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Anaerobiosis and Release from Dormancy in Apple Embryos

LEACHING OF (±) [^{14}C]ABSCISIC ACID AND ITS METABOLITES UNDER AEROBIC AND ANAEROBIC CONDITIONS

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ABSTRACT

An anaerobic treatment released *Pyrus malus* L. cv Golden Delicious embryos from their primary dormancy. It also suppressed the inhibitory effect induced by exogenous abscisic acid (ABA) on after-ripened embryos. For the study of ABA metabolism, a two-step culture method was developed. Embryos in primary dormancy were cultivated aerobically in the presence of [^{14}C]ABA (first culture). Some were directly analyzed to evaluate metabolism of absorbed ABA. The remaining embryos were cultivated on moist cotton without ABA, either in aerobic or anaerobic conditions (second culture). The amounts of ABA and its metabolites were measured both in the embryos and the water-leachates. After the second culture, the embryos showed a spectacular decrease in ABA content, with no difference between anaerobic and aerobic cultures. The amount of ABA glucose ester increased slightly in aerobiosis but diminished markedly in anaerobiosis. Radioactivity of the butanol fraction, which corresponded to polar conjugates, decreased considerably in anaerobiosis, whereas it increased in aerobiosis.

Analysis of the water-leachates indicated that, compared to aerobic conditions, anaerobiosis increased total leaching of radioactive materials (× 4.2) as well as leaching of ABA (× 1.4). In addition, anaerobiosis induced leaching of conjugates, such as ABA glucose ester and butanol-soluble metabolites. We concluded that the anaerobic treatment affects mainly membrane permeability.

Apple embryos have often been used as a model to study embryo dormancy. Many results obtained with this material favor a hormonal regulation of embryo dormancy in which, besides gibberellins, ABA would play a fundamental role. Concerning ABA, the following arguments can be mentioned: (a) in dormant embryos, large quantities of ABA and ABA-GE[1] were detected, mainly in the cotyledons (3, 20, 23); (b) during imbibition and culture *in vitro* at 23°C, ABA-GE was hydrolyzed and ABA migrated from the cotyledons to the axis where it accumulated. There was a relation between the depth of dormancy and the importance of the accumulation of ABA. The greatest quantity of accumulated ABA in the axis corresponded to the deepest dormancy, obtained when the two cotyledons were directly imbibed (15); (c) ABA decreased, then disappeared in the course of the first 4 weeks of the after-ripening treatment in the imbibed seeds (23); (d) after-ripened embryos could be induced to a state of secondary dormancy by exogenous (±) ABA. The concentration of ABA applied needed to be increased in relation with the length of the previous chilling treatment (24).

Tissaoui and Côme (28) showed that apple embryo dormancy can be eliminated at room temperature under the influence of an anaerobic treatment applied to imbibed embryos (from 1 to several weeks in a nitrogen atmosphere). The fact that anaerobic treatment can replace chilling in the release from dormancy led us to question the validity of the hormonal theory and especially the involvement of ABA in this regulation. A similar problem has already been evoked in the case of *Xanthium* seed dormancy, which is also eliminated by anaerobic treatment (7).

In this work, we explored first the possibility that dormancy induced by exogenous ABA might be eliminated by an anaerobic treatment. This having been proved, we used [^{14}C]ABA for further research on the eventual transformations induced by anaerobiosis on [^{14}C]ABA and its metabolites. Two-step experiments were carried out. Embryos were first cultivated aerobically in the presence of (±) [2-^{14}C]ABA, and then in its absence either in aerobic or anaerobic conditions. From the comparison of the data obtained at the end of the first and the second culture, we deduce that anaerobiosis induces considerable modifications in the leaching of ABA and its conjugates.

MATERIALS AND METHODS

Plant Material. Embryos of *Pyrus malus* L. cv Golden Delicious were used in two different physiological states. As dormant embryos, we used those isolated from the fruits either at harvest time or after storage for 1 to 3 months at 14 to 18°C. After-ripened embryos were isolated from fruits which had been stored 5 months at 0°C.

