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Hormones in Pear Seeds. II. Levels of Abscisic Acid, Dihydrophaseic Acid, and Their Metabolites in Relation to Seed Dormancy in Several *Pyrus* Species[1]

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Abstract. **Concentrations of abscisic acid (ABA), dihydrophaseic acid (DPA), and their metabolites were measured in mature pear seeds, using gas-liquid chromatography (GLC). ABA content of *Pyrus communis* L. cv. Bartlett seeds fell during imbibition, but was not affected by temperature (4 vs. 21°C) or time (0 to 4 weeks) of stratification in a moist medium. Levels of DPA and 3 of its metabolites were not correlated with dormancy. The amount of chilling required to break dormancy was not correlated with ABA or DPA content in imbibed, non-stratified seeds of 6 *Pyrus* species.**

Breaking dormancy by cilling has been studied extensively in seeds of apple (*Malus domestica* Borkh.). Luckwill (7) was one of the first investigators to report the occurrence of inhibitory compounds in dormant seeds and their fluctuations during chilling. Subsequently, GA4 and GA7 were identified in immature apple seeds (4, 8), and ABA (11), GA4, GA7, and GA9 (14) in mature seeds. More recently, 9,11-dehydro GA7, GA9, GA_{10}, 13-hydroxy GA_{12}, GA_{15}, GA_{17}, GA_{20}, and GA_{44} have been identified in immature apple seeds (G. V. Hoad, Univ. of Bristol, Personal communication). Letham and Williams (6) tentatively identified zeatin, together with its riboside and ribotide, in apple fruit tissue. Sinska and Lewak (13) reported that GA4, but not GA7, content rose during low temp stratification of apple seeds, although the level returned to the initial concn before the seeds were capable of germination. Rudnicki (11) observed that levels of ABA in diffusates from apple seeds declined during low temp stratification, and suggested that ABA content might be a controlling factor in dormancy. However, Balboa-Zavala and Dennis (1) found the decline in ABA content of apple embryonic axes to be independent of temp, suggesting that factors other than ABA content control dormancy.

In contrast with the work in apple, pear seeds have not been extensively studied until recently. Zeatin has been tentatively identified in immature fruits of Japanese pear (*Pyrus pyrifolia* = *P. serotina*) (10). Strausz (15) investigated the role of an ABA-like inhibitor in dormancy of pear seeds and buds, and noted a positive correlation between inhibitor content and chilling requirement. Gil et al. (5) reported the occurrence of ABA-like, auxin-like, and GA-like substances in immature pear seeds, and Bearder et al. (2) identified a new gibberellin, GA_{45}, in extracts of immature seeds, as well as GA_{25}, ABA, and 4′-dihydrophaseic acid (DPA). In addition, Martin et al. (9) identified phaseic acid (PA), t,t-DPA (or epi-c,t-DPA), and 2 supposed metabolites of DPA (OH-DPA and keto-DPA) in immature seeds, together with GA_{17}, and an unidentified gibberellin, possibly 3β-hydroxy GA_{45}. GA_{17}, ABA, DPA, t,t-DPA, and the metabolites of DPA were also found in mature seeds.

Our purposes were to compare the levels of ABA in mature seeds of species which differed in chilling requirement, and to measure the fluctuations in content of ABA and its metabolites in *Pyrus communis* seeds during the breaking of dormancy by chilling.

Materials and Methods

Seeds of 'Bartlett' pear (*P. communis*) obtained from Morton's Nursery, Yakima, WA, were the primary material used. A second lot of *P. communis* seeds from a Russian source were purchased from Eichenberg and Co., Milton/Mainz, W. Germany. Seeds of *P. longipes* Coss. were collected at Long Ashton Agricultural and Horticultural Research Station, Bristol, England, and those of *P. amygdaliformis* Vill., *P. pashia* Ham., *P. ussuriensis* Maxim., and *P. elaeagrifolia* Pall. were obtained from the Station de Recherches d'Arboriculture Fruitiere, INRA, Angers, France.

