Bul. Agron. 22 (2) : 66 - 77 (1994)

IDENTIFICATION AND ANALYSES OF SHORT CHAIN FATTY ACIDS IN APPLE SEED PARTS DURING STRATIFICATION[1]

Bambang S. Purwoko[2)] and Loyd E. Powell[3)]

ABSTRAK

Penelitian ini bertujuan untuk mengidentifikasi dan menganalisis perubahan kandungan asam lemak rantai pendek (ALRP) pada biji apel selama stratifikasi. Asam-asam heksanoat, oktanoat, nonanoat, dan dekanoat telah diidentifikasi dengan Gas-Kromatografi Mass- Spektrometri. Analisis rutin ALRP dengan Gas-Kromatografi menunjukkan bahwa kandungan ALRP tertinggi ditemukan pada membran nuselus. Konsentrasi ALRP pada kulit biji rendah dan setingkat dengan kandungan ALRP pada embrio.

Asam heksanoat merupakan ALRP terbanyak pada semua bagian biji padahal asam ini dianggap inhibitor yang lemah. Kandungan asam nonanoat dan dekanoat jauh lebih kecil dan biasanya kurang dari 10^{-4} M. Gambaran umum perubahan ALRP selama stratifikasi adalah menurun atau konstan dan kandungannya kurang dari 10^{-3} M, terlalu kecil untuk menjadi inhibitor yang signifikan dalam mekanisme dormansi. Penurunan ALRP terjadi pada suhu 5 ^{o}C dan 20 ^{o}C, padahal hanya perlakuan 5 ^{o}C yang dapat memecahkan dormansi biji apel. Dari studi ini, Dapat disimpulkan bahwa perubahan kandungan ALRP selama stratifikasi tidak mendukung hipotesis bahwa ALRP berperan dalam dormansi biji apel.

ABSTRACT

The objectives of the present studies were to identify the presence of short chain fatty acids (SCFA) and investigate SCFA changes in apple seed parts during stratification. Hexanoic, octanoic, nonanoic, and decanoic acids have been identified using Gas-Chromatography Mass-Spectrometry. Routine analyses of SCFA by Gas-Chromatography showed that the highest SCFA content was found in the nucellus membranes. SCFA content in the seed coats was low and at similar level to SCFA content in the embryos.

In all seed parts, hexanoic acid content was found to be the highest. This acid was considered to be weakly inhibitory. Nonanoic and decanoic acid content was much lower than hexanoic acid content, generally less than 10^{-3} M. The general trend of the SCFA changes was either decrease or constant and less than 10^{-3} M, too small to be significant inhibitor in the dormancy mechanism. It was concluded that SCFA changes during stratification did not support the hypothesis that SCFA had a role in apple seed dormancy.

---

1) Part of first author's dissertation at Cornell University, Ithaca, NY, USA

2,3) Graduate student and Emeritus Professor of Pomology, respectively, at Cornell University, Ithaca, NY, USA

2) Currently at Jurusan Budidaya Pertanian, IPB, Bogor

INTRODUCTION

Short chain fatty acids (SCFA) were reported to have a role in dormancy of several species including in iris bulbs (Ando and Tsukamoto, 1974), wild oat seeds (Berrie *et al.*, 1975 and 1979), and rice seeds (Majumder *et al.*, 1989) . Short chain fatty acids have been shown to inhibit germination and bud growth of several species including wild and common oats (Berrie *et al.*, 1975 and 1979), rice (Majumder *et al.*, 1989), lettuce (Berrie *et al.*, 1975), apple buds (Rogoyski and Powell, 1981), and apple embryos (Purwoko and Powell, 1994). The present studies were done to investigate changes of SCFA content in apple seed parts during stratification of the Northern Spy cultivar and their possible role in dormancy release of apple seed.

