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Suberization: Inhibition by Washing and Stimulation by Abscisic Acid in Potato Disks and Tissue Culture[1]

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ABSTRACT

Wounding of potato (*Solanum tuberosum* L.) tubers results in suberization, apparently triggered by the release of some chemical factor(s) at the cut surface. Suberization, as measured by diffusion resistance of the tissue surface to water vapor, was inhibited by mM concentrations of indoleacetic acid, unaffected by mM concentrations of traumatic acid, severely inhibited at μM concentrations of cytokinin, but stimulated by abscisic acid (ABA) at 10^{-4} M. Thorough washing of potato disks up to 3 to 4 days after cutting resulted in severe inhibition of suberization as measured both by diffusion resistance and by the amount of the octadecene diol generated by hydrogenolysis ($LiAlH_4$) of the tissue. Disks washed after 4 days did not show any inhibition of suberization. High performance liquid chromatographic analysis of the wash from fresh potato disks showed that about 14 ng of ABA was released into the wash per g of tissue. The amount of ABA released increased with time up to 4 to 6 hours of washing. The maximal amount of ABA was washed out after aging for 24 hours and after 2 days of aging ABA could no longer be found in the surface wash of the disks. Addition of ABA to the media of potato tissue cultures resulted in suberin formation whereas control cultures contained little suberin. The effect of ABA on suberization in the tissue cultures was shown to be linearly concentration-dependent up to 10^{-4} M and a linear increase in suberin formation was seen up to about 8 days of culture growth on the media containing 10^{-4} M ABA. From these results it is proposed that during the early phase of wound-healing ABA plays a role in triggering a chain of biochemical processes which eventually (in about 3 to 4 days) result in the formation of a suberization-inducing factor, responsible for the induction of the enzymes involved in suberin biosynthesis.

Suberization involves deposition of a waterproofing polymeric material at the cell wall (6, 11). From the limited amount of chemical information available it has been proposed that the aliphatic components of suberin are esterified to a phenolic matrix resulting in a barrier to diffusion of water vapor (4). The major aliphatic components of suberin of plants thus far examined are ω-hydroxy fatty acids and dicarboxylic acids. In several plants, including potatoes, ω-hydroxyoleic acid and the corresponding dicarboxylic acid constitute the major aliphatic components of suberin (5, 8).

Suberization appears to be the general response of plants to wounding irrespective of the chemical nature of the natural protective polymer of the tissue (2). Wound-healing potato disks undergo suberization and the time course of deposition of suberin in this system has been determined (7). From such studies as well as from studies on the appearance of one of the enzymes involved in the biosynthesis of the aliphatic components of suberin (1), it appeared that the transcription and translation processes involved in the formation of the aliphatic components of suberin occurred 3 to 4 days after wounding. The biochemical events which occur prior to this period are not known. However, since suberization is a wound-induced process, a signal generated at or near the wound is suspected to be responsible for induction and suberization. In this paper we report that thorough washing of the potato disks inhibits suberization and that ABA stimulates suberization in potato disks and in potato tissue culture. It is proposed that ABA triggers the formation of a suberization-inducing factor.

MATERIALS AND METHODS

Materials. Potato tubers (*Solanum tuberosum* L., cv. Russet Burbank or White Rose) were purchased from a local grocery store and stored at 7 C. Cylindrical sections of tissue (2 cm long, 1 cm diameter), cut with a cork borer and razor blade, were quickly rinsed and used as such or after further treatments. The tissue disks were kept at 22 C on rubberized mesh in wide mouth gallon jars, through which 0.6 liter/hr of moist air was passed to provide optimal conditions for suberization. All utensils were autoclaved prior to use.

