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SOLUBLE PHENOLICS AND ALICYCLIC ACIDS IN AGING POTATO TUBER SLICES

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Abstract. Accumulation of soluble phenolic compounds and free alicyclic acids in aging potato tuber slices was followed during 9 days of wound healing. In general, an intensive accumulation of phenolic compounds on the second and third day of incubation in the light was followed by a gradual decrease of the accumulation rate and a stable level was reached before or on the 5th day of aging. It was for the first time that formation of flavonoids in potato tubers was demonstrated. The accumulation rate of total soluble phenolics and especially that of chlorogenic acid was considerably less pronounced in darkness, and no flavonoids were formed in the dark. Free quinic acid was found already in freshly cut material, free shikimic acid started to accumulate after the 16th hr of aging. Their accumulation curves showed a maximum on the third (quinate) and fifth (shikimate) day, when the content of phenolic compounds was reaching their plateau level. It may be concluded that the activity of the shikimic acid pathway in wound-healing potato tubers is not rate-limiting in phenolic biosynthesis.

Key words: *Solanum tuberosum* L., alicyclic acids, flavonoids, phenolics, wounding.

The metabolism of dormant potato tubers can be activated by cutting the tubers into thin slices and incubating them in a moist atmosphere («aging»). In stressed cells at the wound surface repair processes are set in motion among which there occur several types of responses in phenolic metabolism (Rhodes, Wooltorton, 1978), including enhanced synthesis of both monomeric and polymeric phenolic compounds. The major soluble phenolic compound in potato tuber pulp is chlorogenic (caffeoyl-3-quinic) acid together with some of its isomers (Hanson, Zucker, 1963). Its accumulation in potato tuber discs and light dependence of this process have been investigated in detail since the classic studies of M. Zucker and his co-workers (Zucker, Levy, 1959; Zucker, 1963). Information about accumulation of other soluble phenolics in wounded potato tubers is scanty and it concerns only some ferulic acid esters (Bernards, Lewis, 1992) and amides (Negrel et al., 1993).

Wounding induces most, or all, enzymes of the shikimic acid pathway — primary aromatic biosynthesis (Morris et al., 1989). The time courses for the induction of the first enzyme of the shikimate

pathway (3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase, EC 4.1.2.15) and the key enzyme of phenylpropanoid metabolism, phenylalanine ammonia-lyase (EC 4.3.1.5) are similar (Dyer et al., 1989), suggesting co-ordinate regulation for the biosynthesis of aromatic amino acids and phenolic compounds. However, there is no idea yet about the mechanism of such a co-ordination between these two pathways.

In order to study the actual operation of the shikimate pathway one may block it with a specific inhibitor glyphosate (Steinrücken, Amrhein, 1980) and judge about the synthetic capacity of the pathway by accumulation of shikimic acid which usually does not accumulate in considerable quantities under normal physiological conditions. Before starting such experiments, however, potato tubers must be checked for the occurrence of free shikimic acid in normal wound healing tissues not treated with glyphosate. Another alicyclic acid, quinic acid, is of an interest in potato tubers as well, as it is readily formed from shikimate and is a constituent of chlorogenic acid. The aim of the present work therefore was to establish whether at any stage of the aging process in potato tuber slices one may find free shikimic and/or quinic acid, to follow the concomitant changes in phenolic metabolism, and to consider whether these findings might reflect an eventual co-ordination between the primary aromatic acid pathway and the secondary phenylpropanoid pathway.

MATERIAL AND METHODS

Dormant undamaged tubers of potato (*Solanum tuberosum* L., cv. Ants) grown locally and stored at 5°C in darkness were used in this work. Tubers were washed, peeled (about 5 to 8 mm of peel tissue was removed) and cut with a hand microtome into 0.8-mm slices. The slices were rinsed repeatedly in distilled water and laid on a sheet of thick Whatman paper in plastic boxes for aging in air. A constant descending flow of distilled water through the slightly sloping paper decreased the danger of infection and maintained the relative humidity in the covered boxes near to 100% without creating hypoxic conditions. The boxes were kept at 20°C in darkness or under white fluorescent light (fluence rate 40 $W \cdot m^{-2}$). Samples were taken at times indicated in Figures, weighed, fixed in boiling 96% ethanol, ground in a mortar with ethanol, and extracted twice with diluted (45%) ethanol. The combined extracts were analysed for the content of phenolic compounds and alicyclic acids. The results were expressed on a fresh weight basis.