MATERIALS AND METHODS

General Methods

Seeds of apple (Malus domestica), c.v. Northern Spy, were obtained from the Cornell Orchard, Ithaca, New York. Seeds of the 1990 season, hereafter called Northern Spy 1990, were used throughout the experiment of SCFA analyses. Seeds from mature fruits were imbibed overnight. They were then surface sterilized with sodium hypochloride (10 % Clorox), rinsed with deionized water, and placed in a 9-cm petri dish lined with two filter papers (Whatman No. 42) moistened with six ml of deionized water. Half the seeds were stratified at 5 ± 1 ^{o}C in the dark while the other half (controls) were treated in a similar manner, but held at 20 ± 1 ^{o}C.

Seed samples were withdrawn every fifteen days. Twenty seeds and embryos from these samples were germinated in the dark at 20 + 1 ^{o}C. Three replicates for embryo germination were used while six replicates were used for seed germination. The stratification was terminated after 60 days. Germination were considered to have taken place when the radicle reached more than 2 mm. Germination was counted after 14 days.

Forty seeds of Northern Spy 1990 were dissected into seed coats, membranes, and embryos and stored at - 20 ^{o}C until analyzed for SCFA. Three replicates were used. The summary of treatments is as follows :
Stratification temperatures: 5 ± 1 and 20 ± 1 ^{o}C.
Days of stratification: 0, 15, 30, 45, 60.
Three replicates of 40 seeds each for SCFA analysis.

Short Chain Fatty Acid Extraction from Apple Seed Tissues

The extraction method was adapted from Rogoyski (1980). All organic solvents were obtained from the Baker Chemical Company. The extraction and purification scheme for SCFA is shown in Fig. 1.