Potato tissue culture was initiated from pieces of Russet Burbank potato tubers which were cut under aseptic conditions and transferred to growth media. The resulting potato callus was maintained on modified Murashige-Skoog growth medium containing the Murashige-Skoog mineral salts (9) and the following organic constituents (T. Murashige, personal communication): sucrose, 30 g/l; inositol, 100 mg/l; 2,4-dichlorophenoxyacetic acid, 3 mg/l; thiamine-HCl, 0.4 mg/l; kinetin (6-furfurylaminopurine), 0.03 mg/l; 8 g/l Bacto agar (Difco). After the medium was adjusted to pH 5.7 and the agar dissolved, 50-ml quantities were placed in 125-ml Erlenmeyer flasks which were sterilized by autoclaving at 120 C for 20 min. ABA (Sigma Chemical Co.) was mixed with the sterilized media before it cooled into a gel. ABA was added in 200-μl volumes of a 5% DMSO solution (dissolved in DMSO and then diluted with water), which had been sterilized by passing through a Millipore filter, 0.22 μm; to the control culture 200 μl of 5% DMSO was added. The callus cultures were incubated at 27 C in the dark and transferred to fresh medium every 30 days.

Measurement of Suberization as Determined by Diffusion Resistance. The resistance of the tissue surface to diffusion of water vapor (R_t) has been shown to be a good measurement of suberization (7). The diffusion resistance (R_t) of the potato tissue cylinders was calculated from the following formula (10):

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$$R_t = \frac{\rho_t - \rho_a}{E} - R_a$$

where ρ_t = vapor density of the tissue in g/cm³ = $RH_{tissue} \times \rho_v$. RH_{tissue} (the relative humidity of the tissue) is 100 and ρ_v was calculated from $\rho_v = 18e/RT$; e, vapor pressure; T, temperature (°K); and R, the gas constant. ρ_a = vapor density of the air in g/cm³ = $RH_{air} \times \rho_v$, where RH_{air} is the relative humidity of the air. E = evaporation per unit surface area per unit time (g/sec·cm²) as calculated from the measured weight loss of 20 tissue cylinders during 1 hr of exposure to the room atmosphere. R_a = resistance of the air to water vapor diffusion (sec/cm) as determined by measuring water vapor loss of unsuberized tissue using freshly cut tissue cylinders where $R_t = 0$.

Measurement of Suberization by Gas Chromatography. Suberization was measured in terms of the octadecene-1,18-diol generated by hydrogenolysis of the suberized tissue. The tissue cylinders were freeze-dried, ground to a fine powder, and 0.5-g portions of the powder were subjected to hydrogenolysis. The potato tissue cultures were taken from the culture media and soaked overnight in chloroform-methanol (2:1, v/v). The residue was filtered on a small Büchner funnel, washed twice with chloroform-methanol, then twice with tetrahydrofuran, and allowed to air-dry. After grinding the residue to a fine powder, it was weighed and then subjected to hydrogenolysis by refluxing the powdered tissues with excess $LiAlH_4$ in 15 ml of tetrahydrofuran for 48 hr. After decomposing the excess $LiAlH_4$ by adding the reaction mixture to 50 ml of water, 10 ml of concentrated HCl was added and the lipid products were recovered from the reaction mixture by extraction with chloroform (3 × 75 ml). The chloroform extract was evaporated to dryness under reduced pressure, and after the addition of a measured quantity of hexadecane-1,16-diol as an internal standard, the soluble lipid was applied to 0.5-mm layers of Silica Gel G on TLC plates (20 × 20 cm). The chromatograms were developed in diethylether-hexane-methanol (20:5:1, v/v), visualized with 2′,7′-dichlorofluorescein, and the diol fractions were recovered by elution of the silica gel with a 2:1 mixture of chloroform-methanol. The solvent was evaporated under N_2, and the residue was heated with 0.2 ml of N,O-bis(trimethylsilyl) acetamide at 90 C for 15 min. The excess reagent was evaporated under N_2, and aliquots of the resulting trimethylsilyl derivatives dissolved in chloroform were injected into a Varian gas chromatograph equipped with a glass column (183 × 0.3 cm) packed with 5% OV-101 on 80/100 mesh Gas-chrom Q. Part of the effluent of the gas chromatograph was passed through a Biemann separator interface into a Perkin-Elmer Hitachi RMU 6 D mass spectrometer. Mass spectra were recorded at an ionizing voltage of 70 ev.