In the present work, soluble phenolic compounds were defined as the substances detained in a column of polycaprolactam («Ferak», FRG) after applying aqueous extracts (aliquots from the original ethanolic extracts evaporated to dryness and taken up in distilled water) to the column and eluting interfering substances with water. The content of soluble phenolics was calculated from the difference in measured colour reaction of the initial extracts and column effluents (reaction with Folin-Denis reagent, Запрометов, 1974, 75). Chlorogenic acid was used for calibrations.

For revealing the whole set of individual phenolic compounds two-dimensional ascending paper chromatography (I — *n*-butanol-acetic acid-water (BAW), 4 : 1 : 5, upper phase; II — 5% acetic acid; Filtrak FN-15 paper) was used. One-dimensional paper chromatography in BAW was used in quantitative work, followed by spectrophotometrical measurements of diluted ethanolic eluates. Novel flavonoids were characterized by their spectral characteristics (Mabry et al., 1970; Markham, 1982).

Shikimic and quinic acids were determined either in original ethanolic extracts or in aqueous extracts purified from phenolic compounds in a column of polycaprolactam (Тохвер, Пальм, 1986).The two acids were separated by ascending paper chromatography in *t*-butanol-acetic acid-water (3 : 1 : 1) and sprayed first with $NaIO_4$ in acetic acid, thereafter with diluted ethanolic solution of sodium nitroprusside and piperazine adipinate. On the sprayed chromatograms shikimic and quinic acids appeared as yellow spots. They were eluted with water and the absorbance of the eluates was measured at 431 nm. On chromatograms, parallel to potato extracts, standard amounts of authentic shikimic and quinic acids were run and developed, and used as the basis for calculations.

The experiments were run in 4 replicate series. In each series, two sets of 4—5 slices weighing 2 to 3 g were assayed per time of incubation.

RESULTS

Examination of two-dimensional chromatograms of ethanolic extracts revealed a dynamic picture of numerous fluorescent spots. Freshly cut potato pulp contained two isomers of chlorogenic acid together with two weak fluorescent spots of unknown nature. 6 hrs later another pair of isomeric spots showing a blue fluorescence in UV light and having a maximum of absorption 5 nm less than that of chlorogenic acid appeared as shadows. Their content increased during prolonged incubation. During the following days of aging the number of individual compounds increased, reaching its maximum 4 days after cutting. Some substances, however, were present for a few days only. Caffeic acid was found in traces beginning from the 24th hr of incubation. During the 11 days of aging, altogether about 30 individual compounds could be found in extracts from illuminated potato slices. Their number and content in material kept in darkness were less.

Beginning with the 24th hr of illumination three distinct flavonoid spots appeared on chromatograms, though their content did not exceed the lower limit of spectrophotometric measurement till the second or even the third day of illumination. These compounds were not synthesized in darkness. To the best of our knowledge, this is the first report of the occurrence of flavonoids in potato tubers. Therefore we decided to examine the three major flavonoid compounds more closely. Judging by the chromatographic and spectral data presented in Table one may assume that flavonoid No. 3 might be a 3-substituted flavonol with no free hydroxyl groups although $NaOAc/H_3BO_3$ shift in Band I allows a possible B-ring o-dihydroxylation. Both other compounds seem to be flavonol-3-glycosides with free 4'-, 5- and 7-hydroxyl groups. The presence of an additional free 3'-hydroxyl group in compound No. 1 seems possible, as indicated by a shoulder in Band II and the $NaOAc/H_3BO_3$ shift in Band I.