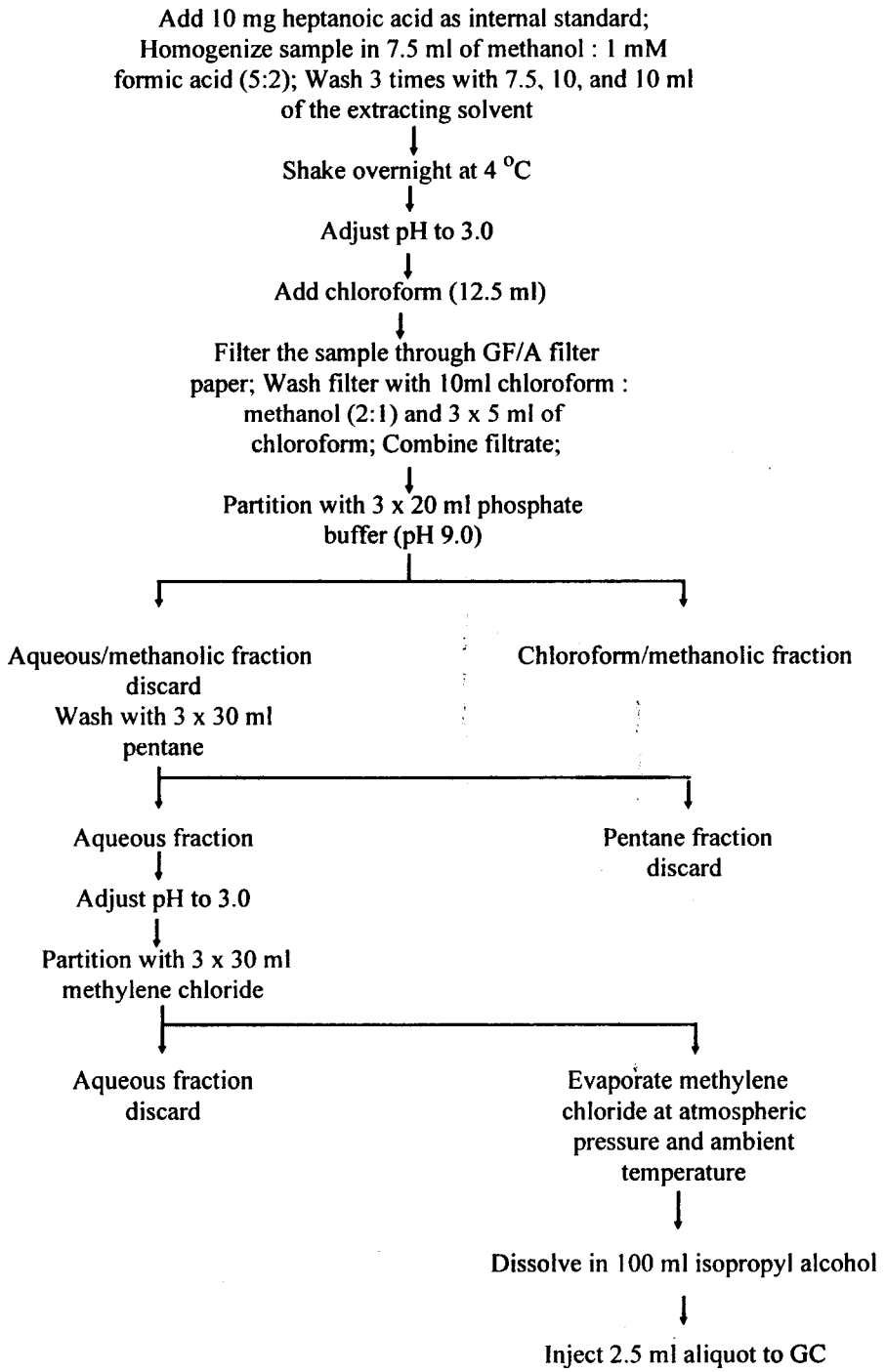

Figure 1. Extraction and purification of SCFA from apple seed tissues for GC analysis.

A Hewlett-Packard Gas Chromatograph, Model 5730A, was used in the chromatographic separation of SCFA. A 3-feet glass coloumn was packed with 10 % SP-216 PS on 100/120 Supelcoport (Supelco). The flow rate of nitrogen carrier gas was 20 ml/min. The flame ionization detector (FID) and injector temperatures were maintained at 250 °C and 200 °C respectively. The oven was programmed from 100 to 190 C at 8 °C/min with no isothermal program at the beginning. The final temperature was held at 190 °C for 8 minutes.

Standard curves were prepared to determine the linear responses of SCFA at different concentrations. Quantitation were based on the recovery of the C7 internal standard. Several injections of each acid (standard) were made every morning on the day of the analysis.

For GCMS identification, SCFA extract was esterified according to Attygalle and Morgan (1986). The extract was dissolved in 25 µL of ether. Ten µL of pentafluorobenzylbromide and 10 µL of triethylamine were added to the sample. The mixture was incubated for 30 minutes. Water (25 µL) was added. The pentafluorobenzyl esters were extracted with 25 µL hexane. A one µL aliquot was injected into the GCMS in the splitless mode.

Identification of SCFA by GCMS utilized a Hewlett-Packard 5890 Gas Chromatograph coupled to a Hewlett-Packard 5970 Mass Selective Detector in the Department of Chemistry, Cornell University. The flow rate of helium as carrier gas was 60 ml/min. A DB-5 capillary column (JNW Scientific) was 25 m in length and 0.22 mm in diameter. The oven was set at 60 °C for an initial isothermal period of 4 minutes and then programmed at 15 °C/min to a final temperature of 260 °C. It was held for 10 minutes at the final temperature. The injector temperature was 250 °C. Electron impact spectra were obtained. The ionization potential was 70 eV.

RESULTS

Short chain fatty acids were routinely analyzed using Gas Chromatography with Flame Ionization detection. Their presence in apple seeds was confirmed using Gas-Chromatography Mass-Spectrometry. Only mass spectra of pentafluorobenzyl esters of reference hexanoic and corresponding acid extracted from apple seeds are shown in Fig. 2. The molecular ions of the pentafluorobenzyl esters of hexanoic, octanoic, nonanoic, and decanoic acids are 296, 324, 338, and 352 respectively. The molecular ions and the ionization fragments of both reference SCFA and SCFA extracted from apple seeds were similar. Thus the existence of those acids was confirmed.