Dry seeds were rinsed rapidly in 70% ethanol, then in tap water, and soaked in distilled water for 20 to 24 hr at 21 ± 1°C. The water was then poured off and the seeds were rinsed several more times with distilled water before being placed on moistened filter papers in 20.3 cm (diam) aluminum pans. The pans were held in loosely closed plastic bags at either 4 ± 1° or 21 ± 1° for 1 to 10 weeks. All seeds showing visible microbial growth were removed at frequent intervals. Samples were removed at weekly or biweekly intervals, blotted, and frozen for subsequent extraction. Similar samples (2 or 4 replications of 25 seeds each) were tested for germination by placing on moist filter paper in petri dishes for 7 days at 21 ± 1°.

The procedures used for extraction and partial purification have been described previously (9). One sample of 10 to 20 g of seeds was analyzed for each species or treatment. An aliquot of the crude acidic butanol fraction was removed for direct electron capture GLC (12), and the remainder was chromatographed, methylated (Me), and trimethylsilylated (TMS), as previously described (9), in preparation for flame ionization GLC. Ethyl acetate, rather than butanol, was used in some additional studies and is so noted when used.

Aliquots of the crude butanol fraction were methylated with ethereal diazomethane (9). The residues were dissolved in ethyl acetate, and aliquots representing 1 to 10 mg-eq. were analyzed for Me-ABA and Me-DPA using a Pye 104 GLC equipped with a ^{63}Ni detector. A glass column (152.4 × 0.35 cm i.d.) was packed with OV-17 (1.5%) plus QF-1 (1.95%) on Gas Chrom Q (100-120 mesh). N_2 flow rate was 40 ml/min at 200°C with detector at 300°. Pulse space was 150 μsec at 4v d.c.

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Two approximately equal peaks occurred in all samples (Fig. 1), one co-chromatographing with Me-ABA (8.8 min), the other having a retention time of 11.8 min, 0.7 min longer than that of Me-phaseic acid. Samples prepared subsequently were chromatographed at similar temp and gas flow rates on 2 additional columns (Table 1), using a Hewlett-Packard 402B GLC, also equipped with a ^{63}Ni detector. The second peak co-chromatographed with Me-DPA, and was clearly separated from Me-t-ABA and Me-t-DPA on 1% XE-60 (Table 1). In comparing species and the effects of stratification, sample contents of both Me-ABA and Me-DPA were estimated by comparison of peak areas with those of known amounts of Me-ABA. Data on relative content of ABA vs. DPA in extracts vs. imbibition water and in various portions of the seed were quantified by comparison of peak heights. A sample of Me-DPA supplied by J. A. D. Zeevaart was used as a standard.

Me and TMS derivatives of TLC eluates were chromatographed on a Pye 104 GLC equipped with a flame ionization detector. The column (152.4 × 0.35 cm) contained 2% SE-33 on Gas Chrom Q (80-100 mesh), and the temp was programmed from 180 to 225°C at 3°/min, using a N_2 flow rate of 60 ml/min.

Quantities of ABA metabolites were estimated by calculating peak areas for compounds previously identifed by gas chromatography-mass spectrometry (9, and Fig. 2). Absolute quantification was not possible, as insufficient quantities of the standards were available.

Results

ABA and DPA content of seeds vs. chilling requirement. Levels of ABA and DPA in imbibed seeds of the various *Pyrus* species showed no clear relationship with chilling requirements (Table 2). *P. ussuriensis* and *P. elaeagrifolia* seeds required 9 and 11 weeks, respectively, for 50% germination, and contained the highest levels of ABA, while 'Bartlett' seeds required the least chilling and contained the lowest level of ABA. However, data for the remaining species do not support the hypothesis that stratification requirement is correlated with ABA content. Both lots of *P. communis* seeds had low levels of ABA, yet the two were at the extremes in terms of chilling requirement. Neither DPA contents nor ratios of DPA to ABA were correlated with chilling requirement (Table 2).