ABA Analysis. ABA was extracted from aqueous potato tissue extracts and isolated by HPLC[3] according to the method of Sweetser and Vatvars (12). An initial ether extraction of the aqueous potato tissue extract, adjusted to pH 8, removed many impurities. The aqueous phase was then adjusted to pH 2.8 and extracted with 3 volumes of ether. The combined ether extracts were evaporated to give the free ABA fraction. The remaining aqueous phase was adjusted to pH 11 and incubated at 60 C for 1 hr under a stream of N_2. After adjusting the pH back to 2.8, this aqueous phase was extracted with 3 volumes of ether to recover the bound ABA fraction.

The free and bound ABA extracts were taken up in methanol and injected into a Waters model ALC/GPC 244 HPLC with a μ-Bondapak C_{18} column (25 × .64 cm). ABA was separated from other impurities by eluting the column with a programmed solvent curve from 25 to 50% (v/v) methyl alcohol-H_2O adjusted to pH 2.6 with H_3PO_4. The ABA fractions were collected, evaporated, and reinjected into the HPLC with 37% methyl alcohol in H_2O (pH 2.6) as the solvent system. A UV detector monitored the absorbance of the column effluent at 254 nm. The ABA peak area was estimated by triangulation and the peak areas were converted to mass using authentic ABA standards.

[3] Abbreviation: HPLC: high performance liquid chromatography.

RESULTS AND DISCUSSION

Effect of Plant Hormones on Suberization of Potato Disks. It is apparent that cutting the potato tuber releases some chemical factor(s) which induces the cells at the cut surface to synthesize suberin. This factor may possibly be one of the plant hormones known to induce plant cell differentiation. Tissue cylinders, cut from potato tubers, were incubated in various concentrations of plant hormones and the suberization of these hormone-treated disks was measured by diffusion resistance of the tissue surface to water vapor (Table I). Indoleacetic acid was inhibitory at fairly high (mM) concentrations but showed little effect at 0.1 mM and lower concentrations. Traumatic acid up to 0.1 mM had little effect on suberization. Cytokinin showed severe inhibition of suberization; even at μM concentrations more than 50% inhibition was obtained under the present experimental conditions. On the other hand ABA showed a significant enhancement of suberization in that with ABA suberization started earlier than in the untreated tissue. For example, with 10^{-4} M ABA suberization reached at least one-third of the maximal level by the 4th day whereas in the control tissue suberization had reached only about one-eighth of the maximal level; ABA did not alter the maximal level of suberization. Optimal amounts of enhancement of suberin synthesis by ABA were observed at 10^{-4} M. Measurement of suberization either as diffusion resistance or as the amount of the aliphatic components of suberin gave identical results.

Effect of Washing Potato Disks on Suberization. Since suberization appears to be a general response to wounding it was suspected that some chemical, produced as a result of wounding at or near the wound, might trigger the suberization process. Furthermore, during our efforts to determine the effect of various chemicals on suberization it was observed that water-treated controls were not as well suberized as the unwashed tissue. Therefore we examined the effect of thorough washing of potato disks on suberization (Fig. 1). Suberization was severely inhibited by a thorough washing of the tissue disks immediately after preparations of the disks. The maximal amount of aliphatic components of suberin found in the tissue and the maximal resistance of the tissue surface to water vapor reflected the inhibition of suberization brought about by wash-

Table I. Effect of Hormones on Suberization in Potato Disks

For each experimental condition 30 disks, which had been aged for 24 hr after cutting, were incubated for 4 hr at 30 C in 20 ml of hormone solution in 250-ml Erlenmeyer flasks on a gyrating waterbath.