Seed and embryo germination during stratification are shown in Table 1. Seeds of Northern Spy 1990 require stratification at 5 °C for more than 60 days for complete germination while excised embryos require 45 days of stratification at 5 °C.

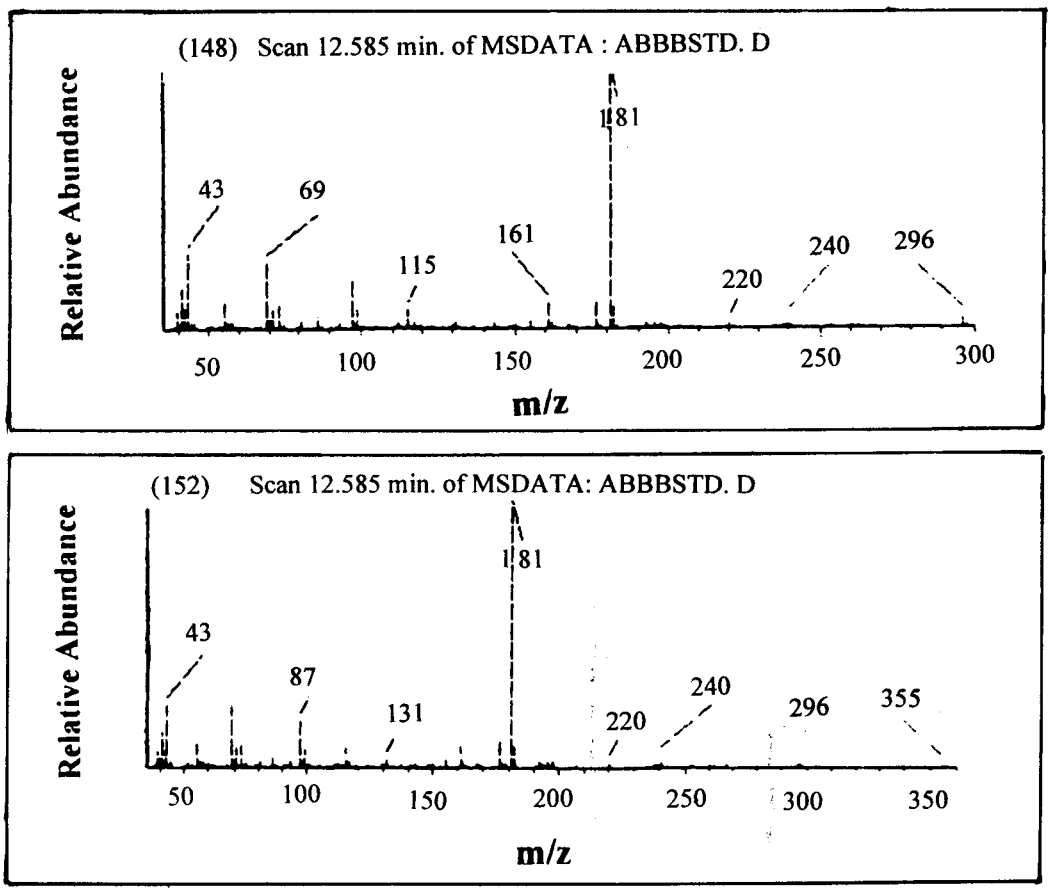

Figure 2. Electron impact (70 Ev) mass spectra of pentafluorobenzyl esters of reference hexanoic acid (A) and hexanoic acid extracted from apple seeds (B).

Table 1. Seed and embryo germination of Northern Spy 1990 during stratification (Perkecambahan biji dan embrio apel Northern Spy 1990 selama stratifikasi).

Days Seed	Germination		Embryo Germination	
	5 °C	20 °C	5 °C	20 °C
	% germination ± SE			
0	0	0	15.0±4.0	15.0±2.3
15	0	0	28.3±4.7	15.0±4.0
30	10.8±3.4	0	86.7±2.3	33.3±2.3
45	60.0±7.6	0	100	30.0±0
60	91.6±6.8	0	100	33.3±6.2

1. Changes of SCFA in the embryo during stratification

Hexanoic acid (C6) in the embryo of Northern Spy 1990 decreased linearly during stratification at both temperatures (Table 3). Hexanoic acid declined from 7.46 to 4.58 μg/g (40 %) over the 60 day period of stratification at 5 °C. At 20 °C, the level of C6 decreased from 7.46 to 5.17 μg/g (30 %) (Table 2).