A full identification of the detected flavonoids was too far from the purpose of the present work. However, we checked three other local potato cultivars (Vigri, Eba, Roosa) for a possible occurrence of flavonoid compounds in their aging tuber slices. Compound No. 1 was easily recognizable in two and No. 2 — in all the three of them, and there were several typical dark purple (turning bright yellow in ammonia vapours) flavonoid spots on chromatograms at and above the position of the compound No. 3. Therefore the occurrence of flavonoids in aged potato slices seems to be a common phenomenon overlooked before.

Chromatographic and spectroscopic characteristics of potato tuber flavonoids

Characteristic		Compound 1	2	3
R_f value in BAW		0.37 to 0.40	0.41 to 0.44	0.50
Spot colour in UV		dark purple	dark purple	dark purple
UV/NH_3		orange yellow	yellow	yellow
Colour after spraying:				
Folin-Denis/NH_3		strong blue	faint blue	blue
Diazotized sulphanilic acid		strong yellow	faint yellow	faint yellow
$FeNH_4(SO_4)_2$		strong greenish-brown	reddish-brown	faint brownish-green
Ethanolic UV spectra:				
λ_{max}, nm	Band II	259, 267sh+	225sh, 245sh, 269	260, 270sh
	Band I	303sh, 362	354	368
Bathochromic shifts, nm:				
NAOH	Band II	23	10	0
	Band I	62	58	2
		No decrease in intensity with time		
$AlCl_3$	Band II	12 to 16; 366 sh	2	0
	Band I	48	44; 305sh, 350sh	0
$AlCl_3$/HCl		No change from $AlCl_3$ spectra		
NaOAc++	Band II	11	7	0
	Band I	48	22	8
$NaOAc/H_3BO_3$	Band II	11— 7= 4	7— 7=0	0
	Band I	48—27=21	22—19=3	8+13=21

\+ — shoulder;
++ — sodium acetate.

The total amount of soluble phenolic substances in aging illuminated slices started to increase soon after cutting and reached a stable level on the 5th day of incubation (Fig. 1, curve *1*). In experiments carried out at the end of the dormancy of tubers (still before any visible growth of sprouts) this stable level of soluble phenolics in illuminated material was twice as high as in late autumn (curve *3*). In darkness the stage of intensive accumulation was less pronounced and did not differ in autumn or spring (curves *2* and *4*).

Accumulation of chlorogenic acid in illuminated slices (Fig. 2, curve *1*) parallelled that of total soluble phenolics accounting for half of the amount of the whole phenolic complex. In tissues kept in darkness, however, after a rise of up to the 40 th hr, chlorogenic acid content started to decline (curve *2*), falling even below its initial value in freshly cut material, and forming by the 9th day of aging less than 10% of total phenolics. Chlorogenic acid accumulation in slices of tubers stored till spring (curves *3* and *4*) revealed no tendency to stop either in the light or in darkness.