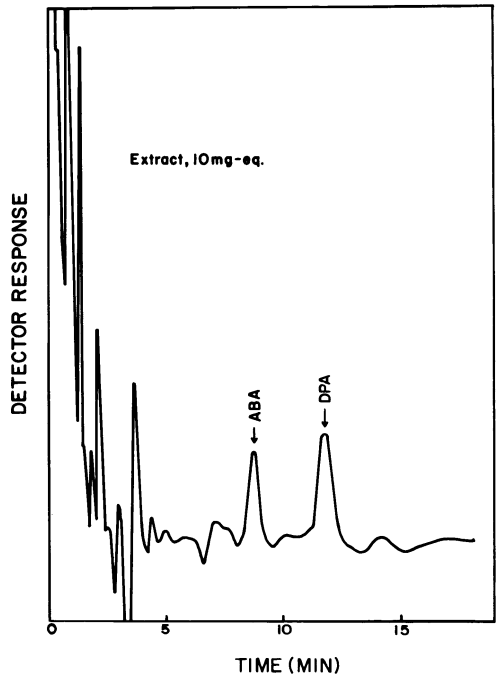

Fig. 1. Electron capture GLC trace of crude methylated acidic butanol fraction of imbibed pear seeds. Column was OV-17 (1.5%) plus QF-1 (1.95%) on Gas Chrom Q (100-120 mesh). Column temp was constant at 200°C.

Table 1. Retention times (min) of methylated derivatives of standard compounds on two columns at 200°C, in comparison with peaks in methylated extracts[z] of imbibed seeds.

Column[y]	Sample	Compound			
		Me-ABA	Me-t-ABA	Me-DPA	Me-t-DPA
2% DC-200	Standards	3.4	4.7	4.7	5.8
	Extract	3.4	—	4.7	—
1% XE-60	Standards	4.4	6.8	6.0	8.4
	Extract	4.4	—	6.0	—

[z]Crude acidic ethyl acetate fraction.

[y]N_2 flow rate 40 ml/min, inlet and detector temp both 240°C, columns 2 mm i.d. × 1.83 m, column support Gas-Chrom Q 80/100 mesh (DC-200) or 100/120 mesh (XE-60).

The content of DPA in non-chilled 'Bartlett' seeds and in various seed portions was subsequently estimated by EC-GLC. The water used for imbibition was also analyzed. The latter contained a small amount of t-ABA and over 8 times as much ABA as the methanol extract (Table 3). However, DPA content was less than 10% of that found in the extract. No Me-t-DPA was observed in the extract of whole seeds. Extraction of imbibed seeds following dissection indicated a higher concn of ABA in embryonic axes and seed coats than in cotyledons; DPA was more abundant in the cotyledons, with relatively little in the seed coats (Table 3).

Changes in ABA and its metabolites in 'Bartlett' seeds during stratification at 4° and at 21°C. Germination at 21° of 'Bartlett' seeds held at 4° increased steadily for the first 3 weeks, then leveled off at 74% thereafter (Fig. 3A); that of seeds held at 21° did not exceed 4% at any sampling date.

When dry seeds were imbibed for 20 hr in distilled water, the level of ABA in the crude acidic butanol fraction dropped 5-fold (corrected for water uptake), while the concn of DPA fell to half the level found in dry seeds (Fig. 3A, B). Subsequent changes in both compounds were negligible regardless of temp,

Table 2. Chilling requirements of *Pyrus* species tested vs. ABA and DPA contents of acidic butanol fractions of methanol extracts of non-stratified imbibed seeds, as estimated by electron capture GLC.

Species	Wk at 4°C required for 50% germination at 20°[z]	ABA (ng/g fresh wt)	DPA (ng ABA-eq/g fresh wt)	DPA/ABA ratio
P. communis cv. Bartlett	1.5	32	36	1.1
P. longipes	3.0	650	110	0.17
P. amygdaliformis	4.0	760	205	0.27
P. pashia	7.0	225	280	1.25
P. ussuriensis	9.0	870	225	0.26
P. elaeagrifolia	11.0	1235	265	0.21
P. communis (Russia)	12.5	138	45	0.33

[z]Values >10 estimated by extrapolation.