There was a constant and low level of C9 in the embryos of Northern Spy 1990 during stratification at both temperatures (Tables 2 and 3). The decanoic acid level in the embryo of Northern Spy 1990 was very low, generally below 0.5 mg/g (Table 2). Table 3 showed that there were no significant differences in any factors analyzed.

Table 2. Endogenous short chain fatty acid content in the embryos of Northern Spy 1990 seeds (μg/g fresh weight ± SE) during stratification at 5 and 20 oC (Kandungan asam lemak rantai pendek pada embrio Northern Spy 1990 (μg/g bobot basah ± SE) selama stratifikasi pada suhu 5 dan 20 °C).

Days	C6	C9	C10
0	7.46±0.39	1.02±0.18	0.23±0.06
15	5.56±0.79	0.94±0.34	0.19±0.06
30	5.09±0.52	1.01±0.14	0.23±0.03
45	5.34±0.18	0.99±0.05	0.28±0.03
60	4.58±0.73	0.63±0.11	0.17±0.03
15	8.71±1.67	0.97±0.02	0.35±0.03
30	7.77±1.13	0.97±0.18	0.28±0.05
45	5.71±1.30	1.19±0.22	0.35±0.11
60	5.17±0.86	1.04±0.07	0.20±0.12

2. Changes of SCFA in the Nucellus Membrane during Stratification

There were significant changes for C6 in nucellus membranes from dormant (0 day) to stratified (15, 30, 45, 60, and 75 days) seeds of Northern Spy 1990 (Table 5). Interaction between temperature and stratification was significant, indicating that changes in C6 followed a different pattern at 5 and 20 °C. In the first 30 days of stratification at 5 °C, C6 decreased from about 14.95 to 8.29 μg/g (Table 4). It then increased to 12.65 μg/g in the next 30 days. At 20 °C there was a 45 % decrease of C6 in the first 30 days. Thereafter, C6 content did not change.

The nonanoic acid changes in the nucellus membranes of Northern Spy 1990 during stratification are shown in Table 4. No significant changes during stratification were observed (Table 5). Table 4 also showed changes in the level of decanoic acid in the nucellus membranes of Northern Spy 1990. There were no significant changes in C10 content during stratification (Table 5).

Table 3. Analysis of variance of short chain fatty acids in the embryos of Northern Spy 1990 seeds during stratification at 5 and 20 °C (Analisis ragam kandungan asam lemak rantai pendek pada embrio Northern Spy 1990 selama stratifikasi pada suhu 5 dan 20 °C).

Source	Mean Square			
	df	C6	C9	C10
0 day vs Stratified	1	5.74ns	0.01ns	0.002ns
Temperature	1	17.27**	0.14ns	0.035ns
Days	3	5.93ns	0.06ns	0.017ns
Linear	1	17.72**	0.02ns	0.009ns
Quadratic	1	0.00ns	0.12ns	0.020ns
LOF	1	0.06ns	0.05ns	0.024ns
Temperature x Days	3	3.01ns	0.06ns	0.004ns
Temp x D-lin	1	7.43ns	-	-
Block	2	8.47*	0.13ns	0.013ns
Error	16	2.00	0.09	0.015

*, **, significant at P = 0.05, 0.01 respectively; ns not significant

3. Changes of SCFA in the Seed Coat during Stratification

The hexanoic acid content in the seed coat of Northern Spy 1990 is shown in Table 6. Eventhough statistically not significant, the trend in C6 content at 5 °C was constant during stratification, except at 45 days where there was an increase. At 20 °C, the C6 level did not change much during stratification. No changes were observed in the nonanoic acid and decanoic acid content in seed coats of Northern Spy 1990 at either 5 or 20 °C (Tables 6 and 7).