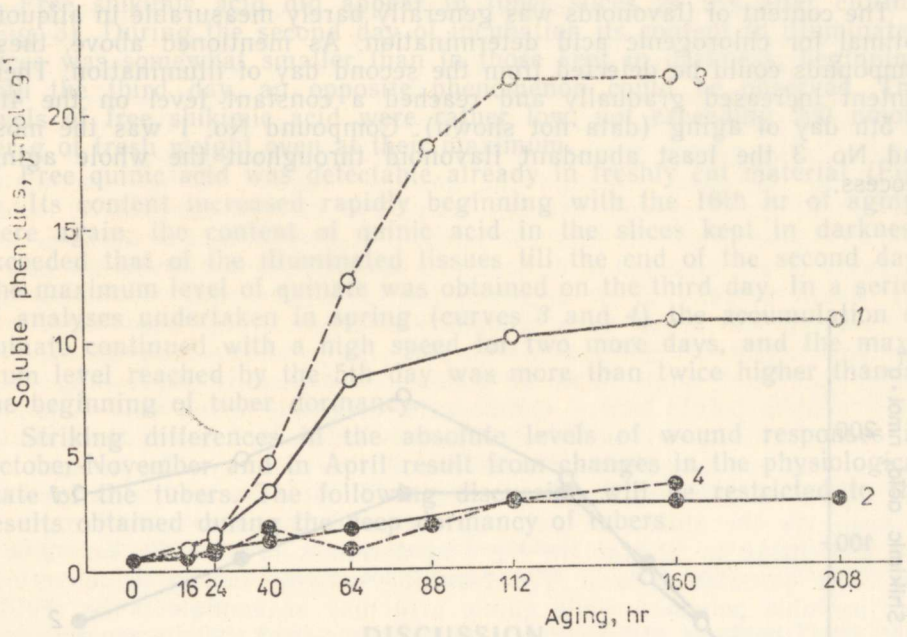

Fig. 1. Accumulation of soluble phenolics in aging potato slices in the light (*1, 3*) and in the dark (*2, 4*) cut from tubers at the beginning (solid line) and at the end (broken line) of storing period.

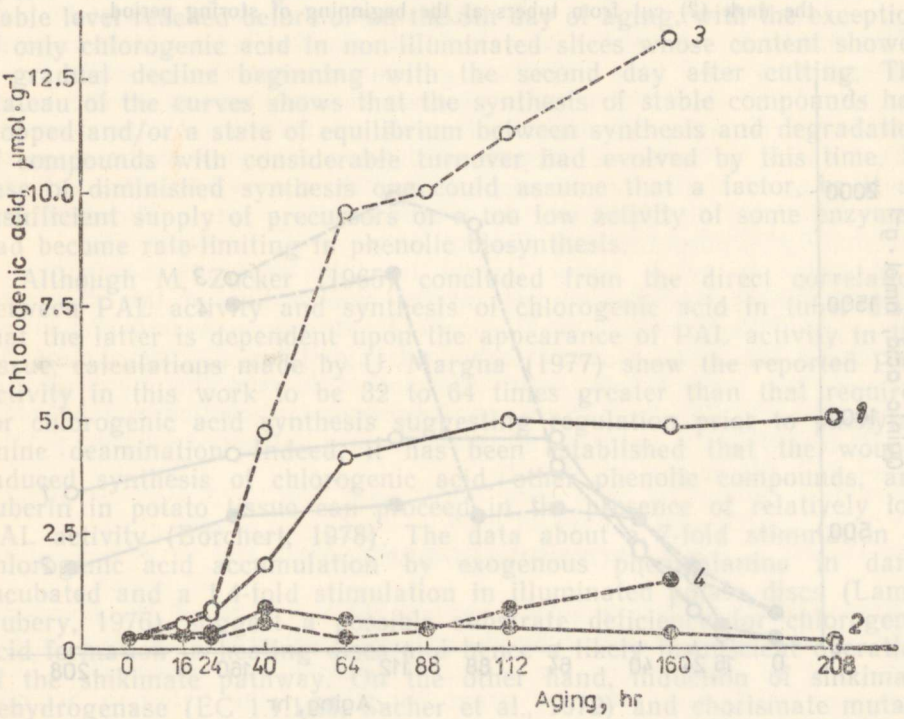

Fig. 2. Accumulation of chlorogenic acid in aging potato slices in the light (*1, 3*) and in the dark (*2, 4*) cut from tubers at the beginning (solid line) and at the end (broken line) of storing period.

The content of flavonoids was generally barely measurable in aliquots optimal for chlorogenic acid determination. As mentioned above, these compounds could be detected from the second day of illumination. Their content increased gradually and reached a constant level on the 4th or 5th day of aging (data not shown). Compound No. 1 was the most and No. 3 the least abundant flavonoid throughout the whole aging process.