For the metabolic experiments, we deliberately used dormant embryos to avoid germinations during the second aerobic culture.

***In Vitro* Cultures.** All cultures, except the germination test illustrated in Figure 1, were realized in aseptic conditions. The embryos, isolated with no previous imbibition of the seeds, were cultivated at 23°C.

The germination tests themselves were carried out under white fluorescent light either on moist filter paper in Petri dishes (Fig. 1; Table I), or on water-agar (agar 8 g/l), each embryo being cultivated separately in a 20- × 200-mm tube containing 15 ml of medium (Fig. 2). In all experiments in the presence of ABA (germination or metabolism studies), agar-solidified media were used, ensuring homogenous absorption of ABA. Embryos were maintained in a vertical position, the distal part of the two cotyledons immersed in the medium (one-third of total length). This mode of culture allowed the greatest uptake of ABA to occur as well as an equal distribution of ABA and its metabolites between the two cotyledons (1). For anaerobiosis and parallel aerobiosis treatments, the embryos were placed flat on moist cotton, conditions previously used by Tissaoui and Côme (28).

To study ABA metabolism, dormant embryos were cultivated

[1] Abbreviations: ABA-GE, 1-abscisyl-β-D-glucopyranoside; PA, phaseic acid; DPA, dihydrophaseic acid; epi-DPA, epi-dihydrophaseic acid; PM, polar metabolites found in the acid fraction; ABA-GS, 1′-O-abscisic acid-β-D-glucopyranoside; DPA-GS, dihydrophaseic acid-4′-O-β-D-glucoside.

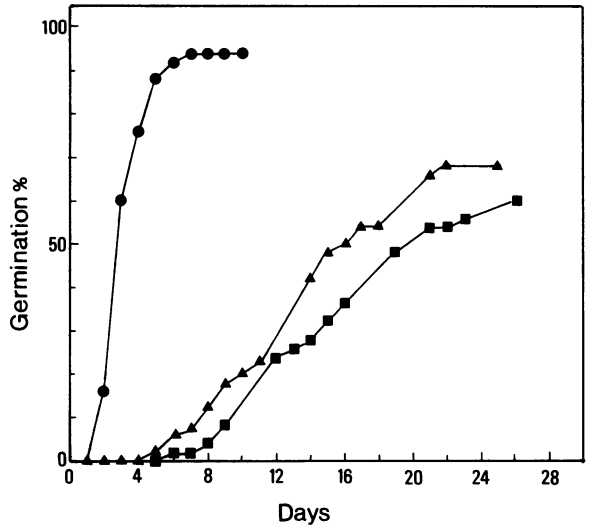

FIG. 1. Effect of an anaerobic treatment on the germination of non after-ripened embryos. During the anaerobic treatment (15 d), embryos had been maintained in darkness at 23°C in an argon atmosphere on water-imbibed cotton. The germination tests were carried out in Petri dishes on filter paper imbibed with water at 23°C under fluorescent light (16 h/d). The embryos were isolated from the fruits either at harvest time (■) or after 1 month of storage at 14 to 18°C (▲, ●). (●), Anaerobically pretreated; (▲, ■), not pretreated.

