorbancy at λ = 540 nm for anthocyanidins formed from leucoanthocyanidins contained in 500 mg of freeze-dried apple leaves after heating with Swain and Hillis (1959) or Govindarajan and Mathew (1965) reagents

Reagent	Time of heating, min.				
	10	20	30	40	50
A — Swain and Hillis	0.286	0.311	0.352	0.355	0.360
B — Govindarajan and Mathew	0.395	0.428	0.478	0.593	0.601
B/A	1.35	1.38	1.36	1.67	1.67

The data presented in Table 1 show that addition of Fe••• ions induces an increase of the yield of the transformation dependent upon heating-time. It can be seen that maximal values of absorbancy were obtained only after a 40 min heating, similarly as in the original method of Swain and Hillis.

Chromatographic separation of anthocyanidins formed in the reaction carried out by both methods indicates that addition of Fe••• ions increases the yield of various dyes to various extents, as seen in Table 2.

Table 2

Ratios of the amounts of anthocyanidins chromatographically separated from the product of leucoanthocyanidin-anthocyanidin transformation carried out according to Swain and Hillis (1959) and Govindarajan and Mathew (1965) for leucoanthocyanidin of apple leaves

Band	R_f values in acetic acid/conc. HCl/water 5:1:5	Absorbancy at λ = 540 nm		B/A
		A — after reaction with Swain Hillis reagent	B — after reaction with Govindarajan Mathew reagent	
I	0.30	0.310	0.450	1.5
II	0.45—0.47	0.190	0.380	2.0
III	0.90—0.93	0.500	0.660	1.3

It has been previously demonstrated (Lewak et al. 1967) that the leucoanthocyanidins of apple-leaves transform under heating with acids into three dyes: cyanidin (band I, table 2), pelargonidin (band II, table 2) and an unidentified compound (band III, table 2). The data presented in Table 2 show that the enhanced yield of anthocyanidins under conditions described by Govindarajan and Mathew results mainly from an increase of transformation of leucopelargonidin moieties into the flavylium salt. The transformation of leucocyanidin is not so effectively catalyzed, and that of other compounds even less.

Similarly, it was shown that the results of the determination of the level of low-and high-molecular weight leucoanthocyanidins depend to a great extent on the manner of preparation of the plant material, although this kind of dependence does not apply to determinations of total leucoanthocyanidin content. The ratios of amounts of high- to low-molecular weight leucoanthocyanidins for the leaves of off-year apple trees cv. Landsberska collected in the same vegetation period but stored differently are given in Table 3.

Table 3

Effect of the time of storage of freeze-dried apple leaves on the polymerization degree of leucoanthocyanidins

Date of collection	Storage conditions	Ratio of the amount of high- and low- molecular leucoanthocyanidins
31.VII.1968 29.VIII. 1968	fresh leaves	0.54 0.69
25.VII. 1968 23. VIII. 1968	freeze-dried leaves, stored for 2 weeks	0.78 0.80
28. VII. 1966 23. VII. 1966	freeze-dried leaves, stored for 6 months	2.18 1.77
2. VIII. 1967 31. VIII. 1967	freeze-dried leaves, stored for 12 months	2.33 4.24

The dependence of the results of determinations on small changes in the composition of analytical reagents and on the time of storage of the material additionally indicates that the methods used in studies of leucoanthocyanidins cannot be trusted without reserve (Lewak 1968). Unfortunately, no better analytical techniques are available and studies of leucoanthocyanidins must further rely on these imperfect methods. They should be, however, very critically interpreted and it should be re-out under strictly standardized conditions.

The fact that the ratio of high- to low-molecular weight leucoanthocyanidins increases during storage of freeze-dried apple leaves indicates that under these conditions the process of condensation of low-molecular flavans to polymeric condensed tannins may take place. This observation confirms the correctness of Freudenberg's suggestion that condensed tannins are formed as the result of nonenzymatic transformations.

The pattern of changes of the total leucoanthocyanidin content in apple leaves during vegetation was independent of the manner of material preparation. The same concerns the changes in the content of low and high-molecular weight leucoanthocyanidins.

On the other hand, the leucoanthocyanidin content in the fractions tested depended to a great extent on the preparation of the material. For this reason we based these investigations on the results obtained with fresh material. The use of freeze-dried material would be more convenient from the technical point of view, however, it is quite possible that the relative increase of the polymeric leucoanthocyanidin content during storage of the leaves does not reflect changes of the synthetic abilities of the plant caused by damage to the cellular structures but, it is rather caused by post-mortal non-enzymatic processes.