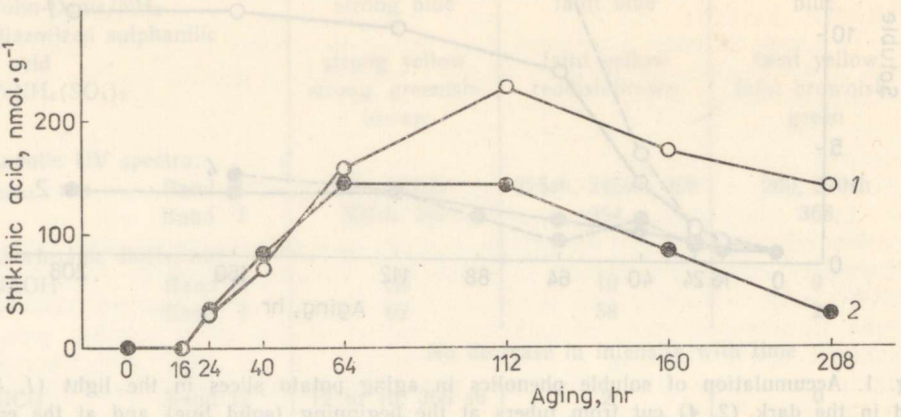

Fig. 3. Accumulation of shikimic acid in aging potato slices in the light (*1*) and in the dark (*2*) cut from tubers at the beginning of storing period.

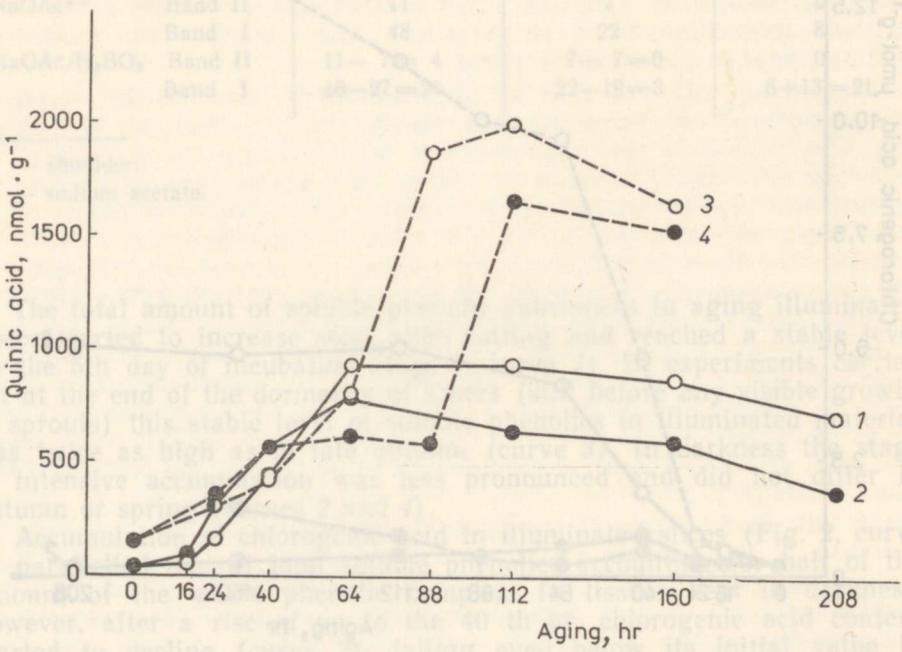

Fig. 4. Accumulation of quinic acid in aging potato slices in the light (*1, 3*) and in the dark (*2, 4*) cut from tubers at the beginning (solid line) and at the end (broken line) of storing period.

Free shikimic acid did appear in tuber slices 24 hrs after cutting (Fig. 3). During the second day of incubation its content in illuminated slices was somewhat smaller than in those kept in darkness; beginning with the third day, an opposite phenomenon could be observed. The levels of free shikimic acid were rather low, not exceeding 200 nmols per g of fresh weight even at their maximum.

Free quinic acid was detectable already in freshly cut material (Fig. 4). Its content increased rapidly beginning with the 16th hr of aging. Here again, the content of quinic acid in the slices kept in darkness exceeded that of the illuminated tissues till the end of the second day. The maximum level of quinate was obtained on the third day. In a series of analyses undertaken in spring (curves *3* and *4*) the accumulation of quinate continued with a high speed for two more days, and the maximum level reached by the 5th day was more than twice higher than at the beginning of tuber dormancy.