Table 4. Endogenous short chain fatty acid content in the nucellus membranes of Northern Spy 1990 seeds (µg/g fresh weight ± SE) during stratification at 5 and 20 °C (Kandungan asam lemak rantai pendek pada membran nuselus Northern Spy 1990 (µg/g bobot basah ± SE) selama stratifikasi pada suhu 5 dan 20 °C).

Days	C6	C9	C10
0	14.95±1.02	2.59±1.12	1.53±0.41
15	10.72±2.55	2.46±0.90	1.19±0.41
30	8.29±1.17	2.47±0.53	2.04±0.34
45	12.91±0.51	2.95±1.17	1.02±0.37
60	12.65±1.21	2.65±1.06	1.12±0.52
15	12.76±0.82	2.20±0.49	1.20±0.29
30	8.48±0.77	2.82±0.57	1.51±0.39
45	8.22±1.01	1.69±0.23	0.78±0.11
60	8.27±0.37	1.41±0.29	0.71±0.13

Table 5. Analysis of variance of short chain fatty acids in the nucellus membranes of Northern Spy 1990 seeds during stratification at 5 and 20 °C (Analisis ragam kandungan asam lemak rantai pendek pada membran nuselus Northern Spy 1990 selama stratifikasi pada suhu 5 dan 20 °C).

Source	Mean Square			
	df	C6	C9	C10
0 day vs Stratified	1	55.16**	0.18ns	0.30ns
Temperature	1	22.88ns	2.18ns	0.53ns
Days	3	8.72ns	0.38ns	1.00ns
Linear	1	0.84ns	0.45ns	0.86ns
Quadratic	1	14.41ns	0.54ns	0.48ns
LOF	1	15.77ns	0.14ns	1.65*
Temperature x Days	3	16.27ns	0.93ns	0.08ns
Temp x D-lin	1	42.66*	-	-
Block	2	0.71ns	5.96*	1.17*
Error	16[x)]	5.07	1.350	.28

x) df for C6 : 15

*, **, significant at P = 0.05, 0.01 respectively; ns not significant

4. Short Chain Fatty Acid Content per Seed during Stratification

The dormant seeds of Northern Spy 1990 contained 416 ng of hexanoic acid per seed (Table 8). During stratification at 5 °C, the C6 level declined generally, but showed an increase at 45 days. The C6 decreased approximately 25 % during stratification at 20 °C.

Table 6. Endogenous short chain fatty acid content in the seed coats of Northern Spy 1990 seeds (μg/g fresh weight ± SE) during stratification at 5 and 20 °C (Kandungan asam lemak rantai pendek padam kulit biji Northern Spy 1990 (μg/g bobot basah ± SE) selama stratifikasi pada suhu 5 dan 20 °C).

Days	C6	C9	C10
0	5.88±0.47	1.89±0.24	1.41±0.22
15	5.35±1.58	1.29±0.53	0.89±0.20
30	5.20±0.18	1.81±0.56	1.20±0.36
45	8.68±1.39	3.28±0.76	1.71±0.36
60	5.15±1.46	2.68±1.69	0.94±0.19
15	4.15±0.99	1.03±0.29	0.94±0.37
30	4.42±1.10	1.23±0.13	0.89±0.15
45	5.73±0.88	2.22±0.87	1.77±1.26
60	4.37±0.61	1.41±0.12	092±0.17

Table 7. Analysis of variance of short chain fatty acids in the seed coats of Northern Spy 1990 seeds during stratification at 5 and 20 °C (Analisis ragam kandungan asam lemak rantai pendek pada kulit biji Northern Spy 1990 selama stratifikasi pada suhu 5 dan 20 °C).