Hormone	Conc.	Diffusion Resistance Day 2	Day 3	Day 4	Day 5	Day 6
IAA	10^{-3}M	3.3	3.5	3.7	4.6	16.3
	10^{-4}M	3.3	3.1	4.7	10.3	33.5
	10^{-5}M	3.5	3.4	6.1	10.1	32.8
Cytokinin	10^{-4}	3.3	3.2	3.8	-	-
	10^{-5}	3.0	3.3	3.8	5.0	6.8
	10^{-6}	3.1	3.2	4.2	8.3	15.6
ABA	10^{-4}	3.1	5.7	10.8	16.8	31.6
	10^{-5}	3.3	3.8	6.0	9.4	21.9
	10^{-6}	3.8	3.5	3.9	6.9	14.5
Traumatic Acid	10^{-4}	3.1	3.3	4.5	9.0	28.6
	10^{-5}	3.4	3.6	5.1	12.1	29.7
	10^{-6}	3.4	3.4	6.1	12.6	21.3
H_2O control		3.5	3.6	4.5	11.9	32.4

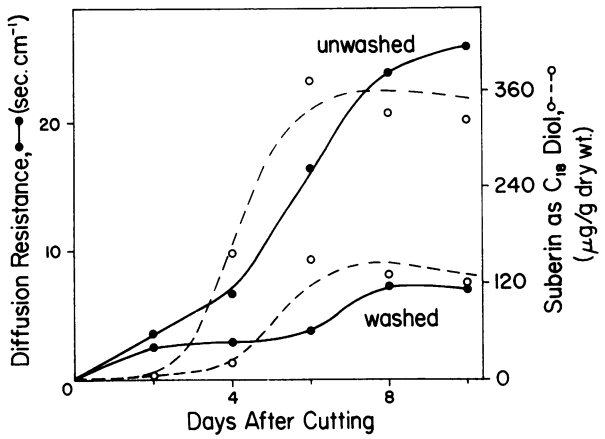

FIG. 1. Time course of suberization in washed and unwashed potato disks. From a well mixed batch of 200 freshly cut disks 100 disks were washed in 150 ml of water for 3 hr at 30 C in gyrating water bath while the other 100 disks served as the unwashed control group. Both groups were subjected to suberizing conditions indicated under "Materials and Methods." At 2-day intervals 20 disks were taken from each group and their diffusion resistance and the amount of octadecene diol generated from them by hydrogenolysis were determined.

ing. These results suggested that a suberization-inducing factor might have been removed by the washing.

If a factor generated at the wound is responsible for suberization, it is essential to determine when this material is generated and how long this factor needs to be present to induce suberization. In order to investigate this, disks were thoroughly washed various periods after slicing and the degree of suberization 8 days after cutting was determined (Table II). The inhibitory effect of washing on suberization was obvious provided the disks were washed within 3 to 4 days after cutting. Little inhibition of suberization could be observed in tissue washed after this period of aging. These results strongly suggest that the factor(s) responsible for induction of suberization is generated at or near the wound but the continued presence of the factor(s) at or near the surface is needed until induction of the enzyme occurs 3 to 4 days after wounding. Experiments with inhibitors of the synthesis of nucleic acids and proteins had already indicated that the transcriptional and translational processes involved in the biosynthesis of the aliphatic components of suberin occurred 3 to 4 days after wounding (1). Time course of incorporation of [1-^{14}C]acetate and [1-^{14}C]oleic acid into suberin components also was consistent with the above concept (3). Measurement of the activity levels of ω-hydroxy acid dehydrogenase, an enzyme involved in the synthesis of characteristic suberin components, namely dicarboxylic acids, also indicated that the induction of this enzyme occurred 3 to 4 days after cutting. Thus, all of these observations collectively suggest that factors responsible for the induction of enzymes involved in the biosynthesis of the aliphatic components of suberin can be removed by thoroughly washing the wound any time prior to the induction.