Striking differences in the absolute levels of wound responses in October-November and in April result from changes in the physiological state of the tubers. The following discussion will be restricted to the results obtained during the deep dormancy of tubers.

DISCUSSION

Accumulation curves of total soluble phenolics and the individual phenolic compounds examined in the present work are all of a similar shape: an intensive accumulation on the second and third days of incubation followed by a gradual decrease of the accumulation rate and a stable level reached before or on the 5th day of aging, with the exception of only chlorogenic acid in non-illuminated slices whose content showed a gradual decline beginning with the second day after cutting. The plateau of the curves shows that the synthesis of stable compounds had stopped and/or a state of equilibrium between synthesis and degradation of compounds with considerable turnover had evolved by this time. In case of diminished synthesis one could assume that a factor, be it an insufficient supply of precursors or a too low activity of some enzymes, had become rate-limiting in phenolic biosynthesis.

Although M. Zucker (1965) concluded from the direct correlation between PAL activity and synthesis of chlorogenic acid in tuber discs that the latter is dependent upon the appearance of PAL activity in the tissue, calculations made by U. Margna (1977) show the reported PAL activity in this work to be 32 to 64 times greater than that required for chlorogenic acid synthesis suggesting regulation prior to phenylalanine deamination. Indeed, it has been established that the wound-induced synthesis of chlorogenic acid, other phenolic compounds, and suberin in potato tissue can proceed in the presence of relatively low PAL activity (Borchert, 1978). The data about a 7-fold stimulation of chlorogenic acid accumulation by exogenous phenylalanine in dark-incubated and a 1.4-fold stimulation in illuminated potato discs (Lamb, Rubery, 1976) suggest a possible substrate deficiency for chlorogenic acid formation in healing discs and hence a likely insufficient operation of the shikimate pathway. On the other hand, induction of shikimate dehydrogenase (EC 1.1.1.25, Sacher et al., 1972) and chorismate mutase (EC 5.4.99.5, Kuroki, Conn, 1988) not dependent on illumination was reported to occur in wounded tuber discs. This should lead to an increase in the levels of phenylalanine and tyrosine, and might be a sufficient prerequisite for accelerated phenolic synthesis.

Free shikimic acid detected in potato slices seems to represent its active metabolic pool reflecting the intensity of the operating pathway. Its occurrence, both in the light and in darkness, would indicate that the shikimate pathway was functioning at a speed not only satisfying the needs of aromatic biosyntheses but enabling even accumulation of free shikimic acid during the whole period of aging examined. This may indeed be the case, although the absolute amount of free shikimate detected at its maximum reaches only 2% of the amount of total phenolics present in the tissues. Further studies with the blockage of the pathway are expected to demonstrate whether the activity of the pathway would actually allow accumulation of surplus free shikimate, or this phenomenon might be a secondary effect resulting from liberation of shikimate from its conjugates with phenylpropanoids during the experiment. The latter point must be considered especially concerning free quinic acid in potato tubers. Quinic acid, although linked with shikimic acid biogenetically by a reversible reaction over 5-dehydroshikimic acid, is not an immediate intermediate of the pathway. The amount of free quinic acid in potato tissues depends not only on its formation *de novo* via the shikimate pathway but on a turnover of chlorogenic acid amounting to 50 $nmol \cdot hr^{-1} \cdot g^{-1}$ in darkness (Taylor, Zucker, 1966) and other quinates as well. The resulting caffeoyl moiety is converted into insoluble polymers while quinic acid may accumulate or be reutilized. Thus the level of free quinic acid may reflect the discrepancy between the utilization of chlorogenate resulting in the liberation of quinate and the reutilization of the latter in synthesis of its conjugates. Irrespective of the source of free quinate, the presence of about 1000 $nmol \cdot g^{-1}$ of this compound in illuminated tissue when the accumulation of chlorogenic acid has stopped before reaching the level of 5000 $nmol \cdot g^{-1}$ suggests that there are still sufficient supplies of quinate available for its synthesis. In the slices kept in darkness the level of chlorogenic acid starts to drop from 900 $nmol \cdot g^{-1}$ on the second day while there are almost 600 nmols of quinate present, and the content of the latter will even increase by 200 nmols during the next 24 hrs. As the decrease in the content of both free alicyclic acids occurs only after the stage of intensive accumulation of phenolics, it may be assumed that the activity of the shikimic acid pathway in wound healing potato slices is not rate-limiting in phenolic biosynthesis.