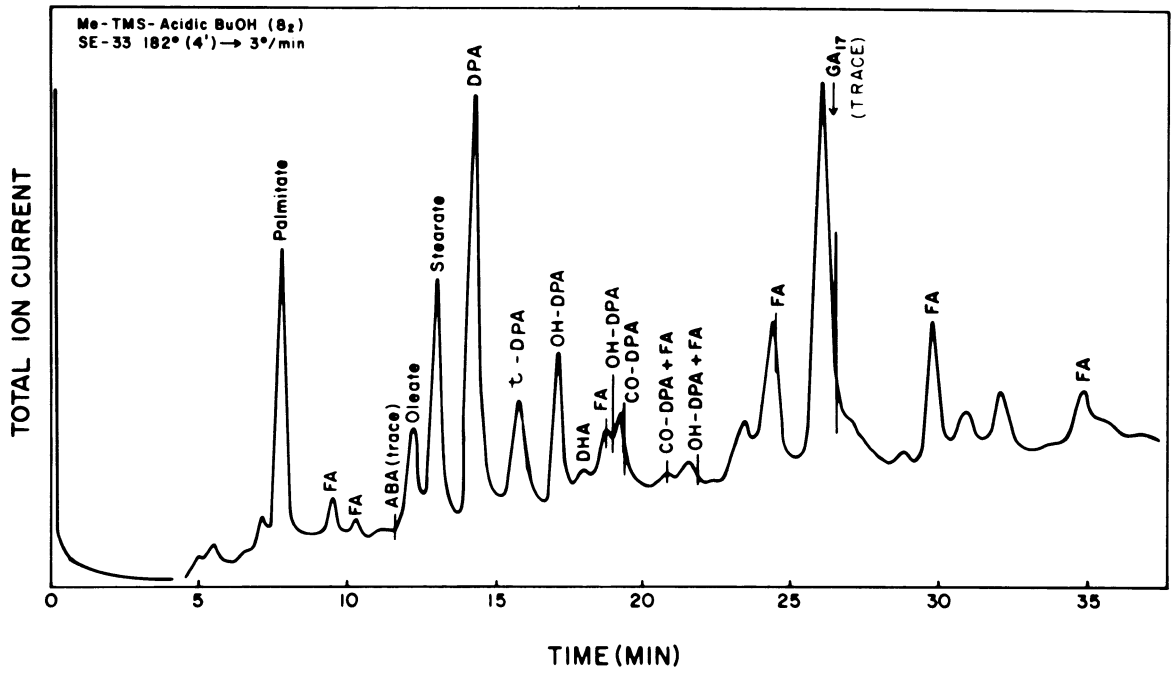

Fig. 2. Total ion current trace of methylated and trimethylsilylated acidic butanol fraction of 'Bartlett' pear seeds stratified for 4 wk at 4°C. Eluate from TLC was chromatographed on 2% SE-33 on Gas Chrom Q. Temp 182° for 5 min, then programmed at 3°/min. Abbreviations not used in text: FA-unidentified fatty acid; DHA-dihydroabietic acid (contaminant).

even during germination. Ratios of DPA to ABA were consistently higher in seeds held at 21°C than in those held at 4° (Fig. 3C).

DPA, OH-DPA and keto-DPA, as measured by flame ionization GLC of chromatographed extracts, increased 28, 100, and 100%, respectively, during imbibition (corrected for water uptake), while that of t-DPA remained constant (Fig. 4). Quantities (arbitrary units based on peak areas) in imbibed seeds were 800, 300, 200, and 125 for DPA, t-DPA, OH-DPA, and keto-DPA, respectively. Levels of ABA were too low for measurement by flame ionization detector. DPA content fell 40% during 4 weeks of stratification, while t-DPA content fell ca. 90%. Neither change was markedly affected by temp or by germination. Levels of the two derivatives fluctuated considerably during stratification (Fig. 4B, C), but did not appear to be related to the breaking of dormancy or to germination.