Fig. 1. Changes in total leucoanthocyanidin content in fresh apple-leaves from on-year (—○—) and off-year (—●—) trees during the vegetation period 1968

The fluctuation of the total leucoanthocyanidin in leaves of apple-trees, cv. Landsberska, during vegetation are less pronounced than in leaves of the previously investigated cv. Macaun. After a rapid increase of their content which takes place during blooming in May, their level remains almost unchanged until September (Fig. 1). During the whole vegetation season the content of high-molecular weight leucoanthocyanidins does not exceed 50% of the total content of these compounds. Significant differences between the content of leucoanthocyanidins in the leaves of on-year and off-years trees can be observed only at the beginning of the vegetation period in May and June. At that time the leaves of on-year plants contain less leucoanthocyanidins than the off-year trees.

At that time the growth of the off-year trees is slower. The slower growth preceeds formation of flower buds for the next year. This process is accompanied by an increased leucoanthocyanidin content as compared to the content of these compounds in the leaves of on-year trees which at the same time exhibited relatively higher growth rates. A pronounced decrease of the content of the compounds studied in the leaves before flower bud formation suggests participation of leucoanthocyanidins in this process. Nevertheless, the character of the curves of leucoanthocyanidin content were similar for both kinds of trees (off-year and on-year) but the comparative total amounts of these substances seemed to be positively correlated with the intensity of flower bud formation of the trees.

Participation of phenolic compounds in the processes of flower bud differentiation was postulated by Spragg (1960). This suggestion was supported by Stenlid (1963, 1968) and Tomaszewski (1964) who studied the mechanisms of control of the IAA level. These mechanisms in plants for which auxins are inhibitors of generative processes, are set in operation by the combined action of IAA-oxidase and phenols. The fact that Grochowska has found considerable amounts of phloridzin in the leaves of apple-trees being in the off-year, together with our results show the complexity of the relations between the occurrence of phenolic compounds and the process of flower bud formation. Leucoanthocyanidins and phloridzin are formed in plants from the same precursors: acyl-CoA and phenylpropane derivatives — metabolites on the biosynthetic pathway of shikimic acid. It is probable that changes of the direction of polyphenol biosynthesis towards increased production of phloridzin or leucoanthocyanidin are related with the differentiation of buds.

Hulme (Jones and Hulme 1961; Hulme and Woolton 1962), Mayer (1964) and other authors studied inhibitory effects of phenolic compounds, especially of the polymeric substances of this group, on the activities of mitochondrial enzymes and terminal oxidases. Their results suggest that phenolic compounds may control energy generation in plants by modyfying the activity of the respiratory enzymes. This may represent one mechanisms of the influence of these compounds on physiological processes. A confirmation of this hypothesis could be based on the demonstration of a correlation between the changes in the content of phenolic compounds and the changes in the respiration rate of plant tissue.

Figure 2 presents changes in respiration rate during vegetation period for leaves of the apple tree, cv. Landsberska, collected from off-year trees, together with the changes in the content of low-and high- molecular weight leucoanthocyanidins in the same leaves. A certain similarity can be found between the oxygen uptake and low-moleculer weight leucoanthocyanidin content curves. This similarity is probably caused by parallel variations in respiration and growth intensity of the leaves. In this latter phenomenon the rate of all biosynthetic processes is reflected, including the changes of leucoanthocyanidin formation, polymerization and destruction. Under no circumstances do these results confirm the hypothesis on the *in vivo* interaction of polyphenols and respiratory enzymes, since according to this hypothesis an inverse correlation should be expected.

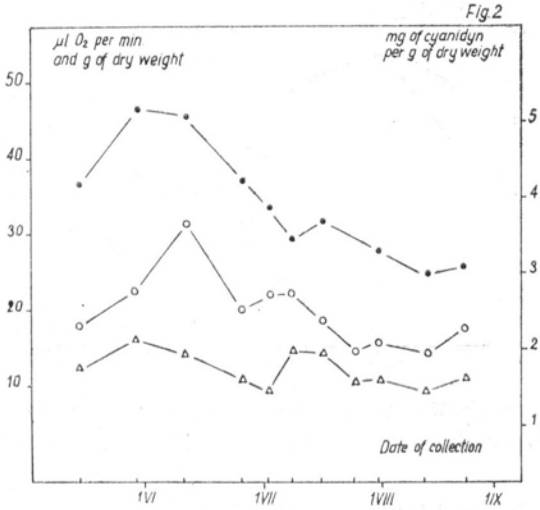

Fig. 2. Changes in respiration rate (●) and in low- (○), and high-molecular weight (△) leucoanthocyanidin content in fresh leaves from the off-year apple-trees during the vegetation period 1968.