In every case quantitation of suberization by diffusion resistance measurements agreed quite well with the results obtained by measuring the amount of octadecene-1,18-diol generated by hydrogenolysis ($LiAlH_4$) of the suberin. However, as can be seen in Table II thorough washing of the disks immediately after cutting resulted in a low diffusion resistance but there was no corresponding inhibition of the formation of the aliphatic constituents. This difference may be due to an inhibition of the synthesis of the phenolic matrix of suberin, which is formed in the early stages of suberization. According to the model proposed for suberin structure the aliphatic components are esterified to the phenolic matrix to generate the waterproofing character of suberin (4). Even though washing during this early period may not severely inhibit the formation of the aliphatic components, inhibition of synthesis of the phenolic matrix would prevent the formation of the suberin layer as the aliphatic components would not be able to cross-link the phenolic polymer and thus waterproof the layer. The possibility that the aliphatic component, for some reason, failed to esterify to the phenolic matrix cannot be ruled out.

Detection of ABA in Potato Tissue Washes. As discussed earlier, ABA has a stimulatory effect on suberization. The inhibition of suberization brought about by thorough washing was partially restored by soaking the washed disks in a solution of ABA for 1 hr at 30 C. The partial restoration of suberization by ABA was concentration-dependent with an optimal level at about 10^{-4} M. These observations suggested the possibility that water-soluble ABA might be removed from the potato disks by washing, resulting in the loss of suberization. To examine this possibility, the wash was examined for ABA by a technique involving ether extraction of the acidified wash and HPLC of the ether extract. A peak was detected by UV absorbance at 254 nm at a retention time equivalent to that of the authentic ABA *cis-trans* standard, the naturally occurring isomer (Fig. 2A). The ABA fractions from several samples were pooled and repurified by HPLC (Fig. 2B) and its identity was confirmed by MS. The mass spectrum of an authentic sample of ABA and that isolated from potato tissue by HPLC showed molecular

Table II. Effect of Washing on Suberization of Aged Potato Disks

The potato disks were cut and aged for the indicated periods under suberizing conditions and at each interval 70 disks (≈100 g) were washed in 150 ml of water at 30C for 3 hr. Diffusion resistance and the amount of suberin as octadecene diol were measured as described in Materials and Methods. Standard devation for diffusion resistance measurements ranged from 6 to 9% and for diol measurements from 7 to 16%.

Day Washed	Diffusion Resistance sec.cm^{-1}	Suberin μg C_{18} diol/g dry wt
0	11	165
1	13	80
2	10	110
3	17	75
4	23	105
5	29	220
6	29	250
Unwashed	29	260

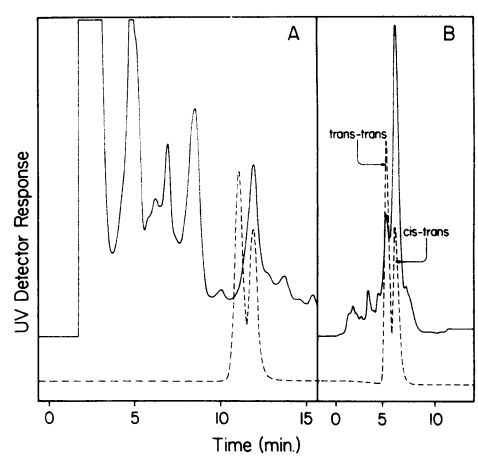

FIG. 2. Isolation of ABA by HPLC. ABA extracts were injected into a Waters HPLC equipped with a μ-Bondapak C_{18} column (25 × 0.64 cm) and eluted at a flow rate of 2 ml/min with a programmed solvent curve from 25 to 50% (v/v) methanol/water adjusted to pH 2.6 with H_3PO_4 for the initial separations (A) and with 37% methanol-water (pH 2.6) for final separations (B). Authentic ABA standards were chromatographed under the same conditions and their retention times are also indicated (broken lines).

ions at m/e 264 and major fragment ions at m/e 246, 208, 190, 162, 131, and 91. Application of this technique to homogenates of fresh potato tuber tissue showed that White Rose potatoes contained 72 ng of ABA/g of tissue, a value which is comparable to the range of ABA content reported for other plant tissues (12). The level of ABA removed by washing of the potato disks increased in a linear fashion up to 4 hr of washing (Table III). When the washes from disks aged for 0 to 6 days were examined for ABA content, only the extracts of disks washed immediately after cutting or those aged for 1 day contained measurable amounts of ABA. The level of bound ABA in all of the tissue-washed extracts was negligible compared to the level of ABA in the free form, and mild alkaline (pH 11) treatment of the tissue surface did not release any additional amount of ABA. These results seem to rule out the possibility that the increase in ABA in the tissue wash was due to the release of ABA from a bound form at the surface unless the linkage involved is stable to mild alkaline treatment and is released by the action of some hydrolytic enzyme. *De novo* synthesis of ABA cannot be ruled out as a possible source as no experiments have been performed to test this possibility.