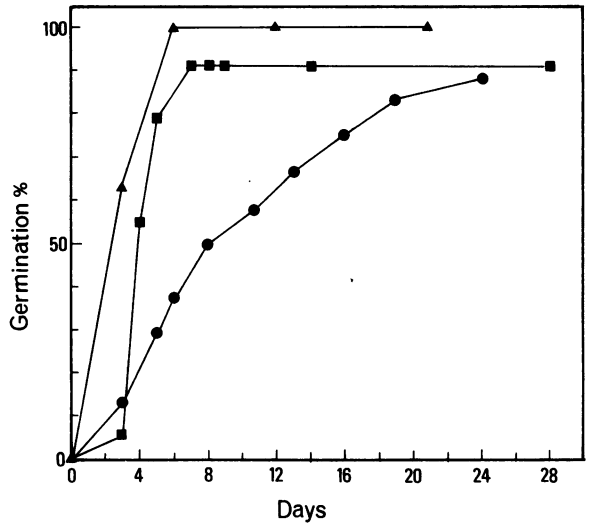

FIG. 2. Effect of an anaerobic treatment on the germination of after-ripened embryos having been induced to dormancy by (±) ABA. After-ripened embryos directly cultivated in aerobic conditions on water-agar (■). Embryos cultivated in aerobic conditions, first 14 d on water-agar containing 4 mg/l ABA, then on water-agar without ABA (●). Embryos cultivated first in aerobic condiions, 14 d on water-agar containing 4 mg/l ABA, then on water-imbibed cotton in a nitrogen atmosphere for 16 d, and finally in aerobic conditions on water-agar without ABA (▲). All cultures were maintained at 23°C either under fluorescent light 16 h/d (aerobiosis) or in darkness (anaerobiosis). The embryos were isolated from fruits which had been stored 5 months at 0°C.

Table I. *Influence of Direct Imbibition of Embryos during Anaerobic Treatment on Further Germination in Aerobiosis*

The embryos were first cultivated in darkness at 23°C in a N_2 atmosphere for 8 d either on water-imbibed cotton or in a water-saturated atmosphere. They were then transferred to aerobic conditions under fluorescent light (16 h/d) at the same temperature. Nonpretreated embryos served as reference. The embryos were isolated from the fruits after 3 months of storage at 14 to 18°C.

Time	Anaerobically Pretreated		Nonpretreated
	Directly imbibed	Maintained in a water-saturated atmosphere	
d		*germination %*	
2	0	0	4
3	41	4	25
5	100	19	46
7		50	52
9		69	55
12		90	60
14		98	60

Table II. *Distribution of the Radioactivity between ABA and Its Metabolites, Identified by Chromatographic R_F, in 20 Embryos after the First Culture*

The embryos, isolated from fruits maintained 2 months at 14 to 18°C, were cultivated 8 d on 20 μM [^{14}C]ABA.

	Free Acids	Acids Released by Alkaline Hydrolysis	Butanol-Soluble Compounds
		cpm	
PM	25,290	9,694	
DPA + epi DPA	91,962	20,052	
PA	5,900	1,726	
ABA	39,524	26,424	
NI[a]	4,418	3,284	
Total TLC	167,094	61,180	116,128
Total radioactivity		344,402	

[a] Not identified.

individually for 8 d in sterile Corning Pyrex tubes (75 × 12 mm) containing 0.2 ml of water-agar (6 g/l of agar) to which was added 2.10^{-5} M of (±) [2-^{14}C]ABA (11.1 μCi/μmol) purchased from Radiochemical, Amersham, England. The medium had a pH of 5.5. According to Kaiser and Hartung (11), this pH, compared to higher ones, promotes the rate of ABA uptake. All the culture tubes, plugged with cotton wool, were maintained in a water-saturated atmosphere in darkness at 23°C. At the end of this 8-d period, the embryos were either directly analyzed or transferred to a medium deprived of ABA for further culture. In the latter case, embryos from the ABA medium were transferred into a sterile rubber-capped glass vial (1,000 ml capacity) and placed flat on water-imbibed cotton (70 ml of water and 20 embryos per vial). They were maintained at 23°C in darkness either in aerobic or in anaerobic conditions for 10 d. In neither case was germination observed during the 10-d period. Anaerobic conditions were obtained by blowing argon (99.99%) through the rubber cap for 30 min by means of a hypodermic needle, air being evacuated by a second needle.

Extraction and Purification. The embryos were ground in a mortar with chilled 80% ethanol (30 ml for 20 embryos) containing 2,6-di-*t*-butyl-4-methyl phenol. The homogenate was stirred overnight in darkness at 4°C and filtered, and the residue reextracted twice with the same volume of chilled 80% ethanol. The filtrate was evaporated under reduced pressure at 40°C. The remaining aqueous extract was adjusted to pH 3 with diluted H_2SO_4 and extracted four times successively with ethyl acetate (v/v) and diethyl ether (v/v). Ethyl acetate was utilized first because DPA is more soluble in this solvent than in diethyl ether (29), which was then used to remove the ethyl acetate remaining in water.