Table 3. ABA and DPA content (ng/g fresh wt) of extracts prepared from non-stratified 'Bartlett' pear seeds, as determined by electron capture gas chromatography on indicated columns.

Extract	ABA	t-ABA	DPA
Expt. 1 (1% XE-60)			
Water used for imbibition	620	51	203
Methanol extract of whole seeds following imbibition[z]	72	–[y]	2,800
Expt. 2 (2% DC-200)			
Methanol extract[z] of:			
Embryonic axes[x]	101	–	1,000
Cotyledons[x]	22	–	2,560
Seed coats[x]	74	–	380

[z]Crude acidic ethyl acetate fraction.

[y]Not detectable.

[x]Wt per seed (mg): embryonic axis=0.8; cotyledons=34; seed coats=26.

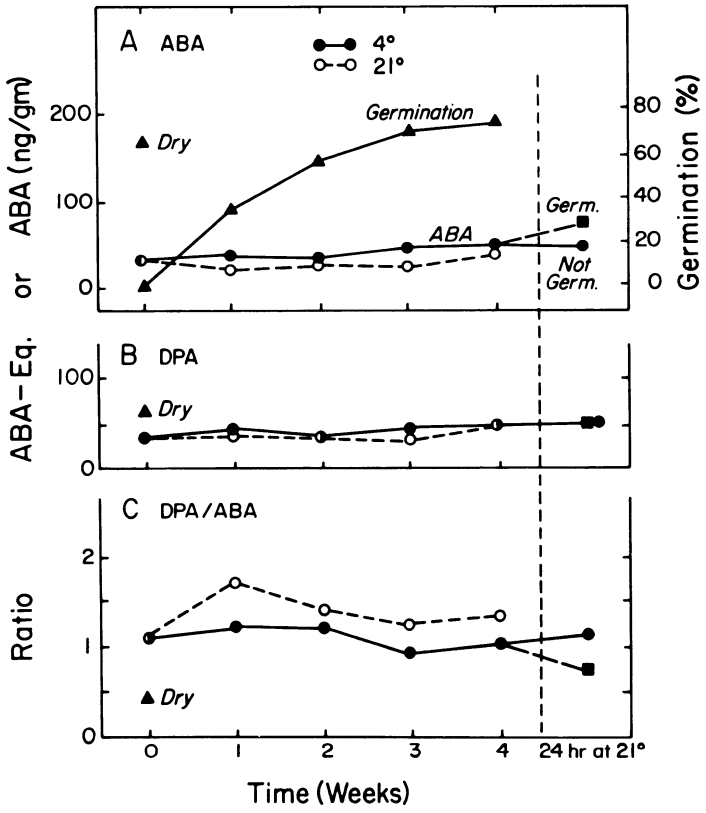

Fig. 3. A. Germination of 'Bartlett' pear seed at 20°C after 0 to 4 wk at 4° vs. content of ABA. Germination of seeds held at 20° did not exceed 4% at any time, and the data are omitted. B. DPA content of same seeds. C. Ratio of DPA to ABA. Values to right of broken line are for seeds held at 4° for 4 wk, then transferred to 21° for 24 hr. Squares indicate values for seeds which germinated within 24 hr; circles indicate values for those which did not.