In order to get a better insight into the processes occurring in wounded tubers, and to comprehend the co-operation of the shikimate pathway and the general phenylpropanoid pathway in wound responses, data about the actual flow of carbon through these pathways must be obtained. We hope to get some information in this line by studying incorporation of labelled precursors into wound phenolics and by estimating the synthetic potential of the shikimate pathway in aging potato tissues with the help of glyphosate.

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LAHUSTUVAD FENOOLSED ÜHENDID JA ALITSÜKLILISED HAPPED VANANEVATES KARTULILÕIKUDES

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Kartulilõikude inkubeerimisel niiskes atmosfääris (nn. vananemisel) jälgiti lahustuvate fenoolsete ühendite ja alitsükliliste hapete akumulatsiooni haavatud kudedes üheksa päeva jooksul. Üldjuhul toimus valgustatud lõikudes teisel-kolmandal päeval intensiivne fenoolsete ühendite kogunemine, mis järk-järgult aeglustus ja saavutas viiendaks päevaks platoo. Esmakordselt tehti kindlaks flavonoidide moodustumine kartulimugulates. Pimeduses oli fenoolsete ühendite, sealhulgas eriti klorogeenhappe, kogunemiskiirus tunduvalt väiksem. Flavonoide ei moodustunud pimedas üldse. Vaba kiinahapet esines juba vastlõigatud materjalis, vaba šikimihape hakkas kogunema esimese päeva lõpul. Nende ühendite kogunemist kajastavatel kõveratel ilmnes maksimum kolmandal (kinaat) ja viiendal (šikimaat) päeval, kui fenoolsete ühendite sisaldus saavutas stabiilse taseme. Toodust võib järeldada, et fenoolsete ühendite biosünteesi vigastatud kartulimugulates ei piira šikimaatse tee aktiivsus.

РАСТВОРИМЫЕ ФЕНОЛЬНЫЕ СОЕДИНЕНИЯ И АЛИЦИКЛИЧЕСКИЕ КИСЛОТЫ В СТАРЕЮЩИХ ОТРЕЗКАХ КЛУБНЕЙ КАРТОФЕЛЯ

Лембе ЛААНЕСТ, Антс ТОХВЕР, Эльмо ПАЛЬМ

Исследовали накопление растворимых фенольных соединений и алициклических кислот в стареющих (выдерживаемых во влажной атмосфере) отрезках клубней картофеля в течение 9 дней после поражения. Как правило, интенсивное накопление фенольных соединений начиналось на свету примерно 24 ч после приготовления отрезков и длилось двое суток, после чего к пятому дню постепенно достигался постоянный уровень этих соединений. Впервые был обнаружен биосинтез флавоноидов в клубнях картофеля. В темноте скорость накопления суммарной фракции растворимых фенольных соединений и в особенности хлорогеновой кислоты была значительно ниже. Флавоноиды в темноте вообще не образовались. Свободная хинная кислота встречалась уже в свежеприготовленных отрезках, накопление же свободной шикимовой кислоты начиналось в конце первых суток. Кривые накопления этих кислот имели максимум на третий (хинат) и пятый (шикимат) день, когда содержание фенольных соединений достигало постоянного уровня. Полученные данные свидетельствуют о том, что активность шикиматного пути в пораженных клубнях картофеля не является лимитирующим при биосинтезе фенольных соединений.