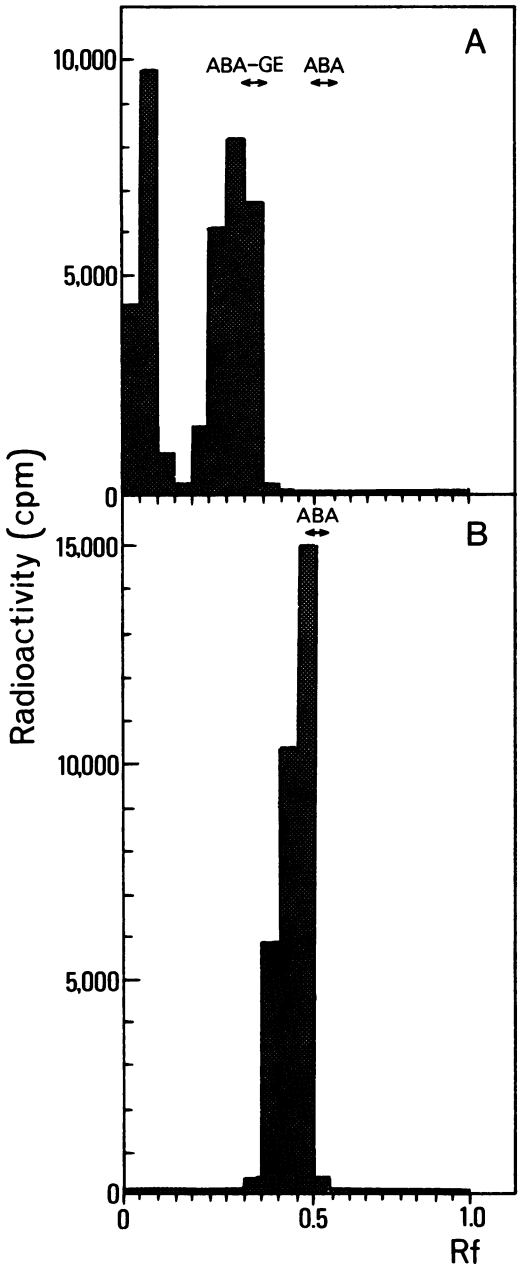

FIG. 3. Demonstration of the presence of ABA-GE. Ten embryos were cultivated for 7 d on 10 μM [^{14}C]ABA. After extraction of free acids by diethyl ether (3, v/v), the aqueous phase was concentrated. The residue was dissolved in a few drops of acetone:methanol (9:1, v/v) and studied in TLC in the solvent $CHCl_3$:CH_3OH:H_2O (75:22:3, v/v/v). One-third of the chromatogram was eluted and directly counted (A). From the remaining two-thirds, only the zone to which ABA migrated (R_F 0.3–0.4) was analyzed. After elution, it was subjected to a mild alkaline hydrolysis followed by a TLC in the same solvent as before (B). The arrows seen under ABA and ABA-GE indicate the R_F locations of the authentic compounds.

The organic fraction was extracted four times alternately with small volumes of saturated sodium bicarbonate solutions and water. The combined extracts, adjusted to pH 7.5, were washed twice with equal volumes of diethyl ether to remove the neutral compounds. The aqueous solution was then acidified to pH 3 and extracted successively three times with ethyl acetate and diethyl ether. This organic phase contained the free acids.

The aqueous fraction remaining after the first partition between organic solvents and water at pH 3 was submitted to a mild alkaline hydrolysis (pH 12, 40°C, 20 h) as described by Milborrow (19).

A considerable proportion of the radioactivity remained in the final aqueous phase after saponification and extraction of the released acids. We used 1-butanol to extract these metabolites.