Source	Mean Square			
	df	C6	C9	C10
0 day vs Stratified	1	0.66ns	0.00ns	0.17ns
Temperature	1	12.26ns	3.77ns	0.02ns
Days	3	8.88ns	2.86ns	0.92ns
Linear	1	1.78ns	4.54ns	0.17ns
Quadratic	1	9.39ns	1.67ns	1.33ns
LOF	1	15.47*	2.37ns	1.28ns
Temperature x Days	3	1.60ns	0.31ns	0.04ns
Block	2	6.54ns	1.77ns	0.81ns
Error	16	3.02	1.66	0.72

*, **, significant at P = 0.05, 0.01 respectively; ns not significant

The nonanoic acid content per seed of Northern Spy 1990 is shown in Table 8. The C9 content during stratification did not change. In general, the C9 level at 5 °C was slightly higher than that at 20 °C. No regular pattern of change was obvious in the decanoic acid content per seed of Northern Spy 1990 (Table 8). During stratification, the C10 amount per seed also appeared to be constant.

Table 8. Short chain fatty acid content per seed of Northern Spy 1990 (ng/seed) during stratification at 5 and 20 °C. (Kandungan asam lemak rantai pendek per biji Northern spy 1990 (μg/biji) selama stratifikasi pada suhu 5 dan 20°C)

Days	C6	C9	C10
0	416.1	77.9	38.7
15	374.6	74.7	30.7
30	335.0	85.2	42.9
45	421.7	109.6	44.4
60	340.8	85.7	28.5
15	464.7	65.2	34.2
30	412.4	74.0	34.3
45	342.9	84.3	41.3
60	317.6	68.9	25.9

DISCUSSION

Apple seed parts were analyzed for their short chain fatty acid content to determine if these substances might be correlated with any physiological roles in dormancy. When apple seed parts were analyzed for SCFA changes during stratification, their concentration in the nucellus membranes was found to be the highest of all seed parts. The concentration of SCFA in the seed coats were lower and similar to that in the embryos. Hexanoic acid was found to be the highest among SCFA in all seed parts. However, hexanoic acid is generally considered to be weakly inhibitory (LePoidevin, 1964; Cathy and Steffens, 1968; Berrie et al., 1975; Ando and Tsukamoto, 1981; Hyodo and Tanaka, 1982; and Purwoko and Powell, 1994). Nonanoic and decanoic acids, found to be the most inhibitory i apple embryo germination (Purwoko and Powell, 1994), were present at much lower amounts and generally existed under 10^{-4} M.

The general picture of SCFA change was that SCFA either decreased during stratification or remained more or less constant. However, throughout the stratification period, the concentration of SCFA was low, much less than 10^{-3} M, and thus probably too low to have any significant inhibitory effects in the dormancy/ germination mechanism.

On a per seed basis, SCFA either decreased or remained relatively constant during stratification. When there was a decrease, the decrease occurred at both 5 and 20 °C. Only stratification at 5 °C caused release from dormancy. In conclusion, the overall change in SCFA during stratification did not support the hypothesis that SCFA have a relationship to dormancy release in apple seeds.

These conclusions are similar to those of other researchers where they failed to find compelling evidence for a regulatory role for SCFA in seed dormancy in wild oat seeds (Metzger and Sebesta, 1982) or iris bulb dormancy (Doss *et al,* 1983) . In contrast, it has been previously reported that SCFA content correlated well with the release of dormancy in wild oat seeds (Berrie et al., 1979), rice seeds (Majumder et al., 1989), and iris bulb (Ando and Tsukamoto, 1974). Berrie et al. (1979) also found that there was a build up of SCFA during maturation of wild oat seeds. Ulbright (1980) claimed that germination of dormant apple embryos was accompanied or preceded by a drop in pentanoic acid. However, her results seemed in part superficial, since she analyzed only the dormant and the germinated embryos.

With respect to changes in SCFA during stratification, there were occasional deviations in the pattern mentioned above. There were a few instances when there were significant but apparently minor increases in SCFA. For example, the trend of SCFA change in seed coats during stratification was either little change or a significant increase at the end of stratification. Since nucellus membranes had higher concentrations of SCFA, and since SCFA have some solubility in water, it is possible that the increase was caused by diffusion from the nucellus membranes to the seed coats.