A similar result was obtained when the changes in oxygen uptake were compared with the content of leucoanthocyanidins of both groups studied in leaves of on-year trees during the vegetation period.

Comparison of the oxygen uptake rate by the leaves of on- and off--year trees (Fig. 3) shows that during the whole vegetation period respiration of the leaves of on-year trees is by 10—20% more intensive than that of the off-year trees.

Comparison of the changes in total leucoanthocyanidin content (Fig. 1) and of the respiration rate (Fig. 3) shows that in the beginning of July a higher respiration rate corresponds to a relatively low leucoanthocyanidin content in the leaves of on-year trees. At the same time in the leaves of the trees in the off-year, the relatively low oxygen uptake is accompanied by a high content of the polyphenols studied. These results may derive also from the differences in growth intensity of the leaves from on- and off-year trees. They indicate, however, correlations between changes in the content of the polyphenols studied and of some metabolic activities in plants, which can play a role in the processes of bud differentiation.

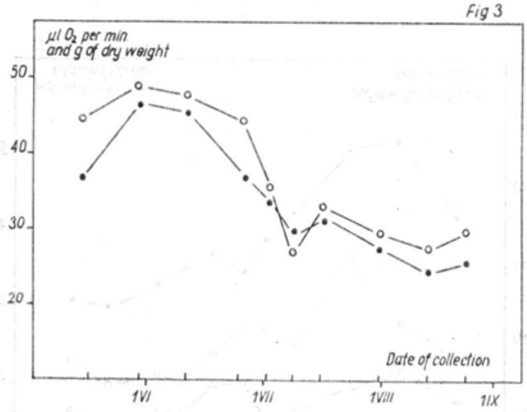

Fig. 3. Changes in respiration rate of fresh leaves from on-year (○), and off-year (●) apple trees during the vegetation period 1968.

This work has been supported in part by Grant FG-Po-236 from the US Department of Agriculture, Agricultural Research Service authorized by Public Law 480.

SUMMARY

1. Comparison of the methods proposed by Swain and Hills (1959) and by Govindarajan and Mathew (1965) for determination of leucoanthocyanidins showed a higher sensitivity of the modification described by the Indian authors. It was also found that alteration of the conditions of the leucoantocyanidin — anthocyanidin conversion causes unequal increases of the yields of various compounds.

2. It was found that low-molecular weight leucoanthocyanidins present in freeze-dried apple-leaves polymerize during their storage.

3. Studies on the changes of leucoanthocyanidin content during vegetation, in fresh leaves of apple tree cv. Landsberska showed that the leucoanthocyanidin level is higher before bud formation in the off-year trees than in the on-year ones.

4. The respiration rate of leaves of on-year trees is by 10—20% higher during the whole vegetation period. This observation cannot be explained by the inhibition of the respiration processes by polyphenols.

Institute of Botany
University Warsaw,
Research Institute of Pomology
Skierniewice

(Entered: April 26, 1969)

REFERENCES

Bielińska M., 1957, Studia nad zawartością azotu i węglowodanów w liściach i pędach jabłoni przemiennie owocujących, Roczn. Nauk Roln. Ser. A 75: 432—520.

Chan B. G., Cain J. C., 1967, The effects of seed formation on subsequent flowering in apple, Proc. Am. Soc. Hort. Sci. 91: 63—68.

Faust M., 1964, The relation between leucoanthocyanins and anthocyanins in McIntosh apples, Proc. Am. Soc. Hort. Sci. 85: 85—91.

Freudenberg K., 1960, Catechine und Hydroxyflavandiole als Gerbstoffbildner, Experientia 16, 101—116.

Govindarajan V. S., Mathew A. G., 1965, Leucoanthocyanidins from anthocyanidins, Phytochem. 4: 985—988.

Grochowska M. J., 1963, Studies on natural growth regulators in apple trees in relation to biennal bearing, Bull. Acad. Polon. Sci. ser. sci. biol. 11; 585—590.

Grochowska M. J., 1964, Identification of the growth inhibitor connected with flower bud formation in apple, Bull. Acad. Polon. Sci. ser. sci. biol. 12: 379—383.

Grochowska M. J., 1968, The influence of growth regulators inserted into apple fruitlets on flower bud formation, Bull. Acad. Polon. Sci. ser. sci. biol. 16: 581—584.

Hancock C. R., Barlow H. W., Lacey H. J., 1961, The behaviour of phloridzin in the coleoptile straight growth test, J. Exp. Bot. 12: 401—408.