After the second culture, the water-leachates and the water used to rinse the cotton were collected. After concentration under reduced pressure, the aqueous phase underwent the same treatment as the embryo filtrates.

Qualitative Analysis of ^{14}C. The reduced organic phases were divided into two equal parts and each chromatographed on Merck precoated silica gel F 254 plates and developed in $CHCl_3$:CH_3OH:H_2O (75:22:3, v/v/v) or in toluene:ethyl acetate:acetic acid (50:30:4, v/v/v). Each TLC was divided into half R_F zones and the radioactivity counted in a water-absorbing dioxane base scintillation fluid (Bray) using a SL Intertechnique Liquid Scintillation Spectrometer. We found that the presence of silica gel in the counting vials reduced the radioactivity counts by no more than 4%, in agreement with previous findings (9, 12). Metabolites were identified by comparison of R_{FABA} with values obtained from the literature.

Biological Test. The biological activity of the butanolic compounds was tested after two chromatographies on Whatman No 1 paper, first in $CHCl_3$:CH_3OH:H_2O (75:22:3, v/v/v) second in toluene:ethyl acetate:acetic acid (50:30:4, v/v/v). The band between the start line and R_F 0.1 was each time eluted by pure methanol. The inhibitory activity was estimated by a straight-growth wheat coleoptile test (15).

RESULTS AND DISCUSSION

Germination Experiments. Dormant embryos treated for 15 d in anaerobiosis (Ar) germinated rapidly when transferred to aerobiosis (Fig. 1). The seedlings obtained no longer showed symptoms of dwarfism.

For maximum effect of anaerobiosis, embryos must be in direct contact with water during the treatment. Non-after-ripened embryos were submitted to an 8-d anaerobic treatment (nitrogen), either water-imbibed or maintained in a water-saturated atmosphere without direct contact with water. After transfer into aerobic conditions on water, germination was clearly enhanced in both cases in comparison with nonpretreated embryos. Germination was, however, much faster when the embryos had been in direct contact with water during the anaerobiosis treatment (Table I).

It was also demonstrated that an anaerobic treatment suppressed the inhibitory effect induced by ABA on after-ripened embryos. These were cultivated on water-agar containing (±) ABA at 4 mg/l. They did not germinate when maintained continuously on this medium. When transferred after 14 d onto water-agar without ABA, these embryos exhibited slow germination (Fig. 2). In this case, part of the previously absorbed ABA was released into the medium (2). Under the effect of the anaerobic treatment (16 d in a nitrogen atmosphere), the germination became as fast as that of after-ripened embryos. The seedlings obtained no longer showed symptoms of dwarfism. Similar results were obtained in an Ar atmosphere.

Analysis of the Embryos after Culture in the Presence of [^{14}C] ABA. In these studies, as well as in the previously described germination experiments, we used (±) ABA. Therefore, the results obtained may not be an accurate reflection of the metabolism of endogenous ABA, the two enantiomers not necessarily being metabolized in exactly the same manner (18, 25, 30).

First, we made certain in a subsidiary experiment that the [^{14}C] ABA of the medium was not metabolized *in situ*. After 12 d of culture, 94% of the remaining activity of the medium was still in the form of [^{14}C]ABA.

The embryos cultivated for 8 d had absorbed 18.5% of the [^{14}C] ABA initially present in the medium, the main part of it (88.5%)

Table III. *Distribution of the Radioactivity between ABA and Its Metabolites, Identified by Chromatographic R_F, in 20 Embryos after the Second Culture*

The embryos, isolated from fruits maintained 2 months at 14 to 18°C, were cultivated 8 d on 20 μM [^{14}C]ABA and then on cotton imbibed with water (70 ml) for 10 d either in aerobiosis or in anaerobiosis.

	Aerobiosis			Anaerobiosis		
	Free acids	Acids released by alkaline hydrolysis	Butanol-soluble compounds	Free acids	Acids released by alkaline hydrolysis	Butanol-soluble compounds
	cpm					
PM	18,480	13,622		8,946	7,628	
DPA + epi DPA	14,152	11,760		10,448	5,832	
PA	2,078	1,562		1,298	1,628	
ABA	3,460	32,942		3,608	8,904	
NI[a]	845	2,007		1,005	2,028	
Total TLC	39,015	61,893	191,344	25,305	26,020	87,620
Total radioactivity		292,252			138,945	

[a] Not identified.