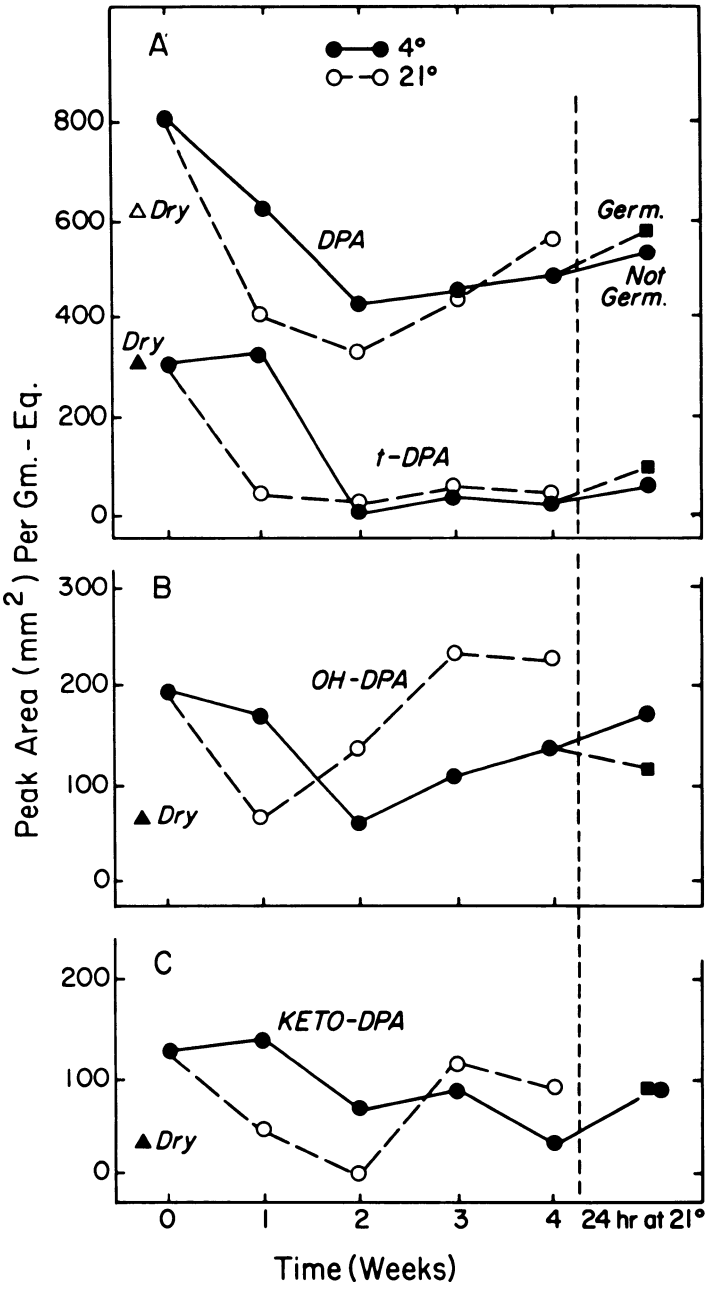

Fig. 4. Relative levels of DPA, t-DPA, OH-DPA, and keto-DPA during stratification of 'Bartlett' pear seeds, as determined by flame ionization gas chromatography. Values to right of broken vertical line are for seeds held at 4°C for 4 wk, then transferred to 21° for 24 hr. Squares indicate values for seeds which germinated within 24 hr; circles indicate values for those which did not.

Discussion

Relative amounts of DPA and ABA differ greatly between Fig. 3 and Table 4. In the former case (OV-17/QF-1 column, Pye 104 GLC), the 2 peaks were of similar area. In the latter (XE-60 or DC-200 column, Hewlett-Packard 402B GLC), the DPA peak was consistently much larger, even though sensitivity to Me-ABA was 5-to 10-fold greater than to Me-DPA. This apparently indicates a differential sensitivity of the ^{63}Ni detectors in the 2 instruments to these 2 compounds.

The discrepancy in values for DPA content as measured by electron capture vs. flame ionization detector is also noteworthy. Electron capture indicated a 50% *decline* in DPA content during imbibition, while flame ionization showed a 28% *increase*. Electron capture measurements revealed little change in DPA content during subsequent stratification, while levels measured by flame ionization detector varied considerably. The differences are probably attributable to losses occurring during TLC, and the electron capture data are therefore more reliable.

Neither method indicated a correlation between DPA or ABA content and dormancy, and levels of DPA derivatives did not change appreciably in response to chilling. ABA fell during imbibition, as was expected in view of the high level in the water used for soaking the seeds (Table 3). The constant level during stratification was not expected, however, on the basis of previous work with apple seeds (1, 11). Bound ABA content of seeds was not measured in this study. However, recent work has indicated parallel variation between free and bound ABA during stratfication of both apple (1) and peach (3) seeds.

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