Effect of ABA on Suberization in Potato Tissue Cultures. If ABA is involved in the induction of suberization during wound-healing, as suggested by the results discussed above, ABA might also trigger suberization in potato tissue culture. To test this possibility ABA was added at different concentrations to the culture media of potato tissue cultures. The tissues grown in the ABA-containing medium showed a slight inhibition of growth and they were brownish in color whereas control cultures were white or slightly colored. After 10 days of growth the soluble lipids were thoroughly extracted from the tissue and the insoluble material was examined for the aliphatic components of suberin after hydrogenolysis ($LiAlH_4$) using a previously developed gas chromatographic technique. Measurable amounts of octadecene-1,18-diol were found in the hydrogenolysates from ABA-treated tissues but very little diol was obtained from control tissue (Fig. 3). The identity of octadecene-1,18-diol was confirmed by MS as previously described (13). This diol is known to be the major aliphatic product derived by reductive cleavage of potato suberin which contain 18-hydroxyoleic acid and the corresponding dicarboxylic acid as major components. The time course of induction of suberization by ABA in the tissue culture (Table IV) indicated that the amount of aliphatic components in the ABA-treated tissue increased up to about 8 days under the present experimental conditions; the amount accumulated during 3 days in ABA-treated tissue was nearly three times as much as that found in 10-day-old control tissue. In the tissue cultures increasing the concentration of ABA to 10^{-4} M resulted in increased amounts of suberin and subsequent increases to 3×10^{-4} M resulted in no further increase (Fig. 4). The inhibition of growth caused by ABA was relatively small when compared to the degree of induction of suberization. These results clearly demonstrate the ability of ABA to induce suberization. As the medium used contained cytokinin, which is inhibitory to suberization, ABA stimulation of suberization might be in part due to the counteraction of suppression of suberization caused by cytokinin. In any case the mechanism by which ABA affects suberization remains unknown.

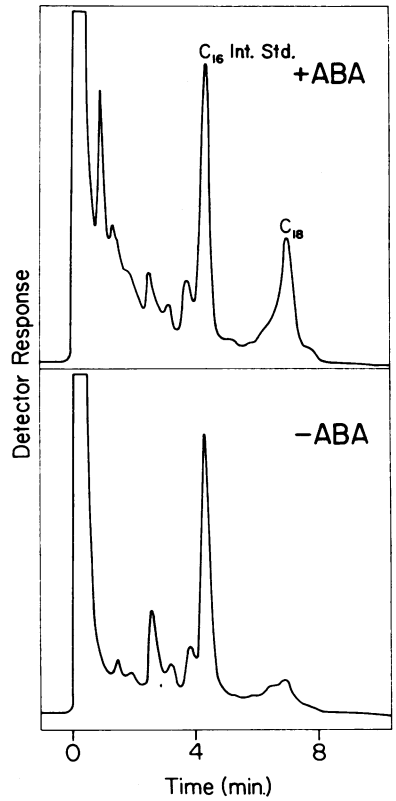

FIG. 3. Gas-liquid chromatogram of the diol fraction (as trimethylsilyl ether) obtained from the hydrogenolysate of ABA-treated and untreated potato tissue cultures. The chromatography was performed on a glass column (183 × 0.31 cm) packed with 5% OV-101 on 80 to 100 mesh Gas-chrom Q. The column temperature was 250 C and the inlet pressure was 30 p.s.i. Equal amounts of hexadecane-1,16-diol were added to each sample before hydrogenolysis as an internal standard.