Table IV. *Distribution of the Radioactivity between ABA and Its Metabolites, Identified by Chromatographic R_F, in the Water-Leachates of 20 Embryos after the Second Culture*

The water-leachates (70 ml) originated from embryo cultures realized under the conditions described in Table III.

	Aerobiosis			Anaerobiosis		
	Free acids	Acids released by alkaline hydrolysis	Butanol-soluble compounds	Free acids	Acids released by alkaline hydrolysis	Butanol-soluble compounds
	cpm					
PM	1,584			5,638	3,234	
DPA + epi DPA	2,974			56,670	13,094	
PA	2,026			4,978	450	
ABA	37,700			51,276	3,564	
NI[a]	2,666			2,955	899	
Total TLC	46,950	620	990	121,517	21,241	60,945
Total radioactivity		48,560			203,703	

[a] Not identified.

being metabolized. The radioactivity was distributed between free acids (ABA, PA, DPA + epi DPA, PM), conjugates releasing free acids by mild alkaline hydrolysis, such as ABA-GE and other compounds (30), and very polar metabolites soluble in 1-butanol (Table II).

The main alkali-hydrolyzable compound proved to be chromatographically identical to ABA-GE. Using the technique described by Dewdney and McWha (6), we isolated among the substances soluble in acetone:methanol (9:1, v/v) one labeled metabolite which migrated at the R_F corresponding to that of ABA-GE in the solvent $CHCl_3$:CH_3OH:H_2O (75:22:3, v/v/v) (Fig. 3A). After mild alkaline hydrolysis, labeled ABA was released (Fig. 3B).

It was not possible to hydrolyze the compounds of the butanol fraction by means of mild alkaline hydrolysis (20). When the compounds of the butanol fraction were applied in aqueous solution to apple embryos (12 h, 23°C), ABA, DPA, and more polar compounds of the acid fraction were produced. This could be due to the presence of ABA-GS, a substance which has already been found in apple embryos as a result of [^{14}C]ABA metabolism (16). Using wheat coleoptile sections, we also observed that the same aqueous extract exhibited a strong growth inhibitory activity. This seemed to indicate that ABA-GS, at least under certain conditions, could release ABA, the enzymes required being present in the embryos.

The results of further experiments indicated that ABA-GS did not represent the totality of the radioactivity measured in the butanol fraction. It is very likely that DPA conjugates, non-alkali hydrolyzable under mild conditions, and among them DPA-GS which has been recently identified (13, 21), are to be found in this fraction.

The polar metabolites found in the acid fraction, PM, migrating at a lower R_F than DPA, were not identified. They might correspond to the polar metabolites previously described by several authors (6, 20, 26). This activity might also be due, at least partially, to one of the polar metabolites of the butanol fraction, slightly soluble in ethyl acetate (16).

Analysis after Culture without ABA under Aerobic and Anaerobic Conditions. The radioactivity remaining in the embryos after culture under aerobic and anaerobic conditions (Table III) represented, respectively, 85 and 40.3% of the initial value (value found after the first culture; Table II). Among the free acids, ABA and DPA (+ epi-DPA) decreased notably. Approximatively one-tenth of the original quantity of ABA remained in both cases. PM decrease was more marked in anaerobiosis than in aerobiosis. The amount of ABA-GE remaining in the embryos increased slightly in aerobiosis but fell to 34% of its original value in anaerobiosis. The radioactivity of the butanol fraction decreased in anaerobiosis

Table V. *Water-Leachates Radioactivity in Three Different Experiments Carried Out in Aerobiosis and in Anaerobiosis (Ar Atmosphere)*

Before culture, the embryos had been maintained in the fruits at 14 to 18°C either 2 months (experiment I) or 1 month (experiments II and III). In experiment I, they were first cultivated 8 d on [^{14}C]ABA at 20 μM and then on water-imbibed cotton for 10 d. In experiments II and III, the first and the second culture lasted, respectively, 12 and 16 d. The concentration of [^{14}C]ABA used in the first culture was 10 μM. The results of experiment I are given in detail in Table IV.