Harley C. P., Moon H. H., Regeimbal L. O., 1959, Evidence that the postbloom apple thinning sprays of naphthaleneacetic acid increase blossom-bud formation, Proc. Am. Soc. Hort. Sci. 72: 57—61.

Holowczak J., Kuč J., Williams E. B., 1962, Metabolism in vivo of phloridzin and other compounds by *Venturia inequalis*, Phytopathology 52: 1019—1023.

Hulme A. C., Wooltorton L, S. C., 1962, Separation of the enzymes present in the mitochondrial fraction from apple peel, Nature 196: 388—389.

Jones J. D., Hulme A. C., 1961, Preparation of mitochondria from the peel of apples, Nature 191: 370—372.

Kamiński W., Pieniążek J., 1968, The effect of different growth regulators on the germination of the apple cultivar Antonovka dehusked seeds, Bull. Acad. Polon. Sci ser. sci. biol. 16: 719—723.

Lewak St., Pliszka B., Eichelberger E., 1967, Leucoanthocyanidins of the leaves of apple-tree, Acta Soc. Bot. Pol. 36: 251—259.

Lewak St., 1968, Determination of the degree of polymerization of leucoanthocyanidins, Phytochem. 7: 665—667.

Marrè È., Servettaz O., 1955, Azione di stimulo della florizina sulla fotosintezi e sulla fotolizi da parte di cloroplasti isolato, Nov. Gior. Bot. Ital. 62: 3.

Mayer A. M., 1964, Factors controling activity of phenolase in chloroplasts from sugar beets, Israel J. Bot 13: 74

Pieniążek S. A., Łazniewska J., Soczek Z., Cegłowski M., Dawydko B., Mrozowski M., 1964, Results from ten-eyar experiments on the control biennal bearing of apple trees on the way of thinning by sodium salt of naphthaleneacetic acid, Prace Inst. Sad. 8: 125—134. (in Polish with English summary).

Sarapuu L., 1964, Floridsine as a β — inhibitor and the seasonal dynamics of its metabolic products in the apple tree, Physiol, rast. 11: 607—614. (in Russian with English summary).

Sarapuu L., 1965, Physiological effect of phloridzin as a β-inhibitor during growth and dormancy in the apple tree, Physiol, rast. 12: 134—145 (in Russian with English summary).

Spragg S. P., 1960, The mobilization of betanin in beetroot. In: "Phenolics in Plants in Health and Disease", Pridham J. B. ed. Pergamon Press, Oxford, p. 17—24.

Stendlid G., 1963, The effect of flavonoid compounds on oxidative phosphorylation and on the enzymatic destruction of indoleacetic acid, Physiol. Plant. 16: 110—120.

Stenlid G., 1968, On the physiological effects of phloridzin, phloretin and some related substances upon higher plants, Physiol. Plant. 21: 882—894.

Swain T., Hillis W. E., 1959, The quantitative analysis of phenolic constitutents, Determination of total tannin, lecuoanthocyanin, anthocyanin in plant extracts, J. Sci. Food. Agric. 10: 63.

Tomaszewski M., 1964, The mechanism of synergistic effect between auxin and some natural phenolic substances. In: "Régulateurs naturels de la croissance végétale", Paris, p. 335—351.

Badanie zmian poziomu leukoantocyjanidyn w liściach przemiennie owocujących jabłoni

Streszczenie

1. Porównanie metod oznaczania leukoantocyjanidyn proponowanych przez Swaina i Hillisa (1959) oraz Govindarajana i Mathew (1965) wykazało większą czułość modyfikacji opracowanej przez autorów indyjskich. Stwierdzono ponadto, że zmiana warunków przekształcania leukoantocyjanidyn w antocyjanidyny powoduje nierównomierne zwiększenie wydajności przekształcenia poszczególnych związków.

2. Stwierdzono, że w trakcie przechowywania liofilizowanych liści jabłoni następuje polimeryzacja niskocząsteczkowych leukoantocyjanidyn.

3. Badając zmiany poziomu leukoantocyjanidyn w świeżych liściach jabołni odm. Landsberska w trakcie wegetacji wykazano w okresie poprzedzającym zawiązywanie pąków kwiatowych wyższą zawartość leukoantocyjanidyn w drzewach nie owocujących w danym roku niż w drzewach owocujących.

4. Oddychanie liści drzew owocujących jest o kilkanaście procent intensywniejsze w czasie całego okresu wegetacyjnego. Wykazano, że nie można tłumaczyć tej obserwacji hamowaniem procesów oddechowych przez polifenole.

Instytut Botaniki Uniwersytetu Warszawskiego
Instytut Sadownictwa, Skierniewice