Table III. Time Course of the Extraction of Abscisic Acid from Potato Disks

	Hours of Extraction[1]	Abscisic Acid[2] ng/g Tissue
Experiment 1	0	6.2
	0.5	8.0
	1	9.0
	2	10.5
	4	14.8
	6 (at pH 11)	14.2
	Days Aged Before Extraction[3]	
Experiment 2	0	13.4
	1	26.0
	2	N.D.
	4	N.D.
	6	N.D.

[1] 125 g of freshly cut potato disks were washed for the indicated periods in 150 ml of water at 30C. For the six hour sample the water used for extracting was adjusted to pH 11 with KOH.

[2] Abscisic acid was determined as described in the Materials and Methods.

[3] The potato disks were cut and aged for the indicated periods under suberizing conditions and at each interval 70 disks (=100 g) were washed in 150 ml of water at 30C for 3 hr.

Table IV. Time course of Induction of Suberization by ABA in Potato Tissue Culture

The low amount of octadecene diol obtained from control with no addition of ABA most probably represents the low amount of suberin present in the starting tissue. 10^{-4} M ABA was used in all cases and the octadecene diol was measured as described in Materials and Methods. Usually experiments were done in triplicate and the tissue samples were pooled prior to analysis.

Day	Suberin μg C_{18} diol/g dry tissue
3	320
4	380
5	400
6	440
8	600
10	560
Control after 10 days	120

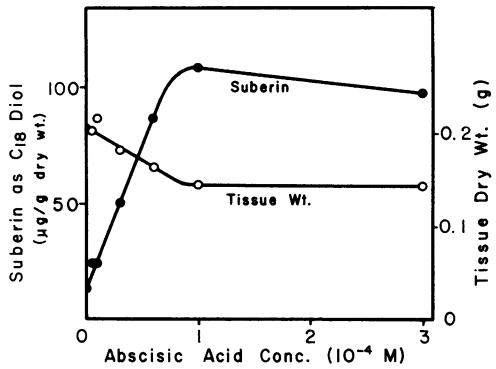

FIG. 4. Effect of increasing ABA concentration on suberization and growth in potato tissue cultures. Suberization was measured by GLC.

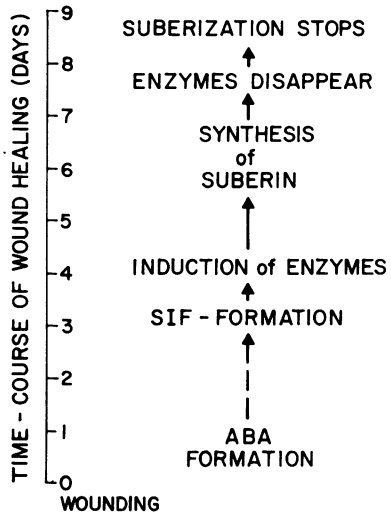

FIG. 5. Tentative proposal depicting the sequence of events which lead to suberization in wounded potato tissue. SIF: suberization-inducing factor.

CONCLUSION

Even though ABA stimulates suberization during the early periods in wound-healing, measurable amounts of ABA can be washed out only prior to aging for 2 days. Thorough washing during the 3rd day does inhibit suberization. It appears clear that removal of ABA is not the cause for inhibition caused by washing during the 3rd or 4th day after cutting potato disks. The partial reversal by ABA of the inhibition caused by washing occurred only when the disks were washed within 1 day after cutting. These results suggest that ABA participates in the early phases of the wound-healing process. It also appears that the suberization-inducing material, washed off the disks during the period immediately prior to the induction of the enzymes, involved in the synthesis of the aliphatic components, is presumably formed as a result of events triggered or mediated by ABA. These events finally lead to the formation of the ultimate enzyme-inducing factor(s). All of the information thus far available concerning wound-healing in potato disks can be summarized as shown in the very tentative proposal shown in Figure 5. Much of the events schematically shown remain to be elucidated at a molecular level.

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