	Experiment I		Experiment II		Experiment III	
	Aerobiosis	Anaerobiosis	Aerobiosis	Anaerobiosis	Aerobiosis	Anaerobiosis
			cpm			
Total radioactivity in the water-leachates (cpm)	48,560	203,703	24,400	93,145	21,119	65,126
Percentage of the total radioactivity						
Free acids	96.68	59.65	92.47	47.70	97.40	59.67
ABA	77.63	25.17	71.34	30.96	76.58	31.38
Acids released by alkaline hydrolysis	1.28	10.43	4.26	33.29	1.18	10.10
ABA		1.75		12.19		2.16
Butanol-soluble compounds	2.04	29.92	3.27	19.01	1.42	30.23

(25%), while it increased in aerobiosis (65%).

The quantitative analysis of the water-leachates (Table IV) showed that anaerobiosis induced an important increase in the total radioactivity released (× 4.2). Among the free acids, ABA and DPA (+ epi-DPA) were the most abundant, especially in anaerobiosis. The amount of leached ABA was 1.4 times greater in anaerobiosis than in aerobiosis. The conjugates hydrolyzable under mild alkaline conditions, as well as the polar metabolites of the butanol fraction, were almost exclusively found in the water-leachates of anaerobically treated embryos. They represented approximately 40% of the total radioactivity eliminated under anaerobic conditions.

These results were confirmed by two further experiments. The water-leachates analyses can be compared in the three experiments (Table V). In aerobiosis, the free acids represented always more than 92% of the total radioactivity released, whereas in anaerobiosis they reached only 48 to 60% of it. The radioactivity of the water-leachates being 3 to 4.2 times higher in anaerobiosis than in aerobiosis, the amount of free acids released (in cpm) was, however, much higher in the former than in the latter case. The same observation can be made concerning ABA. The amount of conjugates released, very low in aerobiosis, was, on the contrary, very high in anaerobiosis.

Thus, anaerobiosis always has the effect of increasing the leaching of free-phase compounds and inducing that of larger molecules such as ABA-GE and butanol-soluble compounds.

These results agree with those from different authors showing that losses of many plants constituents are much greater under anaerobic conditions than under normal aeration (4, 10, 17, 22). In addition, in our case, qualitative differences also exist between the two treatments, since conjugates were released only in anaerobic conditions. Thus, anaerobiosis induces important modifications of membrane permeability, the origin of which has been diversely interpreted (4, 8, 10, 27).

Finally, it seems logical to examine the physiological significance of the described modifications induced by anaerobiosis. This study has shown that the amounts of ABA remaining in embryos after culture, either in aerobiosis or anaerobiosis, were equivalent, although the processes involved were different. Concerning ABA-GE, a potential source of ABA in this material, anaerobically treated embryos contained lower quantities than aerobically treated ones. Similar results were noticed for the polar metabolites soluble in butanol, but the physiological properties of these substances are still unknown.

In the comparison established between anaerobic and aerobic cultures, we did not consider the possibility that the promotive effect of the anaerobic treatment on leaching could persist after transfer to aerobiosis. One of our germination experiments (Table I) demonstrated that the anaerobiosis treatment retained its efficiency when the leaching could only take place after transfer to aerobiosis. The delay in germination then observed between the transfer to aerobiosis and germination might correspond to the time necessary for the completion of some leaching in aerobiosis.

We wonder also whether the effects of the anaerobic treatment do not exert an influence on other hormones implicated in the regulation of embryo dormancy. The hypothesis proposed by Parrish and Davies (22) that the hormone, in their case IAA, might be released from cytologically or chemically bound forms under anaerobic treatment, should be kept in mind for further investigations.

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