CONCLUSION

In summary, the amount of SCFA present in different seed parts or on a per seed basis during stratification was much lower than one millimolar. Given this low concentration and the rather high amount needed for inhibitory activity, it is unlikely that SCFA have a regulatory role in the maintenance and release of dormancy in Northern Spy apple seeds. It is concluded that SCFA are unlikely to have a significant role in dormancy of apple seeds.

LITERATURE CITED

Ando, T. and Y. Tsukamoto. 1974. Capric acid : a growth inhibiting substance from dormant Iris hollandica bulbs. Phytochem. 13: 1031-1032.

Ando, T. and Y. Tsukamoto. 1981. Inhibitory action of saturated fatty acids and their derivatives. Phytochem. 20: 2143-2144.

Attygalle, A.B. and E.D. Morgan. 1986. Versatile microreactor and extractor. Anal. Chem. 58: 3054-3058.

Balboa-Zavala, O. and F.G. Dennis. 1977. Abscisic acid and apple seed dormancy. J. Amer. Soc. Hort. Sci. 102: 633-637.

Berrie, A.M.M., R. Don, D. Buller, M. Alam and W. Parker. 1975. The occurence and function of short chain fatty acids in plants. Plant Sci. Lett. 6: 163-173.

Berrie, A.M.M., D. Buller, R. Don and W. Parker. 1979. Possible role of volatile fatty acids and abscisic acid in the dormancy of oats. Plant Physiol. 63: 758-764.

Cathy, H.M. and G.L. Steffens. 1968. Relation of structure of fatty acid derivatives to their action as chemical pruning agents. Soc. Chem. Ind. Monogr. 31: 224-235.

Doss, R.P., Y. Kimura, and J.K. Christian. 1983. Endogenous levels of abscisic acid and decanoic acid in Dutch Iris bulb and the influence of abscisic acid and decanoic acid on Iris meristems cultured in vitro. Plant Physiol. 72: 713-716.

Hyodo, H. and K. Tanaka. 1982. Inhibition of ethylene production by fatty acids in fruit and vegetable tissues. Plant Cell Physiol. 23: 1237-1243.

LePoidevin, N. 1965. Inhibition of the germination of mustard seeds by saturated fatty acids. Phytochemistry 4: 525-526.

Majumder, M.K., D.V. Seshu, V.V. Shenoy. 1989. Implication of fatty acids and seed dormancy in a new screening procedure for cold tolerance in rice. Crop Sci. 29: 1298-1304.

Metzger, J.D. and D.K. Sebesta. 1982. Role of endogenous growth regulators in seed dormancy of Avena fatua. Short chain fatty acids. Plant Physiol. 70: 1480-1485.

Milborrow, B.V. 1967. The identification of (+)-Abscisin II ((+)-Dormin) in plants and measurements of its concentration. Planta 76: 93-113.

Powell, L.E. 1987. Hormonal aspects of bud and seed dormancy in temperate zone woody plants. HortScience 22: 845-850.

Purwoko, B.S. and L.E. Powell. 1994. Short chain fatty acids, stratification, and gibberellin relationships in apple embryo germination. Bul. Agron. 22 : 19 - 29.

Rogoyski, M.K. 1980. Short chain fatty acids and certain other carboxylic acids as inhibitors of buds in Malus with reference to their site of action. Ph. D. Thesis. Cornell University, Ithaca, New York.

Rogoyski, M.K. and L.E. Powell. 1981. Short chain fatty acids and their inhibition of apple bud growth in vitro. In : D. Lilov, E. Karanov, and L. Iliev eds. : Plant Growth Regulators. Proc. 3rd Int. Symp. Plant Growth Regulators. p. 463-469.

Subbaiah, T.K. and L.E. Powell. 1992. Abscisic acid relationships in the chill-related dormancy mechanism in apple seeds. Plant Growth Regul. 11: 115-123.

Ulbright, C. 1980. Short chain fatty acids as natural plant growth regulators. Ph. D. Dissertation. Washington University, Saint Louis, Missouri.